

Kunitz Trypsin Inhibitor Genes Are Differentially Expressed during the Soybean Life Cycle and in Transformed Tobacco Plants

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We investigated the structure, organization, and developmental regulation of soybean Kunitz trypsin inhibitor genes. The Kunitz trypsin inhibitor gene family contains at least 10 members, many of which are closely linked in tandem pairs. Three Kunitz trypsin inhibitor genes, designated as KTi1, KTi2, and KTi3, do not contain intervening sequences, and are expressed during embryogenesis and in the mature plant. The KTi1 and KTi2 genes have nearly identical nucleotide sequences, are expressed at different levels during embryogenesis, are represented in leaf, root, and stem mRNAs, and probably do not encode proteins with trypsin inhibitor activity. By contrast, the KTi3 gene has diverged 20% from the KTi1 and KTi2 genes, and encodes the prominent Kunitz trypsin inhibitor found in soybean seeds. The KTi3 gene has the highest expression level during embryogenesis, and is also represented in leaf mRNA. All three Kunitz trypsin inhibitor genes are regulated correctly in transformed tobacco plants. Our results suggest that Kunitz trypsin inhibitor genes contain different combinations of *cis*-control elements that program distinct qualitative and quantitative expression patterns during the soybean life cycle.

INTRODUCTION

Soybean seeds contain two major proteinase inhibitor classes—the Kunitz trypsin inhibitor and the Bowman-Birk proteinase inhibitor (Laskowski and Kato, 1980; Ryan, 1981, 1988). These inhibitors are found in other legumes (Norioka et al., 1988), are localized within protein bodies (Horisberger and Tacchini-Vonlanthen, 1983; Vodkin and Raikhel, 1986), and are specific for serine proteases (Laskowski and Kato, 1980; Ryan, 1981). The Kunitz trypsin inhibitor class is the most prevalent soybean protease inhibitor, is represented by a 21-kD protein, and is specific for trypsin (Ryan, 1981; Kim et al., 1985; Tan-Wilson, 1988). By contrast, the Bowman-Birk class consists of several related 8-kD proteins, and inhibits trypsin, chymotrypsin, and elastase (Laskowski and Kato, 1980; Ryan, 1981, 1988; Tan-Wilson, 1988).

Proteinase inhibitors have been proposed to function as storage proteins, regulators of endogenous proteinases, or factors that protect plants from insect attack (Ryan, 1981). Transfer of an alfalfa Bowman-Birk inhibitor gene to tobacco plants showed directly that this proteinase inhibitor can function as an endogenous insecticide (Hilder et al., 1986). Because soybean null lines exist that lack either the Kunitz trypsin inhibitor (Orf and Hymowitz, 1979)

or the Bowman-Birk protease inhibitor (Stahlhut and Hymowitz, 1983), these proteins are not essential for normal growth and development.

We showed previously that the Kunitz trypsin inhibitor gene family is highly regulated during the soybean life cycle (Goldberg et al., 1981; Walling, Drews, and Goldberg, 1986; Jofuku, Schipper, and Goldberg, 1989). These genes are expressed at precise times during embryogenesis, have different expression levels in the embryo cotyledon and axis, and are either inactive or expressed at low levels in mature plant organ systems (Goldberg et al., 1981; Walling et al., 1986). Both transcriptional and post-transcriptional processes regulate Kunitz trypsin inhibitor gene expression (Walling et al., 1986). Jofuku et al. (1989) showed that a null line that lacks Kunitz trypsin inhibitor activity has a frameshift mutation in the major Kunitz trypsin inhibitor gene (KTi3). This mutation prevents KTi3 mRNA from being translated correctly in embryo cells (Jofuku et al., 1989). Other Kunitz trypsin inhibitor mRNAs (e.g., KTi1 and KTi2) are unaffected by the frameshift mutation (Jofuku et al., 1989).

To begin to identify the DNA sequences that regulate Kunitz trypsin inhibitor gene expression, we investigated the organization and developmental regulation of specific Kunitz trypsin inhibitor gene family members. Our studies indicate that the Kunitz trypsin inhibitor family contains at least 10 members, and that some of these genes are

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organized into tandem pairs. We further show that three distinct Kunitz trypsin inhibitor genes (KTi1, KTi2, and KTi3) have different expression patterns during the soybean life cycle and in transformed tobacco plants. Our results suggest that each Kunitz trypsin inhibitor gene contains a unique combination of *cis*-control elements that programs specific expression patterns during embryogenesis and in the mature plant.

RESULTS

Kunitz Trypsin Inhibitor Is Encoded by a Multigene Family

We described previously the isolation of a Kunitz trypsin inhibitor cDNA plasmid, designated as A-37, from a library of mid-maturation stage embryo mRNA (Goldberg et al., 1981). DNA sequence analysis revealed that the mRNA represented by A-37 encoded a protein with only 70% homology to the major Kunitz trypsin inhibitor present in soybean seeds (data not shown). To isolate a cDNA clone representing the major Kunitz trypsin inhibitor mRNA, we constructed a second library using mRNA isolated from immunoselected Kunitz trypsin inhibitor polysomes (Vodkin, 1981; J.J. Harada and R.B. Goldberg, unpublished results). We screened the KTi-enriched embryo mRNA library at a reduced criterion (32°C , $\Delta T_m = -28^{\circ}$) with the A-37 cDNA plasmid and identified a second Kunitz trypsin inhibitor plasmid, designated as pKT3. The pKT3 DNA sequence indicated that it represented the major Kunitz trypsin inhibitor mRNA (Jofuku et al., 1989).

We hybridized the A-37 and pKT3 cDNA plasmids with DNA gel blots to estimate the Kunitz trypsin inhibitor gene copy number. Figure 1A shows that, under moderately stringent hybridization conditions, A-37 hybridized with a single-copy 4.0-kb EcoRI DNA fragment (42° lane). At the same criterion, pKT3 hybridized with two single-copy EcoRI DNA fragments, 5.5 kb and 0.6 kb in length (Jofuku et al., 1989). By contrast, a mixed A-37 and pKT3 probe hybridized with greater than 10 EcoRI fragments under less stringent hybridization conditions (Figure 1A, 22° lane). Together, these results indicate the presence of a large number of Kunitz trypsin inhibitor DNA sequences in the soybean genome, and that genes represented by the A-37 and pKT3 cDNA plasmids are present on different EcoRI fragments.

Kunitz Trypsin Inhibitor Genes Are Organized as Tandem Pairs

We used the A-37 and pKT3 cDNA plasmids to screen two λ Charon 4 soybean genome libraries for Kunitz trypsin inhibitor genes (see Methods). Figure 1B shows that we

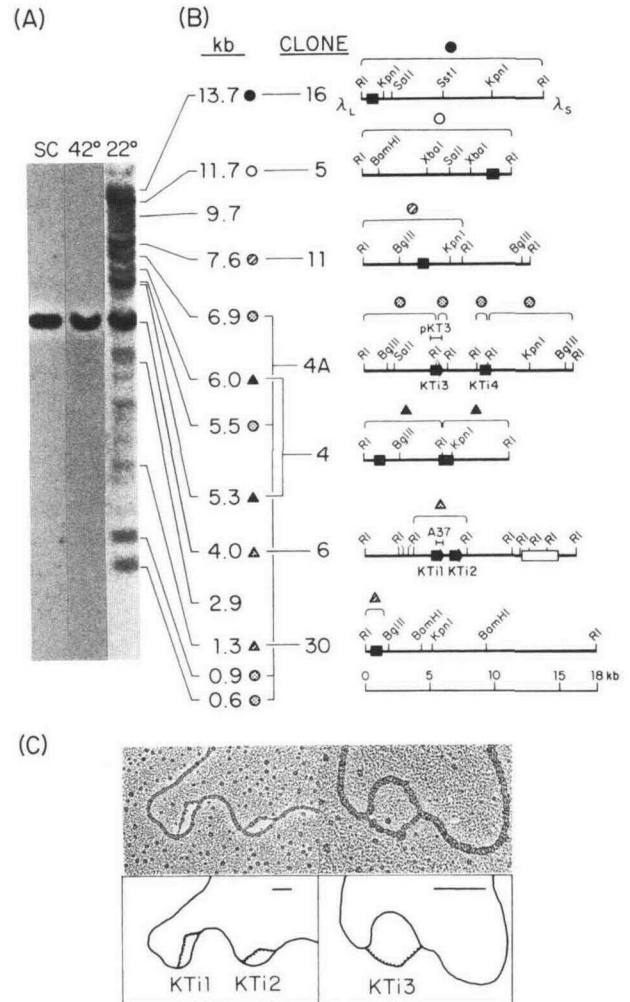


Figure 1. Soybean Kunitz Trypsin Inhibitor Gene Structure and Organization.

(A) Kunitz trypsin inhibitor gene representation in the soybean genome. Dare variety leaf DNA was digested with EcoRI, fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with the Kunitz trypsin inhibitor cDNA plasmid A-37 (Goldberg et al., 1981; Jofuku et al., 1989) at either 42°C ($\Delta T_m = -18^{\circ}$) or 22°C ($\Delta T_m = -38^{\circ}$). SC lane contained a single-copy equivalent of EcoRI-digested λ Clone 6 DNA.

(B) Kunitz trypsin inhibitor gene sequence organization. Boxes and arrows represent gene locations and transcriptional orientations, respectively. The open box represents a non-seed protein gene described previously by Walling et al. (1986). Lines above the λ Clone 6 and λ Clone 4A phages indicate gene sequences represented by the cDNA plasmids A-37 and pKT3, respectively. Brackets indicate the EcoRI DNA fragments that hybridized with labeled A-37 and pKT3 plasmid DNAs at 22°C ($\Delta T_m = -38^{\circ}$). The circles and triangles correlate phage DNA fragments with soybean DNA segments detected by the DNA gel blot shown in **(A)**.

(C) Kunitz trypsin inhibitor gene structure. λ Clone 6 and λ Clone 4A DNAs were hybridized with embryo mRNA under conditions that form R-loops (Fischer and Goldberg, 1982). Bars represent 1 kb.

identified seven recombinant phages that contained 10 distinct Kunitz trypsin inhibitor genes. Collectively, the DNA inserts present in these phages represented more than 145 kb of the soybean genome and contained greater than 90% of the EcoRI DNA fragments detected by A-37 and pKT3 in the DNA gel blot shown in Figure 1A.

Restriction endonuclease mapping and localization of Kunitz trypsin inhibitor DNA sequences within the recombinant phages demonstrated that several genes were clustered in tandem pairs. For example, Figure 1B shows that λClone 6 contains two Kunitz trypsin inhibitor genes, designated as KT1 and KT2, that are separated by less than 0.5 kb and are present on the 4.0-kb EcoRI DNA fragment that hybridized strongly with the A-37 plasmid (Figure 1A, SC and 42° lanes). Similarly, Figure 1B shows that λClone 4A possesses two Kunitz trypsin inhibitor genes, designated as KT3 and KT4. The KT3 gene is present on the 5.5-kb and 0.6-kb EcoRI DNA fragments that hybridized strongly with the pKT3 plasmid, suggesting that KT3 is the major Kunitz trypsin inhibitor gene (Jofuku et al., 1989). λClone 4 also contains two Kunitz trypsin inhibitor gene sequences (Figure 1B). Together, these results indicate that at least six of the 10 cloned Kunitz trypsin inhibitor genes are closely linked as tandem pairs in the soybean genome.

The KT1, KT2, and KT3 Kunitz Trypsin Inhibitor Genes Do not Contain Introns

We characterized the KT1, KT2, and KT3 Kunitz trypsin inhibitor genes by R-loop analysis (Fischer and Goldberg, 1982) to visualize their structures in the electron microscope. Figure 1C shows that these genes do not contain introns detectable by electron microscopy. We sequenced the KT1, KT2, and KT3 genes and their contiguous regions to verify these structures directly. The KT1 and KT2 DNA sequences are presented in Figure 2, while the KT3 gene sequence is presented in Jofuku et al. (1989). A schematic representation of all three genes is shown in Figure 3, along with relevant consensus sequences and a comparison of homologous gene regions. A comparison of the translated protein sequences with the sequence of the major Kunitz trypsin inhibitor protein (Kim et al., 1985) is shown in Figure 4.

Figures 2 and 3 show directly that introns are absent from all three Kunitz trypsin inhibitor genes. Analysis of the protein sequences encoded by the Kunitz trypsin inhibitor genes indicates that the KT3 gene encodes a protein identical to the major Kunitz trypsin inhibitor found in soybean seeds (Figure 4; Jofuku et al., 1989). By contrast, the KT1 and KT2 genes encode proteins with only 70% homology with the major Kunitz trypsin inhibitor protein (Figure 4). Together, these results indicate that the KT1, KT2, and KT3 Kunitz trypsin inhibitor genes have simple structures, and that they encode distinct proteins.

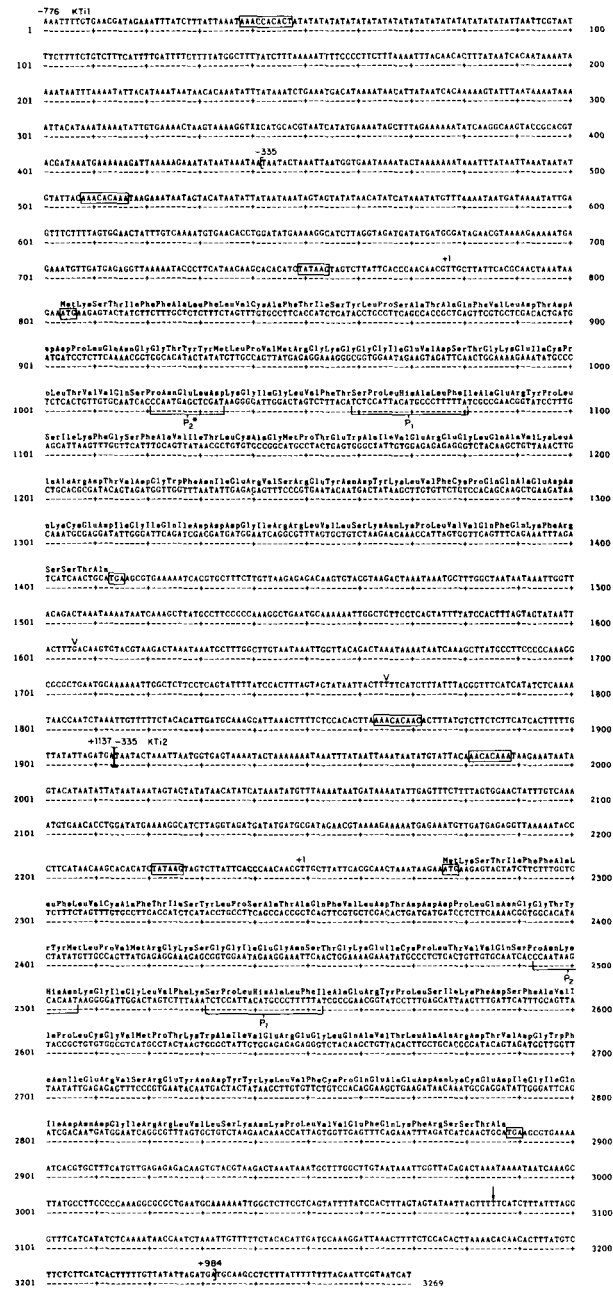


Figure 2. KT1 and KT2 Kunitz Trypsin Inhibitor Gene Sequences.

Boxes enclose TATA-like sequences, translation start and stop codons, and a CACA sequence motif (Goldberg et al., 1989). Brackets at -335, +1137/-335, and +984 enclose sequences with >90% similarity between the two gene regions. 5' and 3' ends of an insertion that arose from a duplication of 3' KT1 gene sequences (nucleotides +674 to +829) are designated by the V. An arrow shows the position at which this insertion sequence would occur within the homologous KT2 gene region (nucleotide +836).

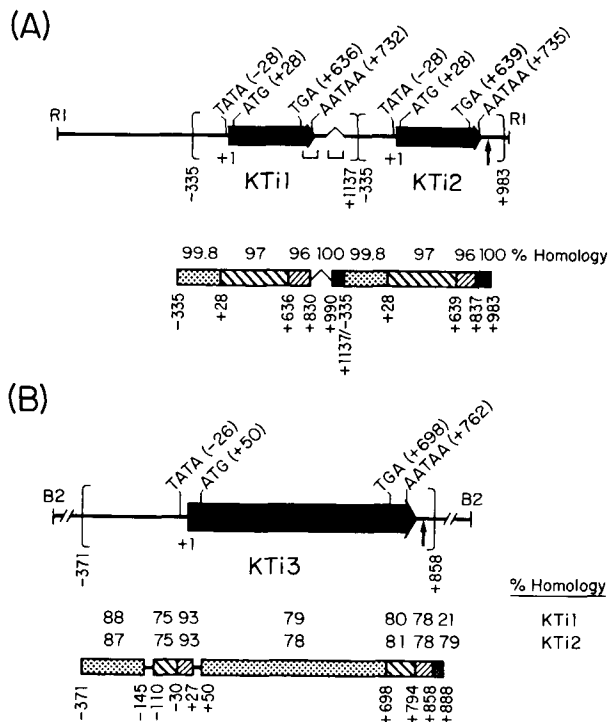


Figure 3. Kunitz Trypsin Inhibitor Gene Similarities.

Relevant consensus sequences and their locations are shown above the genes. Brackets below the gene regions indicate KT1 nucleotides +674 to +829 that are duplicated to form a portion of the 5'-flanking region of KT2. Arrows indicate the KT2 and KT3 3' regions that do not contain the duplicated KT1 DNA segment. Brackets enclosing gene regions indicate sequences that are highly homologous between the KT1, KT2, and KT3 genes.

(A) KT1 and KT2 gene sequence comparison. Boxes filled with similar patterns indicate compared gene regions. Percent homology and nucleotide reference points are shown above and below the designated gene region, respectively.

(B) KT1, KT2, and KT3 gene sequence comparisons. Percent homology of KT3 gene regions with corresponding KT1 or KT2 sequences is shown above the designated region. The 5' and 3' ends of compared gene regions are shown below the designated gene region. Thin lines between regions indicate gaps introduced into the KT3 gene sequence to allow for maximum alignment of all three gene sequences.

The KT1 and KT2 Kunitz Trypsin Inhibitor Genes Are Nearly Identical to Each Other

Figure 3A shows that the KT1 and KT2 genes are closely related to each other. For example, the coding regions of these genes differ by only 18 out of 608 nucleotides, indicating approximately 97% sequence similarity (Figure 3A). In addition, the KT1 and KT2 5'-flanking regions differ by only 1 nucleotide up to position -335. Upstream

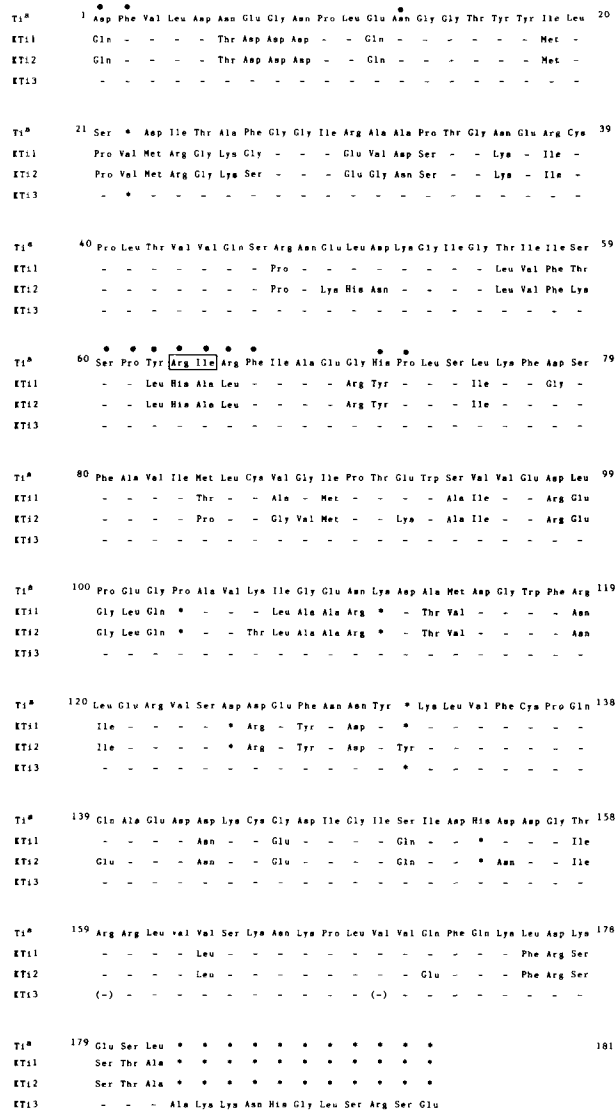


Figure 4. Kunitz Trypsin Inhibitor Amino Acid Comparisons.

The amino acid sequence of the Kunitz trypsin inhibitor *Ti^a* allelic form (Kim et al., 1985) is shown for reference. The translated protein sequences of the KT1, KT2, and KT3 genes are shown below the *Ti^a* sequence. Dashes indicate amino acid sequence identities with the *Ti^a* allelic form. Asterisks designate gaps introduced into a given amino acid sequence to obtain maximum alignment of all four proteins. Closed circles above the sequence designate Kunitz trypsin inhibitor amino acids that form possible contacts with specific amino acids of porcine trypsin (Sweet et al., 1974). Box encloses amino acids Arg-63 and Ile-64. The peptide bond between these two amino acids is cleaved by trypsin (Ozawa and Laskowski, 1966). Substitution and/or deletion of either amino acid inactivates Kunitz trypsin inhibitor activity (Ryan, 1981).

of this position, the KTi1 5' region continues for at least 1 kb, while the KTi2 5' region merges with the KTi1 3' region (Figure 3A). By contrast, Figure 3B shows that the KTi3 coding region is only 80% similar to the corresponding KTi1 and KTi2 gene regions. A slightly higher degree of similarity is found between the KTi3 and KTi1/2 5'-flanking regions. Together, these findings indicate that the KTi1 and KTi2 genes are almost exact copies of each other, and that the KTi3 gene represents a divergent relative.

Kunitz Trypsin Inhibitor Gene Expression Is Regulated during Embryogenesis

We hybridized A-37 plasmid DNA with dot blots containing mRNAs from 14 different embryonic stages to investigate the combined KTi1 and KTi2 Kunitz trypsin inhibitor gene expression patterns during embryogenesis. We showed that, under our DNA/RNA hybridization conditions (42°C, $\Delta T_m = -18^\circ$), there was less than 10% cross-reaction between A-37 and KTi3 mRNA and between pKT3 and KTi1 and KTi2 mRNAs (G. de Paiva and R.B. Goldberg, unpublished results). Figure 5A shows that the KTi1/2 mRNAs accumulated early in embryogenesis, reached a maximum level at mid-maturation [77 days after flowering (DAF)], and then decayed prior to dormancy.

To determine the prevalence of KTi3 mRNA relative to the KTi1 and KTi2 mRNAs, we hybridized A-37 and pKT3 plasmid DNAs with mid-maturation stage embryo mRNA gel blots under the same hybridization conditions. Figure 5B shows that both probes hybridized with a prevalent 0.9-kb embryo mRNA. However, the hybridization signal obtained with the pKT3 probe was approximately fivefold more intense than that obtained with A-37. This result indicates that KTi3 mRNA is more prevalent than both the KTi1 and KTi2 mRNAs at mid-maturation.

To determine whether the difference in KTi1/2 and KTi3 mRNA levels reflected the transcriptional activities of the KTi1/2 and KTi3 genes, we hybridized embryo ^{32}P -nuclear RNA synthesized *in vitro* (Walling et al., 1986) with DNA gel blots containing KTi1/2 and KTi3 gene fragments. Figure 6 shows that ^{32}P -nuclear RNA hybridized approximately fivefold more intensely with the KTi3 DNA fragment than with the KTi1/2 DNA fragment. Together, these data indicate that Kunitz trypsin inhibitor genes are regulated temporally during embryogenesis, that the KTi3 gene is expressed at a higher level than both the KTi1 and KTi2 genes, and that the difference in KTi1/2 and KTi3 gene expression levels is due primarily to a higher KTi3 transcription rate.

The KTi1 and KTi2 Kunitz Trypsin Inhibitor Genes Are Expressed at Different Levels during Embryogenesis

Figures 2 and 3 show that the KTi1 and KTi2 Kunitz trypsin inhibitor genes and their flanking regions are nearly iden-

tical to each other, suggesting that they may have similar expression programs. We used both primer extension and mRNA gel blot studies with two gene-specific oligonucleotide probes, designated as P₁ and P₂, to determine the individual KTi1 and KTi2 gene expression levels in mid-maturation stage embryos. The nucleotide sequences of

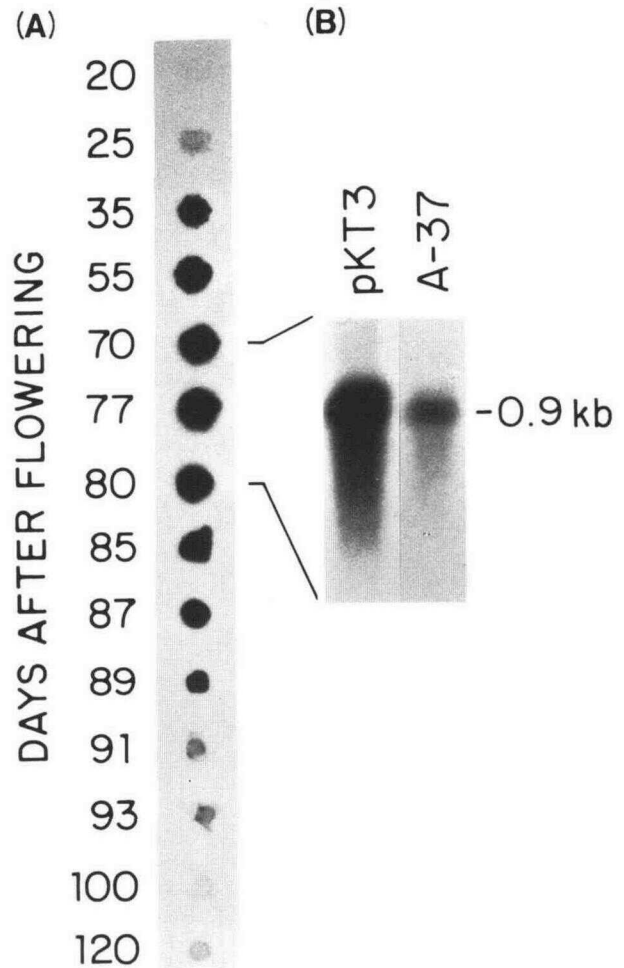


Figure 5. Kunitz Trypsin Inhibitor Gene Expression during Soybean Embryogenesis.

(A) Accumulation of KTi1/2 mRNAs during soybean embryogenesis. 0.2 μg of embryo mRNA from different developmental stages was spotted onto nitrocellulose and hybridized with labeled A-37 plasmid DNA (Figure 1). Soybean embryo developmental stages relative to days after flowering were described by Goldberg et al. (1989).

(B) Representation of KTi1/2 and KTi3 mRNAs in soybean embryos. Two micrograms of soybean mid-maturation stage embryo mRNA was fractionated on methylmercury hydroxide agarose gels, transferred to nitrocellulose, and hybridized with labeled A-37 and pKT3 plasmid DNAs (Figure 1).

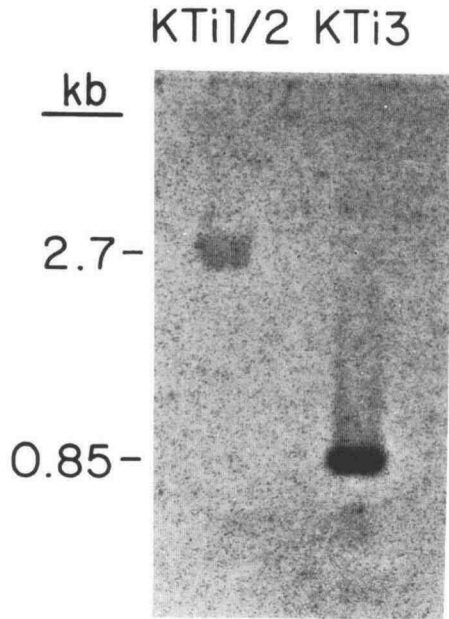


Figure 6. Kunitz Trypsin Inhibitor Gene Transcription in Soybean Embryo Nuclei.

Plasmid DNAs were digested with relevant restriction endonucleases, fractionated by electrophoresis on agarose gels, and transferred to nitrocellulose. The DNA gel blots were then hybridized with ^{32}P -nuclear RNAs synthesized from 60 DAF soybean embryo nuclei. We showed previously that at this developmental stage soybean seed protein gene transcriptional activities reach maximum levels (Walling et al., 1986). KTI1/2 and KTI3 refer to lanes containing 0.6 μg of KTI1 and KTI3 plasmid DNA inserts that are 2.7 kb and 0.85 kb in length, respectively.

the P_1 and P_2 oligonucleotides and their locations within the KTI1 and KTI2 genes are shown in Figure 2 and are schematically represented in Figure 7A.

We hybridized the 22-nucleotide P_1 oligonucleotide with mid-maturation stage mRNA, and then extended the primer with reverse transcriptase in the presence of ^{32}P -TTP. The P_1 oligonucleotide sequence is present in both the KTI1 and KTI2 genes (Figure 2). However, as seen in Figures 2 and 7B, the KTI1 and KTI2 Kunitz trypsin inhibitor genes differ by 1 nucleotide in the region 5' to the shared P_1 oligonucleotide sequence (5'-ACA-3' versus 5'-AAA-3'). Because of the cytosine residue in the KTI1 mRNA sequence, extension of a P_1 oligonucleotide/KTI1 mRNA hybrid will terminate 2 nucleotides before that of the analogous P_1 /KTI2 hybrid in the presence of only ^{32}P -TTP (Figure 7B). Comparison of the P_1 /embryo mRNA primer extension products enables the KTI1 and KTI2 mRNA levels to be estimated individually, even though the two mRNAs differ by only a few nucleotides (Figures 2 and 3).

Figure 7C shows that the expected 23-nucleotide and 25-nucleotide primer extension products were synthesized

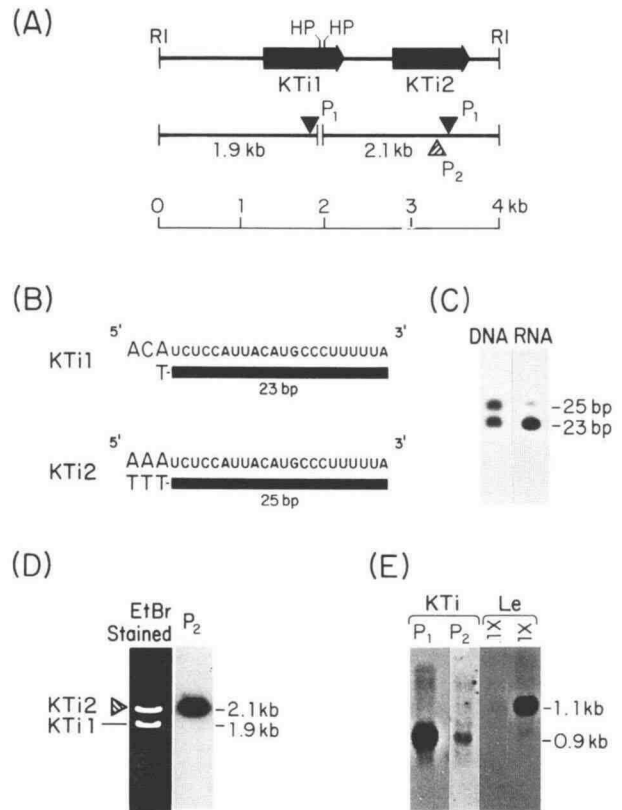


Figure 7. KTI1 and KTI2 Gene Expression in Soybean Embryos.

(A) Organization of the KTI1 and KTI2 Kunitz trypsin inhibitor genes. A schematic representation of the pBR325 recombinant plasmid pE4 is shown for reference. Arrows indicate the sites within the DNA fragments that are recognized by primers P_1 and P_2 (Figure 2).

(B) Experimental strategy for primer extension analysis using P_1 . Dark boxes represent the 22-nucleotide P_1 primer. The 23-nucleotide and 25-nucleotide products expected from extending the P_1 :mRNA hybrids with reverse transcriptase are shown below the corresponding mRNA sequence.

(C) Primer extension analysis. Five micrograms of embryo mRNA were hybridized with primer P_1 , and DNA was synthesized in the presence of only ^{32}P -TTP (lane RNA). A control reaction was carried out using 1 μg of denatured pE4 DNA template (lane DNA).

(D) Hybridization of primer P_2 to DNA gel blots. pE4 DNA was digested with EcoRI and HpaI, fractionated by electrophoresis, and transferred to nitrocellulose. The DNA gel blot was then hybridized with labeled P_2 (lane P_2). KTI1 and KTI2 refer to the 1.9-kb and 2.1-kb EcoRI/HpaI DNA fragments (A).

(E) Hybridization of P_1 and P_2 primers with mRNA gel blots. Five micrograms of embryo mRNA were fractionated on agarose gels, transferred to nitrocellulose, and hybridized with labeled P_1 and P_2 . KTI lanes refer to reactions with P_1 and P_2 . Le lane refers to reactions with a 25-nucleotide primer that represents nucleotides +102 to +127 of the *Le1* lectin gene (Goldberg, Hoschek, and Vodkin, 1983). The 0.1X and 1X lanes contained 0.5 ng and 5 ng of mid-maturation stage (77 DAF) embryo mRNA. 1.1 kb and 0.9 kb refer to lectin and Kunitz trypsin inhibitor mRNA sizes, respectively.

in equimolar amounts using KTi1 and KTi2 DNA as templates, respectively (DNA lane). By contrast, the 23-nucleotide KTi1 mRNA extension product was synthesized at a 20-fold higher level than the 25-nucleotide KTi2 mRNA product in the presence of mid-maturation stage embryo mRNA (RNA lane). Experiments with mRNAs from different embryonic stages indicated that the difference in KTi1 and KTi2 mRNA levels occurred throughout embryogenesis (G. de Paiva and R.B. Goldberg, unpublished results).

To verify this result with a different procedure, we hybridized labeled P_1 and P_2 oligonucleotide probes with embryo mRNA gel blots. Under the hybridization conditions used (see Methods), the 14-nucleotide P_2 oligonucleotide probe should hybridize specifically with KTi2 mRNA because of a 3-nucleotide difference in the corresponding KTi1 mRNA region (see Figure 2). Figure 7D shows that, as expected, P_2 hybridized with KTi2 DNA and did not hybridize detectably with KTi1 DNA (P_2 lane). By contrast, the shared P_1 probe hybridized with KTi1 and KTi2 DNAs with equal intensities (data not shown).

Figure 7E shows that both oligonucleotide probes hybridized with a 0.9-kb embryo mRNA. However, the P_2 probe produced a 10-fold lower hybridization signal with mid-maturation stage embryo mRNA than did the P_1 probe (compare lanes P_1 and P_2), confirming that the KTi2 mRNA is less prevalent than the KTi1 mRNA. By reference to seed lectin mRNA standards (Figure 7E, Le lanes), we estimated that the KTi1 and KTi2 mRNAs represented 0.75% and 0.075% of the mid-maturation stage embryo mRNA mass, respectively. Because the KTi3 mRNA is at least 5 times more prevalent than the KTi1 and KTi2 mRNAs (Figure 5), it represents approximately 4% of the mRNA mass at this developmental stage. Together, these data show that the KTi1 and KTi2 Kunitz trypsin inhibitor genes are expressed at different quantitative levels during embryogenesis even though these genes have nearly identical DNA sequences and flanking regions.

Kunitz Trypsin Inhibitor Genes Are Expressed in Mature Plant Organ Systems

We hybridized Kunitz trypsin inhibitor gene probes with leaf, stem, and root mRNA gel blots to determine whether the Kunitz trypsin inhibitor genes were expressed in organ systems of the mature plant. As seen in Figure 8A, a 4.0-kb KTi1/2 gene probe (Figure 7A) hybridized with a low-prevalence 0.9-kb RNA present in leaf, stem, and root mRNAs. By contrast, Figure 8B shows that the KTi3 gene probe produced a 0.9-kb signal with leaf mRNA but not detectably with stem or root mRNAs. Relative to embryo mRNA standards (Figure 8, E lanes), we estimated that the KTi1, KTi2, and KTi3 mRNAs were present in mature plant organ systems at concentrations at least 1000-fold lower than those observed in mid-maturation stage embryos (compare L, S, R, and E lanes). Together, these results indicate that the KTi1/2 and KTi3 Kunitz trypsin

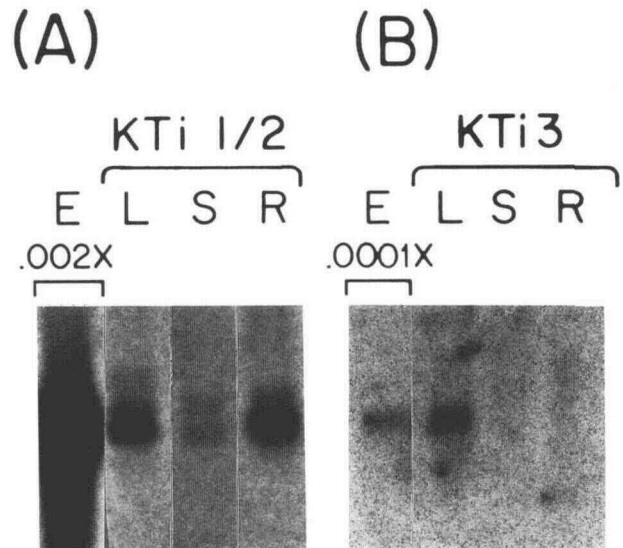


Figure 8. Kunitz Trypsin Inhibitor Gene Expression in Mature Plant Organ Systems.

Five micrograms of soybean leaf (L), stem (S), and root (R) mRNAs was fractionated on methylmercury hydroxide agarose gels and transferred to nitrocellulose paper. The mRNA gel blots were hybridized with Kunitz trypsin inhibitor gene probes and were then subjected to a 42°C washing criterion.

(A) KTi1/2 gene expression in mature plant organ systems. KTi1/2 indicates hybridization with labeled pE4 plasmid DNA (Figure 7). Lane E contained 10 ng of mid-maturation stage embryo mRNA, or the equivalent of 30 molecules of Kunitz trypsin inhibitor mRNA per cell (Figure 7; Goldberg et al., 1981).

(B) KTi3 gene expression in mature plant organ systems. KTi3 represents reaction with labeled pKT3 plasmid DNA (Figure 1). Lane E contained 0.5 ng of mid-maturation stage mRNA or approximately two molecules of KTi3 mRNA per cell (Figures 5 and 7).

inhibitor genes are expressed in specific organ systems of the mature plant in addition to being expressed during embryogenesis.

Kunitz Trypsin Inhibitor Genes Are Expressed Correctly during Tobacco Seed Development

Tobacco Transformants Contain One Kunitz Trypsin Inhibitor Locus

We transformed tobacco plants with the KTi1, KTi2, and KTi3 Kunitz trypsin inhibitor genes to begin to localize sequences responsible for their developmental-specific expression patterns. Figure 9 schematically shows the Kunitz trypsin inhibitor gene regions transferred to tobacco plants. We utilized three DNA fragments: (1) the 4.0-kb EcoRI DNA fragment containing both the KTi1 and KTi2

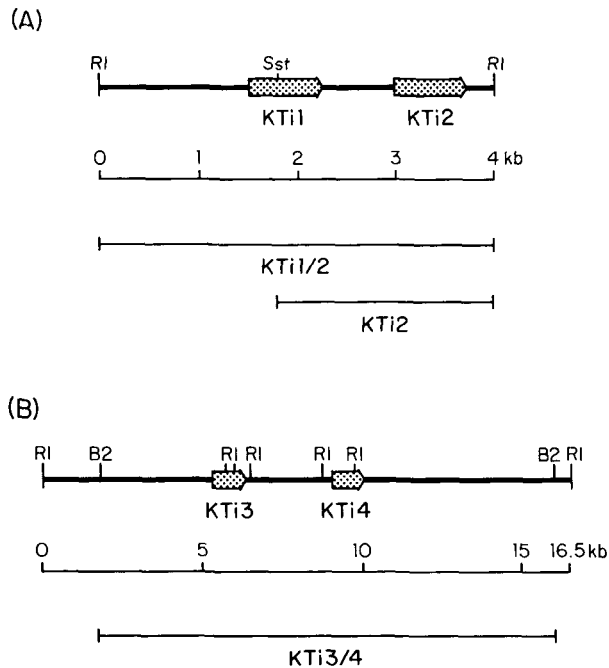


Figure 9. The KTi1/2 and KTi3/4 Kunitz Trypsin Inhibitor Gene Regions.

(A) Schematic representation of the KTi1 and KTi2 gene region. RI and SstI refer to EcoRI and SstI restriction endonucleases, respectively. Boxes represent gene locations, and arrows show transcriptional orientations (Figure 1). Lines below the gene region represent the 4.0-kb KTi1/2 and 2.2-kb KTi2 DNA fragments utilized in the gene transfer studies.

(B) Schematic representation of the KTi3 and KTi4 gene region. RI and B2 refer to EcoRI and BglII restriction endonucleases, respectively. Boxes represent gene locations and transcriptional orientations, respectively (Figure 1). Line below the gene region represents the 12.5-kb BglII DNA fragment used in the gene transfer studies.

genes (Figures 1, 3A, 7A, and 9A), (2) a 2.2-kb SstI fragment containing only the KTi2 gene (Figure 9A), and (3) a 12.5-kb BglII fragment containing the KTi3 gene (Figures 1, 3B, and 9B). In addition to the KTi3 Kunitz trypsin inhibitor gene, the 12.5-kb BglII DNA fragment contains another Kunitz trypsin inhibitor gene, designated as KTi4 (Figures 1 and 9B). The DNA sequence of the KTi4 Kunitz trypsin inhibitor gene has not yet been determined.

We selected several independent transformants for each Kunitz trypsin inhibitor gene region, and then determined genetically the number of Kunitz trypsin inhibitor loci present in each transformant by scoring the kanamycin-resistance (Kn^R) phenotype conferred by linked vector gene sequences (Hain et al., 1985). Seeds obtained from self-

fertilized transformed plants segregated 3:1 relative to the Kn^R marker, indicating the presence of a single Kunitz trypsin inhibitor locus in each transformed tobacco genome (data not shown).

To verify this result directly, we hybridized gel blots containing DNA from transformants containing the KTi1 and KTi2 Kunitz trypsin inhibitor genes (Figure 9A) with the 4.0-kb KTi1/2 gene probe shown in Figures 7A, 9A, and 10A. Figure 10B shows that no detectable signal was obtained with untransformed tobacco DNA (lane C). By contrast, low-copy soybean DNA fragments of the expected size (4.0 kb) were present in transformants containing the KTi1/2 gene region (Figure 10B, RI lanes). In addition, only one DNA fragment was obtained with a restriction enzyme that does not digest either the vector or the KTi1/2 DNA (Figure 10B, Hp lanes). Together, these findings suggest that each tobacco transformant contained a single unrearranged Kunitz trypsin inhibitor gene region.

The KTi1 and KTi2 Kunitz Trypsin Inhibitor Genes Are Expressed Differentially in Tobacco Seeds

We hybridized tobacco seed mRNA gel blots with the 4.0 kb KTi1/2 gene probe shown in Figure 11A to determine whether the KTi1 and KTi2 Kunitz trypsin inhibitor genes were correctly expressed during tobacco seed development. Figure 11B shows that no detectable signal was observed with untransformed tobacco mRNA (C lane). By contrast, the 4.0-kb KTi1/2 gene probe produced a 0.9-kb signal with seed mRNAs from tobacco plants containing either the KTi1 and KTi2 gene (Figure 11B, KTi1/2 lane) or the KTi2 gene alone (Figure 11B, KTi2 lane). As predicted from the results obtained with soybean embryo mRNA (Figure 7), the KTi2 mRNA was present at a lower level than the KTi1 mRNA in the transformed tobacco seeds (compare Figure 11B, KTi1/2 and KTi2 lanes). The difference in KTi1/2 and KTi2 mRNA levels was not paralleled by a similar difference in vector Kn^R mRNA levels in seeds of the same transformants (data not shown). This suggests that the observed mRNA prevalence differences were due to actual differences in KTi1 and KTi2 Kunitz trypsin inhibitor gene expression levels and not due to position effects.

The KTi1 and KTi2 Kunitz Trypsin Inhibitor Genes Are Regulated Temporally during Tobacco Seed Development

We isolated transformed tobacco seed mRNAs at different times during seed development and hybridized each mRNA population with the 4.0-kb KTi1/2 gene probe to determine whether the KTi1 and KTi2 Kunitz trypsin inhibitor genes were temporally regulated during tobacco seed development. As shown in Figure 11C, the KTi1/2 mRNAs

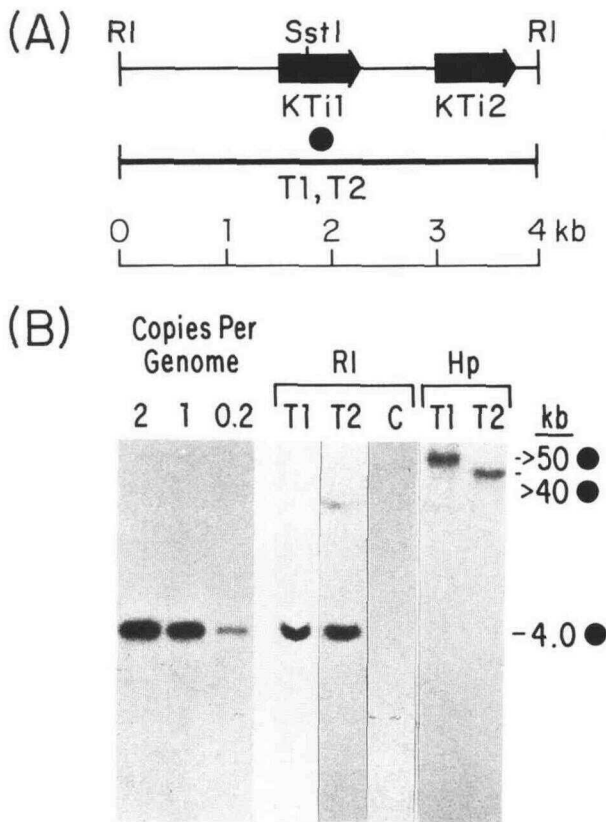


Figure 10. Representation of the KTi1/2 Kunitz Trypsin Inhibitor Gene Region in Transformed Tobacco Plants.

Five micrograms of leaf nuclear DNA from transformed tobacco plants containing the KTi1 and KTi2 trypsin inhibitor genes was digested with the indicated restriction endonucleases, fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with the 4.0-kb EcoRI DNA fragment from plasmid pE4 (Figure 7). (A) Map of relevant KTi1 and KTi2 gene restriction endonuclease sites and locations. RI and SstI refer to the EcoRI and SstI restriction endonucleases, respectively. Boxes and arrows represent gene locations and transcriptional orientations, respectively (Figure 1). The bracketed line below the restriction map indicates the 4-kb KTi1/2 DNA fragment that was transferred to tobacco and is present in KTi1/2 transformants designated T1 and T2 (Figure 9).

(B) Digestion of tobacco DNAs with EcoRI and HpaI. RI and Hp refer to tobacco DNA digested with EcoRI and HpaI restriction endonucleases, respectively. T1 and T2 refer to DNAs from transformed tobacco plants T1 and T2, respectively. C refers to untransformed tobacco DNA. Reconstruction lanes contained 0.2, 1, and 2 copy equivalents of EcoRI-digested λ Clone 6 phage DNA (Figure 1). DNA copy equivalents were calculated using a tobacco genome size of 2.4×10^6 kb (Okamoto and Goldberg, 1985). Sizes of restriction fragments in kilobases are shown to the right of the autoradiograms. Circles refer to tobacco DNA fragments that contain the KTi1/2 gene region shown in (A).

accumulated during the mid- to late-maturation periods of seed development (3 wk, 4 wk lanes). Similar accumulation patterns were obtained with seed mRNAs from several independent tobacco plants containing both the KTi1 and KTi2 genes, or the KTi2 gene alone (data not shown). At their peak prevalences (3 to 4 weeks), we estimated that the KTi1 and KTi2 mRNAs represented approximately 2×10^{-3} and 2×10^{-4} of the levels observed in mid-maturation stage soybean embryos, respectively (compare tobacco and soybean mRNA lanes, Figures 11B and 11C). Taken together, these data indicate that (1) the KTi1 and KTi2 genes are regulated temporally during tobacco seed development, (2) the KTi1 and KTi2 gene expression levels in tobacco seeds are lower than those observed in soybean embryos, and (3) that the KTi1 gene is expressed at a higher level than the KTi2 gene in tobacco seeds.

The KTi3 and KTi4 Kunitz Trypsin Inhibitor Genes Are Expressed during Tobacco Seed Development

We hybridized tobacco seed mRNA gel blots with pKT3 plasmid DNA and the 0.9-kb KTi4 gene probe shown in Figure 11D to determine whether the KTi3 and KTi4 Kunitz trypsin inhibitor genes were also expressed correctly during tobacco seed development. As seen in Figures 11E and 11F, both gene probes produced 0.9-kb signals with seed mRNAs from KTi3/4 transformed tobacco plants (KTi3/4 lanes). Both the KTi3 and KTi4 mRNAs accumulated during the mid- to late-maturation stages of tobacco seed development (Figures 11E and 11F, KTi3/4 3 wk, 4 wk lanes). Densitometric analysis indicated that KTi3 mRNA represented approximately 0.1% of the tobacco seed mRNA mass, and was higher in concentration than that estimated for the KTi1 and KTi2 mRNAs (Figures 11B and 11C). Together, these results demonstrate that each Kunitz trypsin inhibitor gene is regulated temporally during tobacco seed development, and that the quantitative differences in KTi1, KTi2, and KTi3 gene expression levels observed in soybean embryos (Figures 5 and 7) are maintained in developing tobacco seeds.

Kunitz Trypsin Inhibitor Protein Is Present in Transformed Tobacco Seeds

We isolated proteins from mature tobacco seeds and then reacted protein gel blots with antibodies raised against the major Kunitz trypsin inhibitor to determine whether the Kunitz trypsin inhibitor genes were expressed at the protein level. As shown in Figure 12 (T1, T2 lanes), the Kunitz trypsin inhibitor antibodies reacted with several proteins from seeds of transformants containing the KTi3 and KTi4 Kunitz trypsin inhibitor genes (Figure 11D). By contrast, the Kunitz trypsin inhibitor antibodies did not react detect-

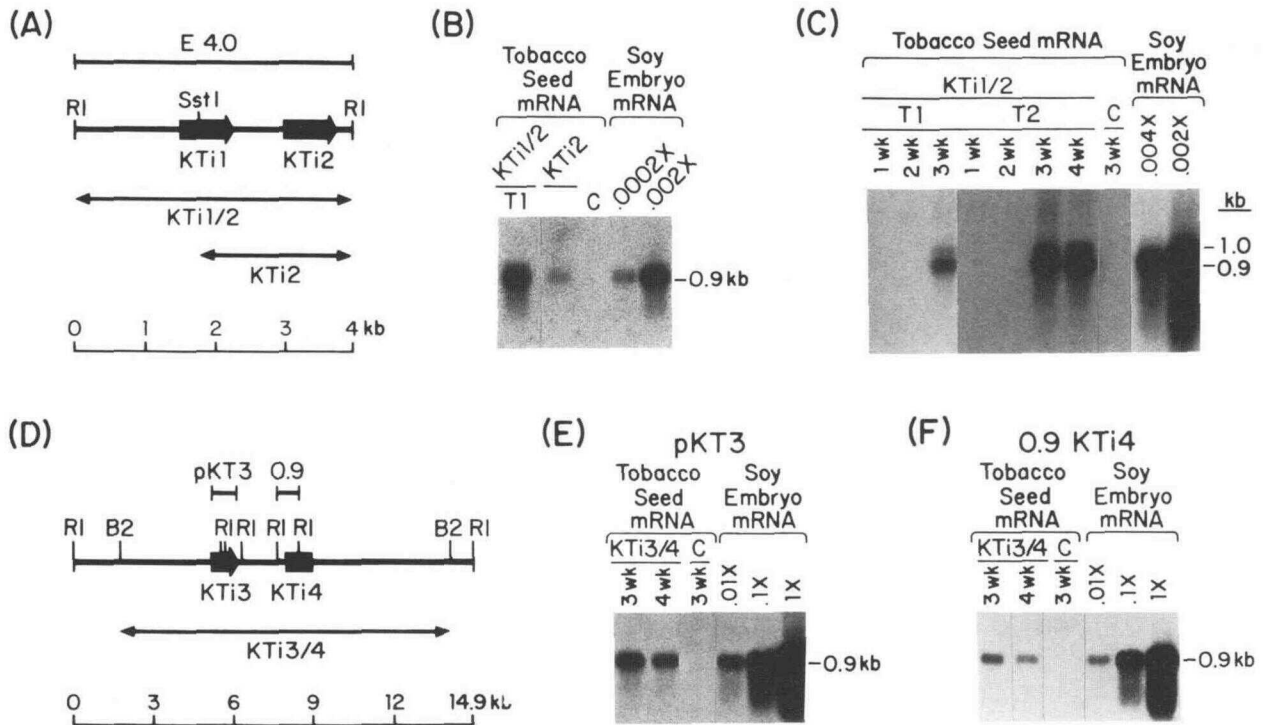


Figure 11. Kunitz Trypsin Inhibitor Gene Expression during Tobacco Seed Development.

(A) The KTi1 and KTi2 gene region. The 4.0-kb KTi1 and KTi2 EcoRI DNA fragment described in Figures 7 and 9 is shown schematically for reference. RI and SstI refer to EcoRI and SstI restriction endonucleases, respectively. Boxes and arrows represent gene locations and transcriptional polarities, respectively. The bracketed line E4.0 refers to the 4.0-kb EcoRI DNA fragment utilized as a hybridization probe in mRNA gel blot experiments shown in **(B)** and **(C)**. KTi1/2 and KTi2 indicate the Kunitz trypsin inhibitor DNA fragments transferred to tobacco plants (Figure 9).

(B) KTi1 and KTi2 gene expression in transformed tobacco seeds. Five micrograms of tobacco seed mRNA harvested 3 weeks after pollination was fractionated by electrophoresis, transferred to nitrocellulose, and hybridized with the E4.0 probe described in **(A)**. KTi1/2 refers to tobacco seed mRNA from transformed plant T1 that contains both KTi1 and KTi2 genes (Figure 9). KTi2 refers to seed mRNA from a pool of five independent tobacco transformants. Genetic analysis indicated that four of the five plants contained the KTi2 trypsin inhibitor gene region described in Figure 9 (data not shown). C refers to untransformed tobacco seed mRNA. The soy embryo .0002X and .002X lanes contained 1 ng and 10 ng of mid-maturation stage embryo mRNA or the equivalent of three and 30 molecules of KTi1/KTi2 Kunitz trypsin inhibitor mRNA, respectively (Figure 7).

(C) KTi1 and KTi2 gene expression in developing tobacco seeds. mRNA gel blots containing 5 μ g of tobacco seed mRNA from different developmental stages were hybridized with the labeled E4.0 DNA fragment. T1 and T2 refer to seed mRNAs from KTi1/2 transformed tobacco plants T1 and T2, respectively (Figure 10). C refers to untransformed tobacco seed mRNA. Wk refers to week after pollination. The soy embryo lanes .004X and .002X contained 10 ng and 20 ng of mid-maturation stage embryo mRNA, and represented Kunitz trypsin inhibitor mRNA prevalences of 0.01% and 0.02%, respectively.

(D) The KTi3 and KTi4 Kunitz trypsin inhibitor gene region. A schematic representation of the KTi3/4 gene region described in Figure 9 is shown for reference. RI and B2 refer to the EcoRI and BglII restriction endonucleases, respectively. Boxes and arrows represent approximate gene locations and transcriptional polarities, respectively. The bracketed lines pKT3 and 0.9 refer to the KTi3 and KTi4 gene probes used in mRNA gel blot experiments described in **(E)** and **(F)**, respectively. KTi3/4 indicates the 12.5-kb DNA fragment used for the gene transfer studies described in Figure 9.

(E) KTi3 gene expression in transformed tobacco seeds. mRNA gel blots containing 0.5 μ g of tobacco seed mRNA were hybridized with labeled pKT3 plasmid DNA. KTi3/4 and C refer to transformed and untransformed tobacco seed mRNAs, respectively. Wk refers to week after pollination. The soy embryo .01X, .1X, and 1X lanes contained 0.005 μ g, 0.05 μ g, and 0.5 μ g of mid-maturation stage embryo mRNA and correspond to KTi3 mRNA prevalences of 0.045%, 0.45%, and 4.5%, respectively (Figures 5 and 7).

(F) KTi4 gene expression in tobacco seeds. mRNA gel blots containing 0.25 μ g of tobacco seed mRNAs were reacted with the 0.9-kb KTi4 gene probe. KTi3/4 and C refer to transformed and untransformed tobacco seed mRNAs, respectively. The soy embryo .01X, .1X, and 1X lanes contained 0.0025 μ g, 0.025 μ g, and 0.25 μ g of mid-maturation stage mRNA, respectively.

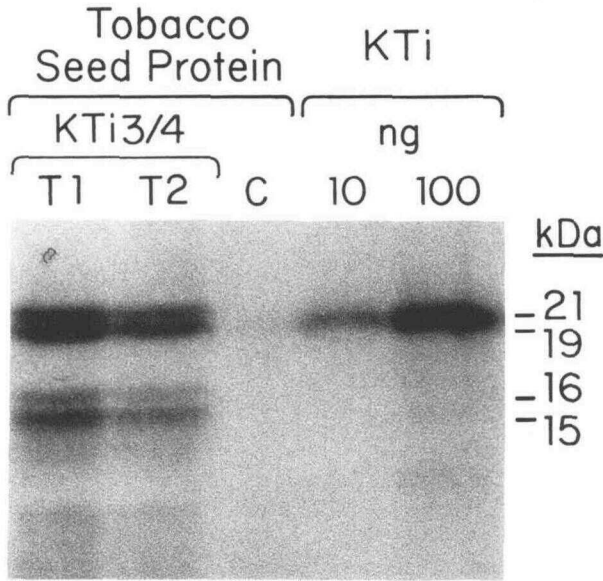


Figure 12. Presence of Soybean Kunitz Trypsin Inhibitor Protein in Transformed Tobacco Seeds.

Sixty micrograms of tobacco seed protein (12 seed equivalents) was fractionated by electrophoresis on polyacrylamide gels, transferred to nitrocellulose, and reacted with Kunitz trypsin inhibitor antibodies. Kunitz trypsin inhibitor protein was detected by using a horseradish peroxidase immunoassay. T1 and T2 refer to seed proteins from KTi3/4 transformed tobacco plants T1 and T2, respectively. C refers to untransformed tobacco seed protein. The KTI lanes contained 10 ng and 100 ng of purified soybean Kunitz trypsin inhibitor protein.

ably with seed proteins from tobacco plants transformed with the KTi1 and KTi2 genes (data not shown), or with proteins from untransformed tobacco seeds (Figure 12, C lane). The 19-kD and 21-kD seed protein bands from KTi3/4 transformants were comparable in size with those obtained with purified Kunitz trypsin inhibitor protein (KTI lanes) and with total soybean seed protein (data not shown). We presume that the 15-kD and 16-kD proteins detected with transformed seeds represent degradation products (Figure 12, T1, T2 lanes). Relative to known Kunitz trypsin inhibitor standards, we estimated that the Kunitz trypsin inhibitor represented 0.2% of the tobacco seed protein mass, or approximately 1/20th of the level found in soybean. Together, these data demonstrate that at least one Kunitz trypsin inhibitor gene (KTI3) is expressed at the protein level in transformed tobacco seeds.

Kunitz Trypsin Inhibitor Genes Are Expressed in Tobacco Organ Systems

We hybridized tobacco leaf, stem, and root mRNA gel blots with Kunitz trypsin inhibitor gene probes (Figures

11A and 11D) to establish whether the Kunitz trypsin inhibitor genes were expressed in organ systems of the mature tobacco plant. Figures 13A and 13B show that the 4.0-kb KTi1/2 gene probe hybridized with 0.9-kb leaf and stem mRNAs from tobacco plants containing both the KTi1 and KTi2 genes (TOB-T; L, S lanes, Figure 13A), or the KTi2 gene alone (TOB-T; L, S lanes, Figure 13B). By contrast, no detectable signals were obtained with either KTi1/2 or KTi2 transformed root mRNAs (TOB-T; R lanes, Figures 13A and 13B), or with untransformed leaf, stem, and root mRNAs (TOB-C lanes, Figure 13A). Figures 13C and 13D show that the KTi3 and KTi4 gene probes produced 0.9-kb signals with tobacco leaf mRNA from plants containing the KTi3 and KTi4 Kunitz trypsin inhibitor genes (TOB-T; L lanes), and did not react detectably with either stem or root mRNAs from the same plants (TOB-T; S and R lanes). Together, these results demonstrate that the Kunitz trypsin inhibitor genes are expressed in tobacco mature plant organ systems, and that the organ system expression programs for individual Kunitz trypsin inhibitor genes differ as in soybean (Figure 8).

DISCUSSION

Many Kunitz Trypsin Inhibitor Genes Are Present in the Soybean Genome

Kunitz trypsin inhibitor genes represent one of several seed protein gene families that are highly regulated during the plant life cycle (Goldberg et al., 1989; Shotwell and Larkins, 1989). Both DNA gel blot studies and characterization of genomic clones indicate that there are at least 10 Kunitz trypsin inhibitor genes in the soybean genome (Figure 1). At least four of these genes (KTI1, KTI2, KTI3, and KTI4) are expressed at the mRNA level in both soybean and transformed tobacco plants (Figures 5, 7, and 11). cDNA clones have been identified that are specific for two additional Kunitz trypsin inhibitor genes that are present on λ Clone 4 and λ Clone 30 phages (Figure 1B; G. de Paiva, K.D. Jofuku, and R.B. Goldberg, unpublished results). Thus, we have evidence that at least six of the 10 Kunitz trypsin inhibitor genes are active in soybean embryos.

Genetic studies indicated that a single gene is responsible for encoding a seed protein with Kunitz trypsin inhibitor activity (Orf and Hymowitz, 1979). Three allelic forms of this gene (Ti^a , Ti^b , Ti^c) encode proteins that differ by only a few amino acids (Orf and Hymowitz, 1979; Kim et al., 1985). Soybean lines have been identified that have reduced amounts of Kunitz trypsin inhibitor protein and lack detectable Kunitz trypsin inhibitor activity (Orf and Hymowitz, 1979). The null phenotype is due to a mutation in the Kunitz trypsin inhibitor structural gene (e.g., Ti^a), and

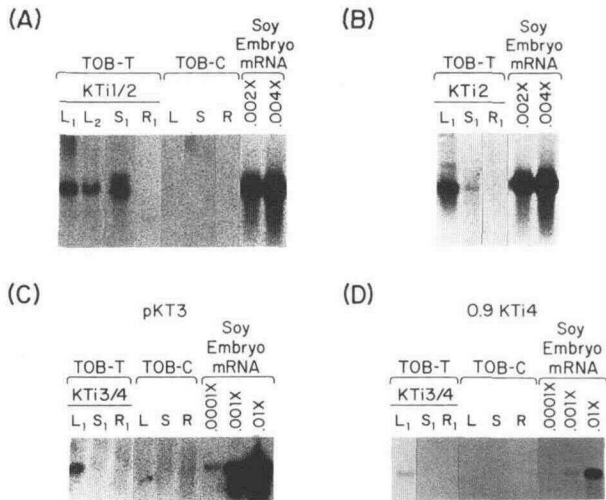


Figure 13. Kunitz Trypsin Inhibitor Gene Expression in Mature Tobacco Plant Organ Systems.

mRNA gel blots containing 5 μ g of tobacco leaf (L), stem (S), and root (R) mRNAs were hybridized with the Kunitz trypsin inhibitor KTi1/2 E4.0, pKT3, and 0.9-kb KTi4 gene probes shown in Figure 11. TOB-T and TOB-C refer to transformed and untransformed tobacco mRNAs, respectively.

(A) KTi1/2 gene expression in transformed tobacco leaf, stem, and root. KTi1/2 L₁, S₁, and R₁ refer to mRNAs from KTi1/2 transformed tobacco plant T1, and L₂ refers to leaf mRNA from KTi1/2 transformant T2 (Figures 10 and 11). The .002X and .004X reconstruction lanes contained 10 ng and 20 ng of soybean mid-maturation stage embryo mRNA and correspond to Kunitz trypsin inhibitor mRNA prevalences of 0.006% and 0.01%, respectively (Figures 5 and 7).

(B) KTi2 gene expression in transformed tobacco leaf, stem, and root. KTi2 L₁, S₁, and R₁ refer to mRNAs from KTi2 tobacco transformant T1 (Figure 11). The .002X and .004X lanes contained soybean mid-maturation stage embryo mRNA and correspond to Kunitz trypsin inhibitor mRNA prevalences of 0.006% and 0.01%, respectively.

(C) KTi3 gene expression in mature tobacco plant organ systems. KTi3/4 L₁, S₁, and R₁ lanes contain leaf, stem, and root mRNAs from KTi3/4 transformed tobacco plant T1. The .0001X, .001X, and .01X lanes contained soybean mid-maturation stage embryo mRNA and correspond to approximately 2, 20, and 200 KTi3 mRNA molecules per cell (Figures 5 and 7).

(D) KTi4 gene expression in tobacco plant organ systems. KTi3/4 L₁, S₁, and R₁ refer to mRNAs from KTi3/4 transformed plant T1. The .0001X, .001X, and .01X reconstruction lanes contained 0.0005 μ g, 0.005 μ g, and 0.05 μ g of soybean mid-maturation stage (77 DAF) embryo mRNA, respectively.

is inherited as a recessive allele (*ti*; Orf and Hymowitz, 1979). These results suggest that most of the Kunitz trypsin inhibitor genes identified in our experiments (Figure 1) do not encode proteins with trypsin inhibitor activity.

DNA sequence analysis of the KTi3 Kunitz trypsin inhibitor gene (Figure 4; Jofuku et al., 1989) indicates that it encodes a protein identical to that of the Kunitz trypsin inhibitor *Ti*^a allelic form (Kim et al., 1985). A null line that lacks Kunitz trypsin inhibitor activity has three mutations in the KTi3 gene (Jofuku et al., 1989). These mutations cause a frameshift that results in premature termination of KTi3 mRNA translation, and lead to a 100-fold reduction of KTi3 mRNA in soybean embryos (Jofuku et al., 1989). The KTi1 and KTi2 mRNAs are unaffected by the KTi3 gene mutation (Jofuku et al., 1989), and are probably responsible in part for the residual Kunitz trypsin inhibitor cross-reacting material found in mutant seeds (K.D. Jofuku and R.B. Goldberg, unpublished results).

The KTi1 and KTi2 gene sequences (Figure 2) indicate that they encode proteins with only 70% similarity to the Kunitz trypsin inhibitor *Ti*^a form (Figure 4). Inspection of the KTi1 and KTi2 protein amino acid sequences (Figure 4) shows that they lack the Arg-63 and Ile-64 amino acids responsible for trypsin inhibitor activity (Laskowski and Kato, 1980; Ryan, 1981). Nor do they contain other amino acids at these sites that would cause the KTi1 and KTi2 proteins to have chymotrypsin or elastase inhibitor activities (Ryan, 1981). These findings correlate well with the genetic analysis of Kunitz trypsin inhibitor null lines (Orf and Hymowitz, 1979), and strongly suggest that the KTi3 Kunitz trypsin inhibitor gene is responsible for encoding most of the Kunitz trypsin inhibitor activity and protein found in soybean seeds.

Several Kunitz Trypsin Inhibitor Genes Are Tandemly Linked

We do not know yet how all 10 Kunitz trypsin inhibitor genes are organized with respect to each other in soybean chromosomes because we have not attempted to link the genomic clones by either DNA walking studies (Harada, Barker, and Goldberg, 1989; Nielsen et al., 1989) or by genetic studies with restriction fragment length polymorphisms (Cho, Davies, and Nielsen, 1989). Analysis of individual Kunitz trypsin inhibitor genomic clones (Figure 1B), however, demonstrated that several Kunitz trypsin inhibitor genes are linked in tandem pairs. The most striking example of this form of organization is the KTi1 and KTi2 gene pair (Figures 1B and 1C). These genes are approximately 0.5 kb apart, are in the same transcriptional orientation, and have >95% nucleotide sequence similarity in their 5', coding, and 3' regions (Figures 2 and 3). The KTi1 and KTi2 Kunitz trypsin inhibitor genes also show extensive similarity to the KTi3 5', coding, and 3' regions (Figure 3). These results suggest that the KTi1, KTi2, and KTi3 genes probably originated from the duplication of an ancient relative, and that the KTi1 and KTi2 gene pair probably reflects a recent duplication and/or gene conversion event.

Kunitz Trypsin Inhibitor Genes Have Different Expression Programs

A significant aspect of our results is the observation that individual Kunitz trypsin inhibitor genes have different qualitative and/or quantitative expression programs. All Kunitz trypsin inhibitor genes investigated encode 0.9-kb mRNAs that are temporally regulated in soybean embryos and in seeds of transformed tobacco plants (Figures 5, 7, and 11). In both soybean and tobacco, however, individual Kunitz trypsin inhibitor mRNAs accumulate to different levels during seed development (Figures 5, 7, and 11). The KTi3 mRNA is the most prevalent (Figures 5 and 11), and is followed in prevalence by the KTi1 and KTi2 mRNAs (Figures 7 and 11). Transcription studies demonstrated that the difference in KTi3 and KTi1/2 mRNA levels is due primarily to a difference in the transcription rates of their corresponding genes (Figure 6).

Kunitz trypsin inhibitor genes are expressed at low levels in organ systems of soybean (Figure 8) and transformed tobacco plants (Figure 13). The reduced organ system expression levels are due in part to lower Kunitz trypsin inhibitor gene transcription rates in the leaf, root, and stem (Walling et al., 1986). Expression of the Kunitz trypsin inhibitor gene family in mature plant organ systems is similar to that observed for the lectin gene (Okamuro, Jofuku, and Goldberg, 1986; Goldberg et al., 1989), but differs from the glycinin and β -conglycinin storage protein genes that are expressed only during embryogenesis (Harada et al., 1989; Nielsen et al., 1989).

KTi1 and KTi2 mRNAs are present in the soybean leaf, stem, and root (Figure 8). In transformed tobacco plants these mRNAs are detected in both the leaf and stem but not the root (Figure 13). The absence of KTi1 and KTi2 mRNAs in transformed tobacco roots may indicate that the KTi1 and KTi2 genes are expressed at levels undetectable by our mRNA gel blot procedure, that position effects altered the KTi1 and KTi2 expression programs, or that the hybridization signal obtained with soybean root mRNA was due to expression of a related Kunitz trypsin inhibitor family member. By contrast with these findings, KTi3 and KTi4 mRNAs are detectable only in the leaf of both soybean (Figure 8) and transformed tobacco plants (Figure 13). We conclude from these observations that individual Kunitz trypsin inhibitor genes can have different qualitative expression programs in mature plant organ systems.

In situ localization studies shown in the accompanying paper (Perez-Grau and Goldberg, 1989) indicate that other differences occur in Kunitz trypsin inhibitor gene expression programs. These studies showed that KTi3 mRNA is more prevalent than the KTi1/2 mRNAs in the soybean embryo axis. In addition, KTi3 mRNA accumulates in specific globular stage embryo cells long before the KTi1 and KTi2 mRNAs are visualized. Finally, KTi3 and KTi1/2

mRNAs are distributed differently within embryo cotyledon cells, and have distinct spatial accumulation programs.

Control of Kunitz Trypsin Inhibitor Gene Expression—A Simple Hypothesis

What are the molecular events responsible for specifying the individual Kunitz trypsin inhibitor gene expression programs? The simplest hypothesis is that all Kunitz trypsin inhibitor genes share elements that program their expression during embryogenesis (Goldberg, 1986). Quantitative differences in expression levels (e.g., KTi3 versus KTi1 and KTi2 genes) could be due to the number of embryo *cis*-control elements and/or the presence of additional elements that enhance gene transcription rates in embryo cells. By contrast, expression in mature plant organ systems may be due to the presence of other control elements that are specific for cells within the leaf, root, or stem (Goldberg, 1986). Different combinations of these elements would cause Kunitz trypsin inhibitor genes to have different expression programs in mature plant organ systems.

At the present time we have no evidence for or against this hypothesis. Recent experiments with the cauliflower mosaic virus 35S gene indicate, however, that a hierarchy of discrete control elements is responsible for programming 35S gene expression to specific tobacco cell and tissue types (Benfey, Ren, and Chua, 1989). The Kunitz trypsin inhibitor gene family provides a unique opportunity to dissect control elements responsible for programming gene expression at all stages of the plant life cycle. What these elements are, and how they interact with regulatory gene products, remain to be determined.

METHODS

Isolation of Kunitz Trypsin Inhibitor Phages and Plasmids

The isolation and characteristics of the A-37 Kunitz trypsin inhibitor cDNA plasmid were described previously (Goldberg et al., 1981; Jofuku et al., 1989). The pKT3 cDNA plasmid representing the major Kunitz trypsin inhibitor gene KTi3 was isolated from a soybean mid-maturation stage embryo cDNA library (J.J. Harada and R.B. Goldberg, unpublished results) as described previously (Jofuku et al., 1989). Recombinant λ Charon 4 phages containing Kunitz trypsin inhibitor genes were isolated from a soybean Forrest variety EcoRI genome library (Fischer and Goldberg, 1982), and an AluI/HaeIII linker library of soybean Dare variety DNA. The linker library was constructed according to the procedure of Maniatis et al. (1978).

DNA Isolation and Labeling

Plant, phage, and plasmid DNAs were isolated as described previously (Jofuku and Goldberg, 1988). Synthetic oligonucleo-

tides were synthesized and purified according to established procedures (Matteucci and Caruthers, 1981; Vorndam and Kerschner, 1986). DNAs were labeled by nick translation under conditions specified by Bethesda Research Laboratories. Oligonucleotides were 5' end-labeled as described by Maniatis et al. (1978).

Polysomal mRNA Isolation

Soybean and tobacco polysomal poly(A) mRNAs were isolated as described (Cox and Goldberg, 1988).

Seed Protein Isolation

Tobacco seed proteins were extracted according to Sano and Kawashima (1983).

Filter Hybridization Studies

DNA and mRNA gel blot experiments were carried out according to the procedures of Wahl, Stern, and Stark (1979) and Thomas (1983), respectively. mRNA gel blot studies with oligonucleotide probes were performed as described by Woods et al. (1982) and by Whitehead et al. (1983). Dot blot experiments were carried out as described by Thomas (1983). Protein gel blot studies were performed as described by Johnson et al. (1984).

R-Loop Analysis

R-loops were formed between Kunitz trypsin inhibitor phage DNAs and soybean mid-maturation stage embryo mRNAs as described previously (Fischer and Goldberg, 1982).

Primer Extension Analysis

A greater than 50-fold mass excess of a 22-nucleotide synthetic oligonucleotide primer (P_1 ; Figure 2) was hybridized with mid-maturation stage embryo mRNA or with denatured pE4 plasmid DNA (Figure 7) at 42°C in 10 mM Tris (pH 7.6), 300 mM NaCl, and 0.5 mM EDTA. The primer was then extended by reverse transcriptase using only ^{32}P -TTP (>600 Ci/mmol) as described by Murray, Hoffman, and Jarvis (1983). Labeled primer extension products were subjected to electrophoresis on a 20% polyacrylamide gel under denaturing conditions, and then visualized by autoradiography.

^{32}P -Nuclear RNA Synthesis

Synthesis of labeled RNA from isolated soybean embryo nuclei was carried out as described by Walling et al. (1986) and Cox and Goldberg (1988).

Tobacco Transformation and Plant Regeneration

Tobacco leaf discs and protoplasts were transformed with the KTi1/2, KTi2, and KTi3/4 gene regions (Figure 9) according to the

procedures of Horsch et al. (1985), Okamoto et al. (1986), and Barker, Harada, and Goldberg (1988).

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