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Developmentally regulated expression of the bean β-phaseolin gene in tobacco seed

(foreign gene expression/Mendelian segregation/electrophoretic immunoblot analysis)

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ABSTRACT Recombinant phage λ177.4 contains a gene for β phaseolin, a major storage glycoprotein of French bean seed. A 3.8-kilobase *Bgl* II–*Bam*HI fragment containing the entire 1700-base-pair coding region, together with 863 base pairs of 5′ and 1226 base pairs of 3′ flanking sequence, was inserted into the A66 Ti plasmid of *Agrobacterium tumefaciens* and used to transform tobacco. The level of phaseolin in the seeds of plants regenerated from cloned tissue was 1000-fold higher than in other tissues. The molecular weight of the phaseolin RNA transcript in tobacco seeds was identical to that found in bean seeds. The phaseolin protein in tobacco seed was glycosylated and appeared to undergo removal of the signal peptide. However, a large proportion of the phaseolin was cleaved into discrete peptides. These same peptides were formed as phaseolin was degraded during tobacco seed germination. The phaseolin gene appeared to be inserted as a single copy, and the proportion of phaseolin per genome copy in tobacco seeds (up to 3% of the total embryo proteins) resembled that in the bean seeds (40% of total seed protein, expressed from about 14 copies per diploid genome). Furthermore, the transplanted gene was turned on during tobacco seed development, and its protein product, phaseolin, was localized in the embryonic tissues. Finally, the phaseolin gene was inherited as a Mendelian dominant trait in tobacco.

The transfer of foreign genetic information to broad-leafed plants by means of Ti plasmid vectors is now well established (1). Intronless genes and prokaryotic genes for antibiotic resistance placed under the control of transferred DNA promoters appear to be functional and maintained through meiosis (2–7). The gene encoding the bean (*Phaseolus vulgaris* cv. Tendergreen) seed protein β phaseolin, which contains five introns, gives rise to immunologically detectable levels of protein in sunflower callus when placed under its normal flanking promoter regions or when placed under the octopine synthase promoter (8). We report here that the phaseolin gene is expressed at a much higher level in the seeds of transformed tobacco plants, accumulating to levels of >1% of the total protein. Furthermore, the phaseolin gene product in tobacco behaves in a manner similar to that in bean in both structural and biological properties. These observations indicate the conservation of DNA regions specifying tissue-specific expression across evolutionarily diverse botanical families.

MATERIALS AND METHODS

Transformation of Tobacco Stem Tissue and Regeneration of Recombinant Plants. Inverted stem segments (4) of *Nicotiana tabacum* var. Xanthi were inoculated with *Agrobacterium tumefaciens* strain A66 containing the 3.8-kilobase (kb) genomic fragment of the β-phaseolin gene (8). The resulting shoot-callus pieces were freed of *Agrobacteria* (4) and maintained on Murashige and Skoog medium (MS) without hormone supplement. The tissue was cloned by the feeder plate method (9) and then placed in liquid culture to induce shoot-stem elongation (10). The shoots were grafted (11) onto 6- to 8-week-old *N. tabacum* var. Xanthi plants and grown at 22°C with a 16-hr photoperiod. Flowers were self-pollinated, and the seeds were allowed to mature.

Quantitative and Qualitative Protein Assays. Proteins were extracted and quantified as described (8). Phaseolin was quantitated in tissues by dot-immunobinding assay (12). Protein patterns were analyzed after fractionation on a 13% polyacrylamide gel (13) or by two-dimensional gel electrophoresis (14) followed by electrophoretic immunoblot analysis with polyclonal antiserum to phaseolin (15). Antigen–antibody complexes were visualized by treating the filters with ^{125}I-labeled *Staphylococcus* protein A, followed by autoradiography.

For immunodetection of concanavalin A-bound proteins, tissue extracts were incubated with concanavalin A-Sepharose beads followed by elution of the bound fraction with 1% $NaDodSO_4$. The bound and unbound fractions were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis followed by immunoblot analysis as described earlier.

Isolation of RNA and Blot-Hybridization Analysis. Total RNA was isolated from leaves as described (16). RNA from developing seeds was prepared by isolation of polysomes followed by phenol extraction of the polysome pellet (17). RNA was fractionated on agarose-formaldehyde gels, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled fragment of either phaseolin or the octopine synthase gene (8).

Isolation of DNA and Quantitation of the Gene Copy Number. DNA was isolated from leaves of recombinant plants of the R_2 generation (18). The DNA was digested with appropriate restriction enzymes, fractionated on 1% agarose gels, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled fragment of a phaseolin cDNA clone (19). The gene copy number was determined by comparing band intensities with copy number reconstructions on the same gel (20).

RESULTS

Quantitation of Phaseolin in Various Transformed Tissues. Transformed calli, seedlings, and seeds were assayed for phaseolin by dot-immunobinding assay (Table 1). The

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Abbreviations: kb, kilobase(s); bp, base pair(s).

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Table 1. Quantitation of phaseolin in transformed tissues

Tissue	Independently transformed samples	Phaseolin per μg of total protein	
	No.	pg	$\%_{avg}$
Callus	10	0.01–10	0.002
Seedlings	10	0.05–12	0.002
Seed lots (15 seeds)	6	5,000–20,000	1

Measured amounts of total protein from tissues were subjected to quantitative dot-immunobinding assay (see the legend of Fig. 8). The amount of phaseolin in each sample was calculated by subtracting the radioactive values obtained for the control tissue from the values obtained for the corresponding transformed tissue.

amount of phaseolin in calli and seedlings was ≈0.002% of the total protein, whereas the amount in transformed seeds was up to 1000-fold higher, consistent with phaseolin being expressed at the high level expected of a developmentally regulated gene.

Qualitative Analysis of Phaseolin Protein in Tobacco Seeds. Phaseolin genes in bean are expressed as a group of closely related polypeptides that are heterogeneous in molecular mass and isoelectric charge. On one-dimensional gels, the phaseolin polypeptides from bean seeds separated into three major bands (Fig. 1*A*, lane 1). The lower two bands (48 and 45.5 kDa) are glycosylated (48 kDa) and nonglycosylated (45.5 kDa) forms of the β-phaseolin gene product (19). Fractionation of proteins from transformed tobacco seeds by $NaDodSO_4$/polyacrylamide gel electrophoresis followed by immunoblot analysis with phaseolin antibody showed that ≈30% of the immunoreactive protein comigrated with an authentic β-phaseolin gene product from bean (48 kDa) and the remainder migrated with apparent molecular masses of 29, 27, 26, and 25 kDa (Fig. 1*A*, lane 3). Lane 2 of Fig. 1*A* contained protein from normal tobacco seeds, showing that there are no tobacco proteins that crossreact with phaseolin antibodies. Analysis of the tobacco storage proteins in normal and transformed seeds showed no quantitative or qualitative differences (data not shown).

Two-dimensional electrophoretic separation of proteins from the transformed tobacco seeds further confirmed the presence of full-length authentic β-phaseolin (Fig. 1*B*, lane 3). More than one spot was present, probably resulting from deamidation or differences in the extent of glycosylation; however, the complexity was considerably less than that of native β phaseolin (Fig. 1*B*, lane 1), which is probably encoded by three or four genes (20). The smaller peptides seen in the one-dimensional gels (Fig. 1*A*, lane 1) separated as four distinct spots on two-dimensional gels (Fig. 1*B*, lane 3).

The 48-kDa protein from both bean seeds and transformed tobacco seeds bound to concanavalin A, whereas the 45.5-kDa protein did not (Fig. 2). This suggests that the 48-kDa protein is glycosylated in both bean and tobacco seeds and that the 45.5-kDa protein is the nonglycosylated form of β phaseolin. Of the four smaller products, only the 29-kDa protein was glycosylated, suggesting that it is derived from the COOH-terminal region of phaseolin, which contains two base sequences characteristic of N-glycosylation sites (19).

Since the concanavalin A data suggests that the 29-kDa immunoreactive peptide is derived from the COOH-terminal region of the protein, the small molecular mass proteins cannot result from premature termination of translation. Coextraction of purified phaseolin with normal tobacco seeds did not result in the appearance of 29- to 25-kDa proteins, thus ruling out the possibility that the latter resulted from degradation of phaseolin during the process of protein isolation. Furthermore, the discrete size of these small molecular mass immunoreactive peptides supports the notion that specific cleavage events take place in the tobacco seeds.

Localization of Phaseolin Within Tobacco Seeds. A mature bean seed consists almost entirely of embryonic tissues, whereas a tobacco seed is comprised of 30% embryonic tissues and 70% nonembryonic tissues. Although the major tobacco storage proteins were synthesized and accumulated in both the embryonic and nonembryonic tissues of the tobacco seed (data not shown), native phaseolin accumulated mainly in the embryonic tissue (Fig. 3). The nonembryonic tissue contained mainly the 29- to 25-kDa peptides. Tobacco seeds have protein bodies both in the endosperm and in the embryo; however, phaseolin was found only in the protein bodies of the embryo (M. Chrispeels, personal communication). This, along with the results obtained from preliminary pulse–chase experiments, would suggest that phaseolin is synthesized only in the embryo and that the small amount of native phaseolin in the endosperm is a result of contamination during dissection of the seed. If phaseolin is synthesized only in the embryonic tissue, then our estimate of phaseolin in the seed relative to the total seed protein (1%) should be corrected by 3-fold because embryo protein represents only one-third of the total tobacco seed protein (data not shown). Therefore, the β-phaseolin gene in tobacco accounts for at least 3% of the total embryonic protein.

Analysis of Phaseolin and Octopine Synthase RNA Transcripts. The high levels of phaseolin in seed can be attributed either to a high rate of protein synthesis reflected by high levels of phaseolin mRNA or to greatly reduced rates of protein degradation in seed. To address these possibilities, RNA was isolated from transformed tissues, fractionated on a formaldehyde-agarose gel, blotted onto nitrocellulose, and hybridized with a ^{32}P-labeled fragment of octopine synthase (8) or phaseolin cDNA (19). The size of the transcript [1700 base pairs (bp)] in the transformed tobacco seeds when probed with phaseolin cDNA was identical to phaseolin

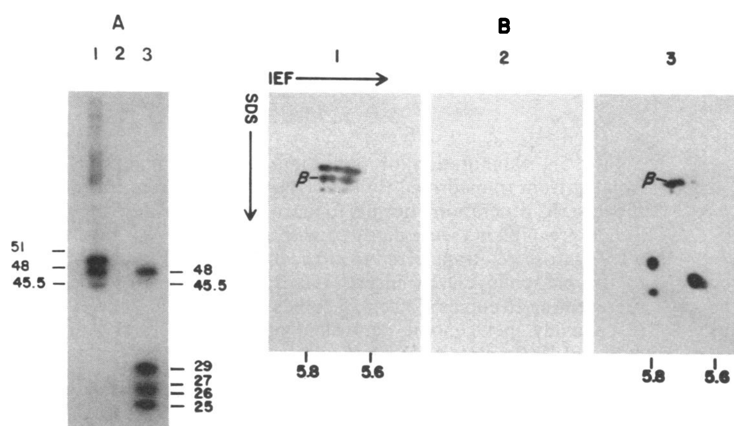


FIG. 1. Immunological detection of phaseolin polypeptides synthesized in transformed tobacco seeds. (*A*) Proteins were fractionated by $NaDodSO_4$/polyacrylamide gel electrophoresis, and the phaseolin peptides were detected by immunobinding assay. Lanes: 1, 100 ng of purified phaseolin; 2, aliquot of total protein extracted from 15 untransformed seeds; 3, aliquot of total protein extracted from 15 transformed seeds. Molecular sizes (shown in kDa) were calculated from the mobilities of bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and soybean trypsin inhibitor (21 kDa) included in the same gel. (*B*) A second aliquot of the same protein samples used in *A* was subjected to two-dimensional gel electrophoresis, and the phaseolin peptides were detected by the immunobinding assay. IEF, isoelectric focusing; SDS, $NaDodSO_4$.

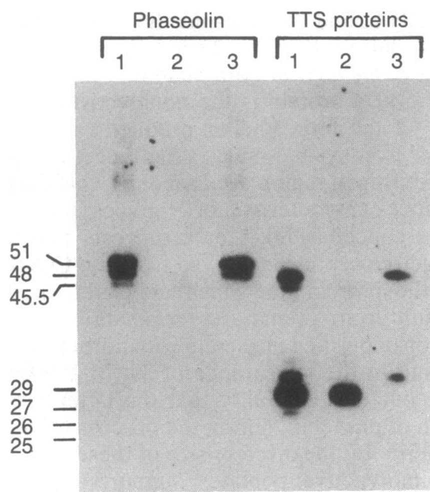


FIG. 2. Immunological detection of concanavalin A-bound and unbound fractions of purified phaseolin and proteins from transformed tobacco seeds. Purified phaseolin and proteins extracted from transformed tobacco seeds (TTS) were incubated with concanavalin A-Sepharose, and the bound and the unbound fractions were analyzed for peptides immunoreactive with phaseolin antibody as described. Lanes: 1, protein extracts that were not incubated with concanavalin A-Sepharose; 2, proteins that did not bind to the concanavalin A-Sepharose; 3, proteins that bound to concanavalin A-Sepharose. Molecular sizes are shown in kDa.

mRNA in bean seeds, suggesting correct transcriptional and posttranscriptional processing. The level of octopine synthase (a transferred DNA marker gene) mRNA was the same in both the transformed leaves and seeds (Fig. 4A), whereas the level of phaseolin mRNA was several orders of magnitude higher in the seeds (Fig. 4B). From reconstruction blots, the phaseolin transcript in seed was ≈0.01% of the total RNA, whereas the octopine synthase transcript was <0.0025%. Assuming poly(A)$^+$ RNA is 1% of the total RNA in tobacco seeds, then the phaseolin message was ≈1% of the total poly(A)$^+$ RNA in tobacco seeds. This is possibly an underestimated value for the level of phaseolin RNA, since RNA was isolated from seeds obtained from young capsules (16–20 days after anthesis), and phaseolin synthesis in tobacco commenced around day 16 after anthesis and peaked only at

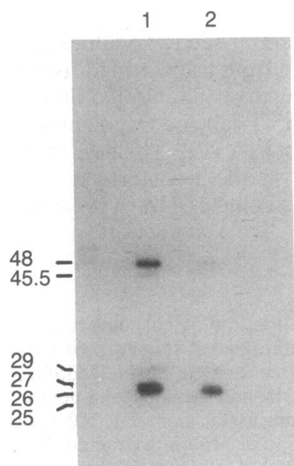


FIG. 3. Localization of phaseolin within the transformed tobacco seed. Mature tobacco seeds were dissected to separate out the embryo (with cotyledons) and the nonembryonic tissue. Proteins were extracted from the seed parts with phosphate-buffered saline. Then each protein fraction (10 μg) was analyzed for phaseolin as described. Lanes: 1, proteins from embryonic tissue; 2, proteins from nonembryonic tissue. Molecular sizes are shown in kDa.

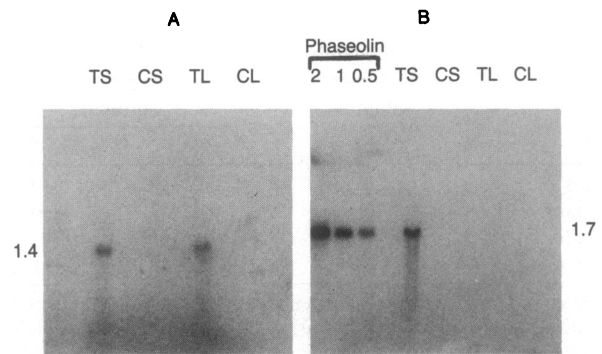


FIG. 4. Detection of phaseolin and octopine synthase transcripts in transformed tissue. Replicate blots were prepared with the following RNA samples and hybridized with the octopine synthase fragment (*A*) or the phaseolin cDNA fragment (*B*) (8): phaseolin, (lanes labeled 2, 1, and 0.5 in *B*) total bean cotyledon RNA containing 2-ng, 1-ng, and 0.5-ng equivalents of phaseolin RNA; TS, 10 μg of total RNA from developing seeds of transformed plants; CS, 10 μg of total RNA from developing seeds of normal plants; TL, 10 μg of total RNA from leaves of transformed plants; and CL, 10 μg of total RNA from leaves of normal plants. Molecular sizes (shown in kb) were calculated from mobilities of alfalfa mosaic virus RNAs included in the same gel. *A* was exposed for 5 days with a screen, while *B* was exposed for 14 hr with a screen.

day 28 after anthesis (see Fig. 6). The much higher level of phaseolin mRNA in seeds compared to leaves strongly suggests that transcription of the phaseolin gene in tobacco is developmentally regulated.

Quantitation of Gene Copy Number. Phaseolin RNA comprises ≈40% of the total poly(A)$^+$ RNA in the mature bean cotyledons, and there are about seven copies of the phaseolin gene per haploid genome in the bean cultivar Tendergreen (20). Therefore, on average, each gene copy gives rise to ≈3% of the total poly(A)$^+$ RNA in the bean seed, assuming that all copies of the phaseolin gene are equally expressed. Based on genomic blots (Fig. 5), we estimate that there is only one copy of the phaseolin gene per diploid genome in the tissues derived from transformed seeds. Thus, the single transferred β-phaseolin gene gives rise to comparable levels of phaseolin mRNA in transformed tobacco seeds. Comparisons on an absolute basis are difficult to make because of the structural diversity between the two seeds.

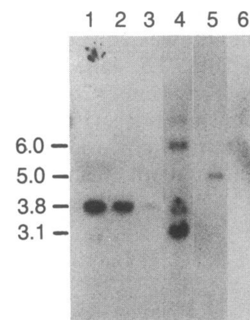


FIG. 5. Quantitation of phaseolin DNA sequences in DNA isolated from transformed tissues. DNA isolated from transformed tobacco (R_2 generation), normal tobacco, and *Phaseolus vulgaris* cv. Tendergreen plants were digested with *Bam*HI/*Eco*RI, resolved on a 1% agarose gel, transferred to nitrocellulose paper, and hybridized to the phaseolin cDNA insert. Lanes: 1–3, reconstruction lanes representing 10 copies (230 pg), 5 copies (115 pg), and 1 copy (23 pg), respectively, per genome equivalent of AG-PvPh3.8 (19) digested with *Bgl* II/*Bam*HI; 4, DNA (6 μg) from leaves of *P. vulgaris*; 5, DNA (10 μg) from leaves of transformed tobacco plants; 6, DNA (10 μg) from leaves of control tobacco plants. Molecular size is shown in kb.

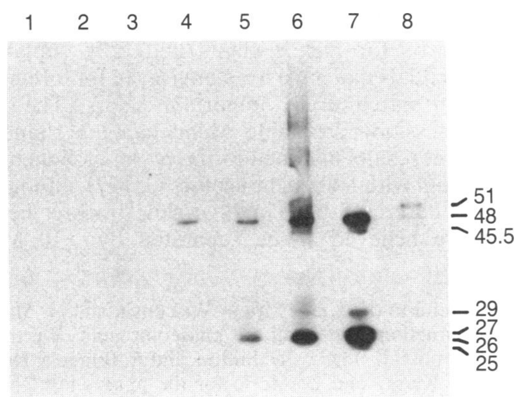


FIG. 6. Onset of phaseolin synthesis in developing transformed tobacco seeds. Seeds were collected at different times after fertilization, and their proteins were extracted. Samples were taken at 12, 14, 16, 18, 22, 25, and 28 days after pollination, and 50 μg of their protein were subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis followed by immunoblot analysis (lanes 1–7). Purified phaseolin (100 ng) is shown in lane 8, and molecular sizes are shown in kDa.

Accumulation and Utilization of Phaseolin Protein in Tobacco Seeds. During normal bean seed development, phaseolin first appears 16 days after anthesis and reaches a maximum level by day 31 (21). Similarly, phaseolin in tobacco seeds first appeared on day 16 after anthesis and reached a maximum level by seed maturity (Fig. 6), unlike the native tobacco storage proteins that first appeared by day 9 and accumulated to high levels by day 15 (data not shown; ref. 22). The proportion of the 29- to 25-kDa proteins relative to the authentic β phaseolin increased with seed maturity, suggesting that as the seed matures, the newly formed phaseolin is less protected.

As bean seeds began to germinate, the family of phaseolin proteins (45.5–51.0 kDa) started to disappear, and degradation products of 20–29 kDa began to appear (Fig. 7*A*). By day 6 little undegraded phaseolin remained and the degradation products began to accumulate. A similar sequence of events occurred for phaseolin in tobacco. Because only one gene product is involved, the pattern is somewhat simpler. The full-length β-phaseolin protein band disappeared over the first 6 days of germination followed by a slower disappearance of the degradation products (Fig. 7*B*). These data are consistent with the utilization of phaseolin during tobacco seed germination. They also show that phaseolin does not continue to be produced in the germinating seeds. Taken together with the fact that the expression of phaseolin in the developing tobacco seeds follows the same pattern as it does in the bean seed, these observations leave little doubt that phaseolin expression is developmentally regulated in tobacco, being synthesized and accumulated only during seed development and maturation.

It appears that some of the degradation peptides of phaseolin in germinating bean seed comigrated with the small molecular weight immunoreactive peptides in tobacco (Fig. 7*A*, lane 7). However, we cannot be certain that they are identical because the pattern in germinating bean seed is more complex than is the pattern in germinating tobacco.

Inheritance of the Phaseolin Gene in Tobacco Seeds. Dot-immunobinding assay of 64 individual transformed seeds of the first generation (R_1) (Fig. 8) showed that 25% of the seeds lacked all traces of phaseolin, whereas the remaining 75% had 0.3–2% of phaseolin. By grouping the latter into two categories, one that contains from 10 to 20 ng of phaseolin and the other from 2 to 9 ng of phaseolin per μg of total protein, the ratio obtained is consistent with the 1:2:1 segregation expected if the phaseolin gene were functioning as a Mendelian dominant trait. The observed variation in the amount of phaseolin in individual seeds of either category could be explained on the basis that the embryonic tissue, where phaseolin is made, varies in relative amount from seed to seed. Based on the morphology of A66-transformed tobacco (10), $\approx$75% of the seeds (178/247) gave rise to nonrooting plants (transformed) and 25% to normal plants (untransformed). Again, this is consistent with transferred DNA being inherited as a single dominant Mendelian trait.

Examination of seeds from two plants of the R_2 generation following selfing showed an $\approx$3:1 ratio for the presence and absence of phaseolin, respectively, implying that the plants are heterozygous for the phaseolin gene. The level of phaseolin in seeds of the R_2 generation was comparable to the level in the seeds of the R_1 generation.

DISCUSSION

We conclude from this study that a 3.8-kb DNA fragment containing the phaseolin coding region flanked by 863 bp of 5′ and 1226 bp of 3′ flanking DNA includes all of the sequences necessary for correctly regulated expression of this gene. It is especially interesting to note that the phaseolin promoter, which normally functions in the cotyledons of the bean seed, similarly functions only in the embryonic tissue (which includes the cotyledons) in the tobacco seed. Tobacco storage proteins, on the other hand, are made both in the embryonic tissue and in the endosperm. The tissue specificity function encoded in the phaseolin gene promoter thus differentiates between the different tissues in a seed and this, in turn, implies differences in inducing signals in the two tissues. Clearly, many further experiments are required to define the DNA region(s) that determine tissue-regulated expression and also to identify the factors that induce the turning on (and off) of this gene.

The 3.8-kb DNA fragment has been shown to be correctly transcribed and the transcript correctly processed in transformed sunflower callus (8). The fact that transformed tobacco seeds contain a 1.7-kb phaseolin mRNA as do bean seeds would strongly suggest that correct excision of introns and poly(A) addition to the primary transcript has occurred.

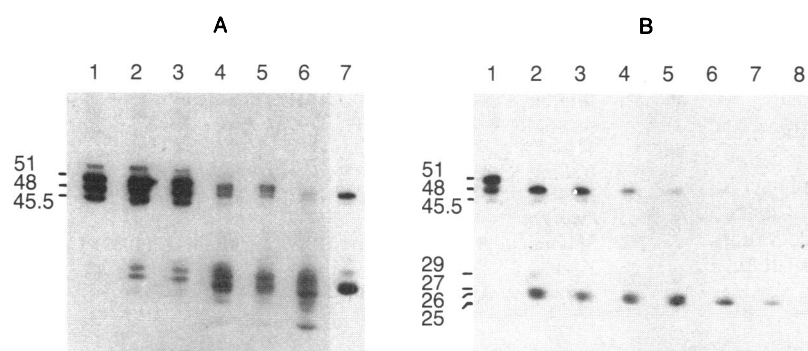
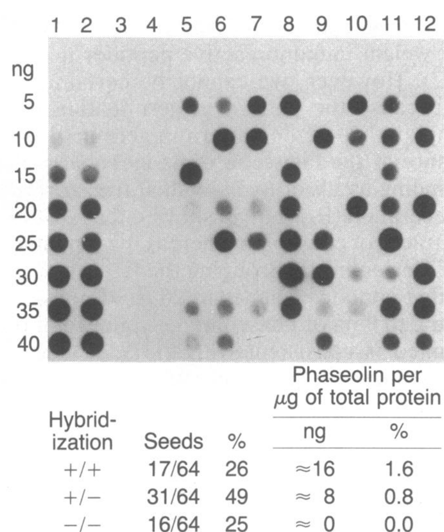


FIG. 7. Fate of phaseolin in germinating bean and tobacco seeds. Seeds were allowed to germinate on sterile agar plates for different time periods, and their proteins were extracted. Proteins were then analyzed by the immunobinding assay for phaseolin peptides. (*A*) Germinating bean seeds. Lanes: 1–6, Total protein (5 μg) from bean seeds after 0, 2, 4, 6, 8, and 10 days of germination; 7, total protein (50 μg) from transformed tobacco seeds (lane 7 was exposed twice as long as the other lanes). (*B*) Germinating tobacco seeds. Lanes: 1, purified phaseolin (100 ng) from bean seed; lanes 2–7, proteins (50 μg) extracted from transformed seeds after 0, 1, 2, 3, 4, and 5 days of germination; 8, protein (50 μg) from normal seeds. Molecular sizes are shown in kDa.



Hybrid-ization	Seeds	%	Phaseolin per µg of total protein: ng	%
+/+	17/64	26	≈16	1.6
+/−	31/64	49	≈ 8	0.8
−/−	16/64	25	≈ 0	0.0

FIG. 8. Quantitation of phaseolin in individual transformed tobacco seeds. Proteins from individual seeds were extracted, and 2 µg of total protein was subjected to dot-immunobinding assay. Lanes: 1 and 2, purified phaseolin in duplicate at 5, 10, 15, 20, 25, 30, 35, and 40 ng as indicated; 3 and 4, normal tobacco seeds; 5–12, transformed tobacco seeds. Discs corresponding to each dot were cut out, the radioactivity was determined, and the amount of phaseolin was estimated from a standard curve obtained by plotting cpm/min vs. ng of purified phaseolin per dot. The seeds were then categorized as +/+, high phaseolin; +/−, intermediate phaseolin; −/−, phaseolin, and the number of seeds over the total (64), the percentage of seeds, and the amount of phaseolin per µg of total protein in each category were determined.

The closely corresponding migration of phaseolin protein from bean and tobacco in one- and two-dimensional electrophoretic separations indicates that glycosylation events are very similar in these two environments. It also indicates that the hydrophobic NH_2-terminal signal sequence of β phaseolin is correctly processed in the foreign environment of tobacco.

Our data suggest that the smaller molecular weight immunoreactive peptides result from degradation of full-length β phaseolin rather than premature termination of translation. It has been deduced from sequence data that the two glycosylation sites in phaseolin are at the carboxyl end (19); therefore, premature termination of translation cannot result in a 29-kDa peptide that is glycosylated. Further evidence for posttranslational degradation comes from the fact that during germination, the authentic size β phaseolin disappears prior to the low molecular mass peptides, suggesting that the latter came from the full-size phaseolin. Incubation of native phaseolin with extracts of germinating untransformed tobacco seeds yields peptides, giving the degradation pattern typical of the transformed seeds (data not shown).

Processing of phaseolin in bean is known to go through several stages, each mediated by membrane-associated functions, resulting in it being sequestered in protein bodies (24). It appears that phaseolin in transformed tobacco seeds is also sequestered in protein bodies (M. Chrispeels, personal communication). It is important to note that the synthesis of phaseolin in tobacco seeds did not significantly decrease the amount of tobacco storage proteins.

Although proteins involved in germination-specific processing have been identified (25), it remains to be determined if specific proteolytic cleavage sites within the phaseolin molecule exist. If phaseolin is cleaved within the tobacco seed, as it is in the bean seed, this would further accentuate the commonality of processing systems despite the structural differences between tobacco seed and mature bean seed.

The results presented here confirm the value of Ti plasmids as gene vectors for higher plants and fully support the practical potentials that have been envisaged for foreign gene expression in agriculturally important plants. The tissue-specific expression and stable Mendelian inheritance observed parallel results obtained for insertion of cloned genes into *Drosophila* with P element vectors (26, 27), although the experiments described here involve gene transfer between taxa that are believed to be separated by >10 Myr of evolution (23).

We are grateful to the University of Wisconsin and N. Murai for the A66 construction containing the phaseolin gene. We thank J. Pitas, L. Blomquist, J. Hawley, D. Sutton, and A. Binns for technical assistance; R. Klassy and D. Merlo for the phaseolin cDNA and octopine synthase fragment; D. Talbot for the phaseolin antisera; and L. Hoffman and J. Cramer for critical evaluation of results. This is Agrigenetics Advanced Research Division Manuscript no. 34.

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