RESEARCH ARTICLE

Phaseolin Gene from Bean Is Expressed After Transfer to Sunflower Via Tumor-Inducing Plasmid Vectors

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The insertion of genes encoding beneficial traits (such as disease resistance, yield improvement, and stress tolerance) into well-adapted crop plants is potentially of great agricultural importance. The demonstration that the soil bacterium *Agrobacterium tumefaciens* can insert specific regions of indigenous tumor-inducing (Ti) plasmids into plant nuclear DNA has stimulated extensive research into the use of these plasmids as vectors for gene transfer (*1*). Furthermore, because *A. tumefaciens* can infect many hosts (*2*), this organism is suitable for genetic engineering in numerous plants.

Although Ti plasmids of 140 to 235 kilobase pairs (kbp) are known to encode the essential traits for oncogenicity (*3*), the molecular mechanism for tumor induction is only now beginning to unfold. No functions are known for much of the Ti DNA, but the virulence region and the 21 to 25 bp adjacent to the transferred DNA (T-DNA) of the Ti plasmid appear to participate in its integration into plant cells (*4*). No specific integration sites in the plant genome have been identified (*5*).

Abstract. *Sequences coding for the bean seed protein phaseolin were inserted into transferred DNA regions of tumor-inducing plasmids. Constructions were devised in which the coding region of phaseolin was fused in the correct reading frame with the coding region of octopine synthase and placed under the transcriptional control of the octopine synthase promoter. Other plasmids were prepared to permit expression of the phaseolin-encoding sequences from the flanking phaseolin promoter region. The RNA transcribed in sunflower cells transformed with these constructions was characterized by hybridization procedures, S1 nuclease mapping, and by translation in vitro of extracted RNA. These tests showed that the genomic intervening sequences were correctly excised. Immunoreactive phaseolin polypeptides were detected by enzyme-linked immunosorbent assay and by antibody hybridization to electrophoretically separated protein extracts of sunflower tissues isolated from crown gall tumors and of transformed sunflower cells grown in tissue culture. These results demonstrate the expression of a plant gene after transfer to a taxonomically distinct botanical family.*

Transferred DNA encodes genes for opine synthesis (*6*) and encodes several oncogenes for tumor morphology (*7*). A Ti plasmid–encoded gene for nopaline synthase was expressed after passage through two seed generations of tobacco (*8*). Several bacterial, animal, and unrelated plant genes have been inserted into various restriction enzyme sites of the T-DNA region and successfully transferred into the plant genome (*9*). A yeast alcohol dehydrogenase gene inserted into T-DNA was also maintained through meiosis in tobacco, but it, like other transferred genes, was not expressed either at the RNA or protein level (*8*).

One method that has resulted in foreign gene expression has been to form hybrid genes, in which the coding sequence of a foreign gene is linked to a promoter sequence derived from a Ti plasmid. For example, transformation of kanamycin-sensitive petunia cells with Ti plasmids containing the Tn5-derived neomycin phosphotransferase II (NPTII) gene ligated to the nopaline synthase promoter yielded antibiotic-resistant callus tissue (*10*). The nopaline synthase promoter also activates the transcription of other hybrid genes (*9, 11*).

We now describe two series of experiments with Ti plasmid constructions. In one, DNA coding for phaseolin was fused to—and expressed under control of—a region of T-DNA that includes the octopine synthase (OCS) gene promoter. Other constructions involved the insertion of the entire phaseolin gene into T-DNA so that expression resulted from its own promoter.

Features of the Phaseolin Gene and Protein

Phaseolin is a glycoprotein that constitutes up to 50 percent of the storage protein in bean (*Phaseolus vulgaris* L.) cotyledons, where its expression is under strict developmental control (*12*). It is encoded by a family of 7 to 14 genes containing limited base substitutions and deletions. These genes are expressed as a group of closely related polypeptides that are heterogeneous in molecular size and isoelectric charge (*13*). Nucleotide sequence analysis of a genomic clone (λPVPh177.4) and a corresponding phaseolin complementary DNA (cDNA) clone established the order of the 1990 bp from the cap site to the polyadenylation [poly(A)] site. The gene contains 80 bp of 5′ untranslated DNA, 1260 bp of protein-encoding DNA interrupted by five introns (a total of 515 bp) and 135 bp of 3′ untranslated DNA (*14*). Sequences important for effective and accurate eukaryotic transcription (CCAAT and TATA) (C, cytosine; A, adenine; T, thymine) are present upstream from the cap site, and AT-rich regions flank the gene. The deduced amino acid sequence revealed a very hydrophobic amino-terminal region typical of signal sequences involved in protein translocation through membranes (*14*). Amino-terminal analysis has confirmed that the first 24 amino acids are not present in the mature protein (*15*).

Two subclones of λPVPh177.4 in pBR322 were used for insertion into the T-DNA region of Ti plasmids. Clone AG-pPVPh3.0 contains a 3-kbp Eco RI–Bam HI fragment (Fig. 1D). Because the Eco RI end of this fragment starts at phaseolin codon 14, the phaseolin pro-

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moter region and about one-half of the putative signal peptide are absent (Fig. 1E). The Bam HI site is 1100 bp 3′ to the poly(A) addition site. The Eco RI–Bam HI fragment is, therefore, useful for placing the phaseolin coding region under the control of other promoters. The second subclone (AG-pPVPh3.8) contains the complete phaseolin gene, including its promoter region, on a 3.8-kbp Bgl II–Bam HI fragment (see Fig. 2C). The Bgl II site is located 800 bp 5′ to the

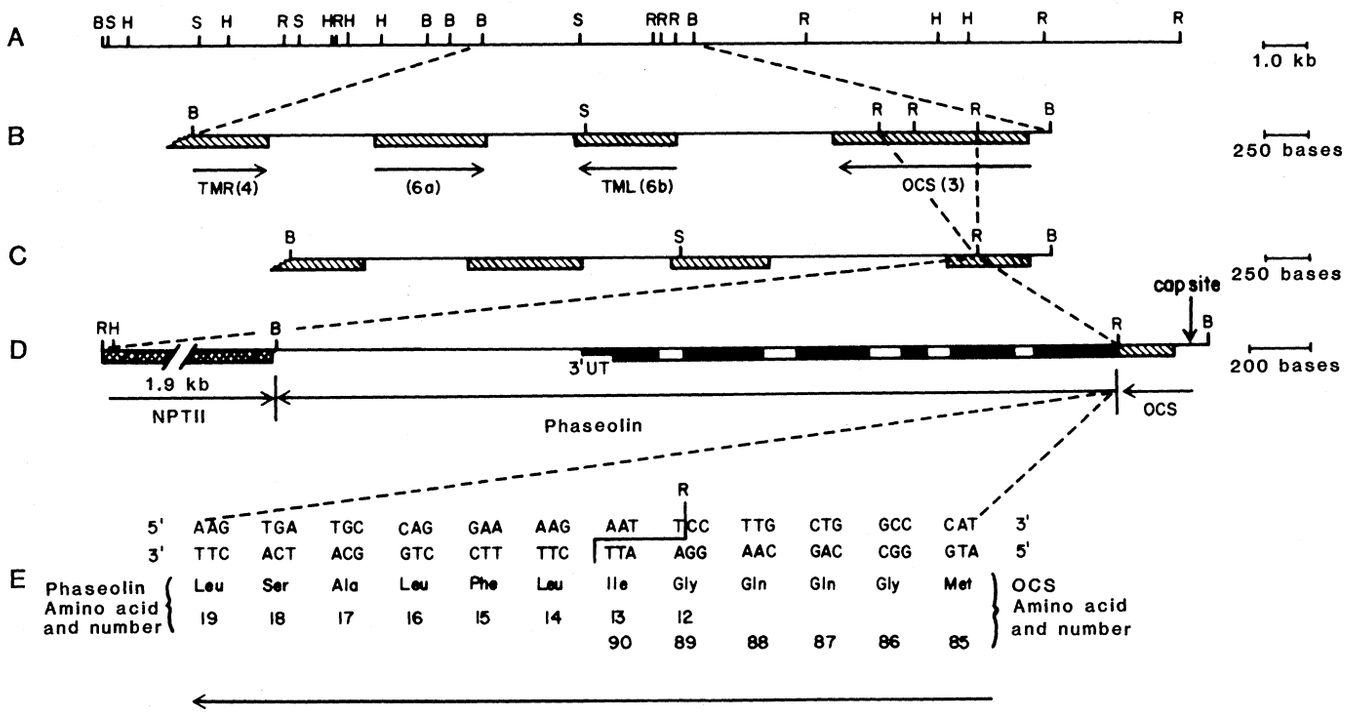

Fig. 1. Construction of a T-DNA fragment containing an OCS-phaseolin fusion gene. (A) Map of the T region of pTi15955 showing locations of recognition sites: *B*, Bam HI; *R*, Eco RI; *S*, Sma I; and *H*, Hind III, as deduced from the complete nucleotide sequence of the T-DNA (*27*). (B) Bam HI fragment 17a [as numbered in (*20*)] was cloned in a derivative of pBR322 in which the Eco RI recognition site had been destroyed (*28*). Diagonally hatched bars denote three known and one putative (6a) T-DNA transcripts (*27, 29*). *TMR* and *TML* refer to the tumor morphology loci *tmr* and *tml*, respectively, defined by Garfinkel *et al.* (*7*). *OCS* marks the location of the octopine synthase gene (*30*). Horizonal arrows indicate direction of transcription. (C) Cleavage by Eco RI followed by religation resulted in a derivative of OCS in which the two internal Eco RI fragments had been deleted. (D) A 3.0-kbp Eco RI–Bam HI fragment containing all of the phaseolin coding region except the first 33 nucleotides of the first exon and a 1.9-kbp Eco RI–Bam HI fragment containing the entire NPTII gene (to provide a selectable marker for subsequent constructions) (*31*) were mixed and ligated into the Eco RI site of the modified OCS gene. The cross-hatched bar denotes the NPTII region; solid bars show phaseolin exons; and open bars show phaseolin introns. (E) Nucleotide sequence across the Eco RI fusion site. Replacement (*19*) of the Bam HI fragment 17a of pTi15955 with the engineered Bam HI fragment 17a containing the OCS-phaseolin and NPTII sequences resulted in pTiF12A.

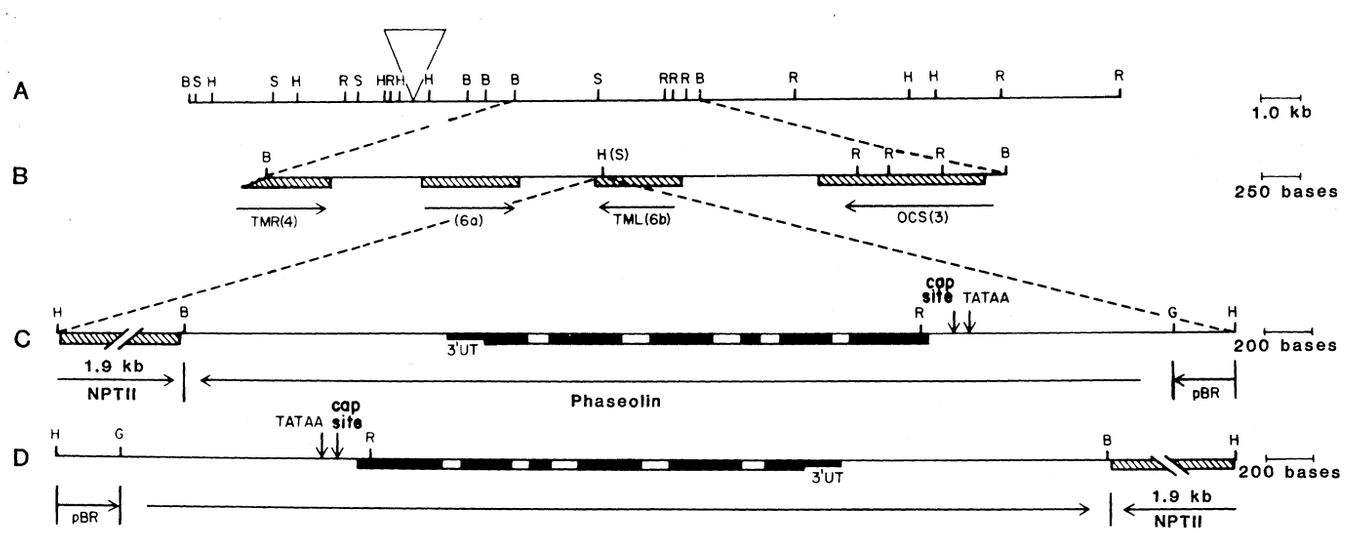

Fig. 2. Construction of engineered T-DNA fragments containing complete phaseolin genes. (A) Map of the T region of pTi15955 and pTiA66. Restriction sites are indicated as in Fig. 1 and in addition, *G* = Bgl II. The T region of pTiA66 differs from that of pTi15955 by the presence of a 2.7-kbp insertion in Bam HI fragment 8 [inverted triangle (*32*)]. (B) The single Sma I site present in Bam HI fragment 17a was converted to a Hind III site (*33*), and the modified fragment was ligated into the Bgl II site of the shuttle vector pRK290. (C and D) A 6.0-kbp Hind III fragment from pKSKB3.8 (*34*), which carries the NPTII and phaseolin gene sequences and 346 bp of pBR322 DNA (*pBR*), was cloned into the new Hind III site of the shuttle vector in both orientations. The shuttle vectors were then used to transfer the NPTII and phaseolin sequences to pTi15955 or pTiA66, resulting in pTi529R and pTi539L, respectively.

messenger RNA (mRNA) cap site; the Bam HI site is the same as in AG-pPVPh3.0. Hence, the second subclone was used to test the ability of the phaseolin promoter to function in transformed tissue.

Fusion of the OCS and Phaseolin Genes

The OCS gene is activated persistently in plant tumors, in axenic tumor tissue culture, and in the specialized cells of regenerated plants (*16*). Neither the OCS nor the related nopaline synthase gene contains intervening sequences (*17*). To circumvent any problems associated with tissue-specific developmental regulation of the phaseolin promoter, the phaseolin gene was placed under control of the OCS promoter.

Both OCS and phaseolin are synthesized from codons in identical reading frames relative to the sequences comprising the first 5′ Eco RI site of each gene. Using these Eco RI sites, we were able to construct a chimeric gene comprised of the first 264 bp of OCS and all but the 33 bp at the 5′ end of the phaseolin coding sequence. Transcription of this chimeric gene from the OCS promoter resulted in an mRNA encoding the first 88 amino acids of OCS, and all but the first 11 amino acids of phaseolin. A clone (pKSB17KB3.0) was constructed in pBR322 containing the fused OCS-phaseolin gene and the NPTII gene flanked by T-DNA sequences (legend to Fig. 1).

The 9.1-kbp Bam HI fragment of a partial digest of pKSB17KB3.0 containing the OCS-phaseolin, NPTII, and T-DNA sequences was ligated into the Bgl II site of the plasmid pRK290 (*18*). This resulted in a plasmid (pKSOSKB3.0) capable of replication in both *Escherichia coli* and *A. tumefaciens*; pKSOSKB3.0 was used as a "shuttle plasmid" to transfer the engineered T-DNA region into *A. tumefaciens*. Nucleotide sequence analysis of the region spanning the Eco RI site verified that the correct reading frame was achieved. The wild-type Bam HI fragment 17a of plasmid pTi15955 was replaced with engineered T-DNA sequences containing the NPTII and phaseolin sequences by means of the shuttle plasmid and modifications of a marker exchange system based on plasmid incompatibility (*19*). The resulting Ti plasmid carrying the OCS-phaseolin and NPTII genes was designated pTiF12A. The predicted absence of the large (12.5 kbp) Hind III fragment 1 (*20*) that was used as a target site for insertion was confirmed by comparison of the restriction patterns of pTi15955 with those of pTiF12A (Fig. 3A). As a consequence of the insertion, two new Hind III fragments (10.7 and 6.2 kbp) were detected and, as anticipated, the 10.7-kbp fragment contained the phaseolin gene.

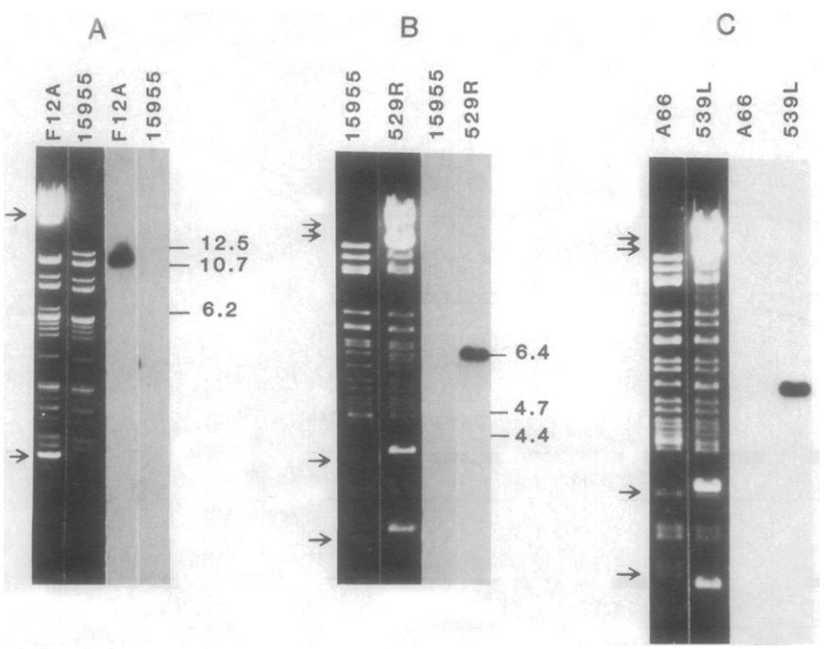

Fig. 3. Proof of constructions. Photographs of ethidium bromide–stained agarose gels are shown on the left of each panel. On the right of each panel are the corresponding blot hybridization (*35*) data obtained with nick-translated phaseolin clone probes. Arrows on the left designate fragments resulting from the pPH1J1 excluding plasmid (*19*). The sizes of fragments of interest are shown on the right. (A) Hind III digest of fusion construction pTiF12A compared to pTi15955. (B) Bam HI digest of genomic construction pTi529R compared to pTi15955. (C) Bam HI digest of genomic construction pTi539L compared to pTiA66.

Complete Phaseolin Gene Construction

An additional set of constructions involved insertion of the complete phaseolin gene into the putative large tumor locus (tml) of pTi15955 (Fig. 2A). This locus was selected because the tml gene is the most actively transcribed T-DNA gene in sunflower tumor callus (*21*) and because its central location within the T region made it likely that the inserted DNA would be stably maintained in plant cells.

Bam HI fragment 17a was isolated from pTi15955, modified by replacing the Sma I site with a Hind III site, and cloned into pRK290 (Fig. 2A). The resulting plasmid (p111) was cleaved with Hind III and ligated to a 6.0-kbp Hind III fragment containing the entire sequences for phaseolin and NPTII (Fig. 2C). Shuttle vectors based on pRK290 containing the phaseolin and NPTII genes, flanked by T-DNA, were obtained. The phaseolin gene was inserted either in the same transcriptional polarity as the flanking sequences of the tml gene ("right" orientation: p121) or reversed ("left" orientation: p122) (Fig. 2, C and D, respectively). The Bam HI 17a fragment of pTi15955 was replaced with the engineered T-DNA fragment by means of p121 and marker exchange. The resulting Ti plasmid carrying the phaseolin sequence in the "right" orientation was designated pTi529R. A similar exchange using p122 resulted in a pTiA66 derivative plasmid carrying the phaseolin gene in the "left" orientation (pTi539L).

Both genomic constructions were verified by restriction endonuclease analysis and hybridization with phaseolin genomic clone probes. Comparison of Bam HI digests of pTi529R with pTi15955 revealed the expected loss of a 4.7-kbp Bam HI fragment 17a and the appearance of 6.4- and 4.4-kbp fragments in pTi529R (Fig. 3B). The presence of the phaseolin sequence in the 6.4-kbp Bam HI fragment was verified by hybridization with a phaseolin probe (Fig. 3B). Digestion of pTi539L with Bam HI resulted in the expected loss of the wild-type 4.7-kbp Bam HI fragment 17a and the appearance of new 6.6- and 4.1-kbp fragments (Fig. 3C). As predicted, the 6.6-kbp Bam HI fragment contained the phaseolin sequence.

Expression of the OCS-Phaseolin Fusion Gene

Agrobacterium tumefaciens harboring engineered Ti plasmids was used in two ways to transform sunflower tissues. (i)

Stationary phase cultures were inoculated into stems of 3- to 5-day-old seedlings; 15 to 30 days later the resulting crown gall tissues were harvested and examined for expression of the gene product. (ii) Sunflower stem sections were inoculated by a tissue culture technique (*8*). Hereafter, we designate transformed tissues according to the various Ti plasmids with which they are incited. Thus, "A66 control tissue" was incited with pTiA66, "F12A fusion tissue" with pTiF12A, "529R genomic tissue" with pTi529R, and "539L genomic tissue" with pTi539L.

Transcriptional activity of the OCS-phaseolin sequences was confirmed by RNA hybridization blots (Fig. 4). Poly(A) RNA was extracted from transformed tissues, separated on formaldehyde-agarose gels, transferred to nitrocellulose, and hybridized to nick-translated probes of cloned phaseolin coding regions, phaseolin intron 4, or the OCS gene (Fig. 4). An 1850-base transcript that hybridized to both the phaseolin coding region probe and the OCS probe (but not to the intron probe) was present in F12A fusion tissue (Fig. 4A). The transcript is larger than the 1400-base OCS mRNA present in A66 control tissues and the 1700-base authentic phaseolin mRNA by the expected amounts. A very weak signal (requiring 6 days of autoradiographic exposure for detection) was observed for a 2000-base transcript in F12A fusion tissue RNA. This signal may represent a small amount of OCS-phaseolin RNA that was not completely processed. From reconstruction blots with known quantities of phaseolin mRNA (data not shown), we estimate the 1850-base transcript observed in F12A fusion tissue represented about 0.5 percent of the total poly(A) RNA. We conclude that the fusion construction expresses substantial amounts of RNA of the size predicted for OCS-phaseolin fusion mRNA.

Poly(A) RNA isolated from F12A fusion tissue directed the translation in vitro of a 52-kilodalton (kD) polypeptide that was immunoprecipitable with antibody to phaseolin (Fig. 5). Authentic phaseolin mRNA directed the translation of three polypeptides that migrated with apparent molecular sizes of 43, 45, and 47 kD. The phaseolin gene in AG-pPVPh3.0 used in this construction codes for the 45-kD form of phaseolin (*14*). The fusion peptide should be 76 amino acids (about 7 kD) larger. Therefore, the size of the 52-kD protein detected by in vitro translation of fusion RNA agrees closely with the predicted size of the OCS-phaseolin fusion peptide. We were not able to detect any fusion protein in F12A fusion tissue by the enzyme-linked immunosorbent assay

Table 1. Protein expression of the phaseolin gene in transformed sunflower tissues. Phaseolin was detected by ELISA (*26*) with monospecific polyclonal antibodies to phaseolin. Samples (0.1 g) of cultured stem tumor tissues (*8*) were hand homogenized with 0.3 ml of buffer and centrifuged, and 0.1 ml of the supernatant was added to each of duplicate wells. The lower limit of detection was 1 ng per gram fresh weight. Values for phaseolin protein content are the means ± the standard deviations of the mean. The highest values observed for 529R and 539L tissues were 24 and 16.8 ng per gram fresh weight, respectively. Tissues used as controls were derived from inoculations of hypocotyl sections of many individual sunflower seedlings with nonengineered *A. tumefaciens* strains 15955 or A66.

Tissue	Tumors tested (number)	Phaseolin positives		
		Number	Percent	Phaseolin (ng/g)
539L genomic	41	33	80	8.4 ± 4.5
529R genomic	30	24	80	6.8 ± 5.9
F12A fusion	34	0	0	None
Controls	60	1	0	1.3

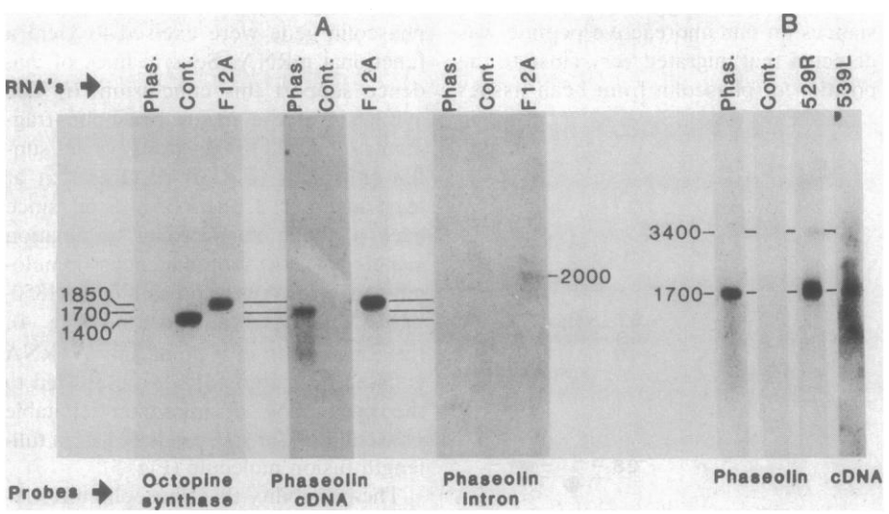

Fig. 4 (left). Detection of phaseolin RNA transcripts in transformed sunflower tissues (*36*). (A) OCS-phaseolin fusion construction. Replicate blots were prepared with the following RNA samples and hybridized with phaseolin cDNA, OCS clone DNA, or phaseolin intron DNA: *Phas.*, 10 ng of bean cotyledon midmaturation total RNA; *Cont.*, 1 μg of A66 control tissue poly(A) RNA; *F12A*, 1 μg of OCS-phaseolin F12A fusion tissue poly(A) RNA. (B) Phaseolin genomic construction: *Phas.*, 10 ng of bean cotyledon midmaturation RNA; *Cont.*, 2 μg of A66 control tissue poly(A) RNA; *529R*, 2 μg of poly(A) RNA from 529R genomic tissues; *539L*, 2 μg of poly(A) RNA from 539L genomic tissues. Blots were hybridized with a phaseolin cDNA probe. Molecular weights were calculated from the mobilities of alfalfa mosaic virus RNA's included on the same gel.

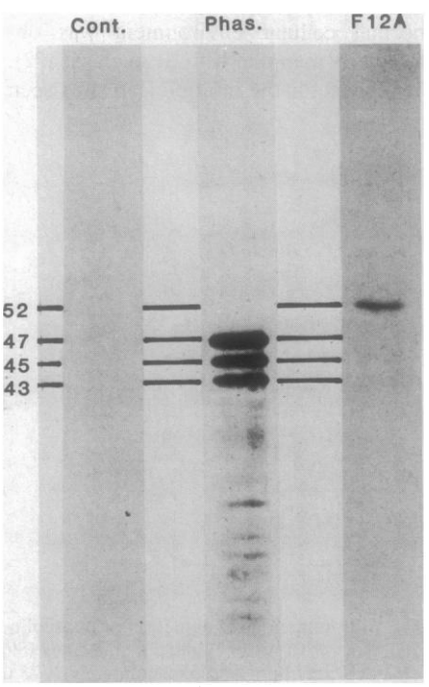

Fig. 5 (right). Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis of immunoprecipitated products from translation in vitro of the fusion RNA. Poly(A) RNA was isolated from transformed tissues and translated in the reticulocyte lysate system (*37*). The translation products were immunoprecipitated with phaseolin antibody and analyzed by SDS–polyacrylamide gel electrophoresis followed by autoradiography (*38*). *Cont.*, A66 control tissue poly(A) RNA; *Phas.*, poly(A) RNA from bean (cv. Contender) cotyledons; *F12A*, F12A fusion tissue poly(A) RNA. The molecular weights were calculated from the mobilities of protein standards included on the same gel.

(ELISA) (Table 1). However, we were able to detect in F12A fusion tissue a 26-kD protein that reacted to antibody to phaseolin (Fig. 6A).

Expression of the Phaseolin Genomic Construction from its own Promoter

The phaseolin probe was hybridized to a 1700-base transcript in poly(A) RNA that was isolated from sunflower tissues transformed with *Agrobacterium tumefaciens* cells harboring pTi529R or pTi539L (Fig. 4B). The relative abundance of these putative phaseolin transcripts was, however, considerably lower than that of the OCS-phaseolin fusion mRNA. From reconstruction blots (data not shown), we estimate that the transcript in 529R genomic tissues comprise only about 0.025 percent of the total poly(A) RNA.

Although the data in Fig. 4B suggest that higher levels of mRNA accumulate when the phaseolin gene is in the "right" orientation, most other experiments suggest that there is no significant difference in expression of RNA in 529R and 539L genomic tissues (data not shown). This distinction is important because the possibility existed that expression might result from readthrough from adjacent T-DNA promoter sequences. Confirmation that transcripts found in transformed tissues were initiated in a manner similar to that for the phaseolin promoter in its normal cellular environment was obtained by mapping with S1 nuclease (*22*). The basis for the multiple cap sites seen in transcripts isolated from bean cotyledons and also found here for phaseolin transcription in transformed sunflower tissue (Fig. 7) is not well understood; however, the 5′-flanking DNA for the phaseolin gene contains several closely spaced promoter sequences (*14*). A trace signal of about 3400 bases was observed in tissues containing the genomic construction (Fig. 4B); it is also present in small amounts in authentic *Phaseolus* cotyledon RNA preparations. This transcript does not hybridize to probes corresponding to 5′ or 3′ regions flanking the phaseolin gene and therefore cannot be attributed either to abnormal upstream promotion or aberrant transcription termination.

Protein expression in transformed tissues containing the entire phaseolin gene was detected by ELISA procedures and by protein blotting. The lower limit of phaseolin detection by ELISA was 1 ng per gram fresh weight of transformed tissue. The highest level detected was about 24 ng per gram fresh weight, but considerable variation was observed among the many samples tested (Table 1). Only one false positive reading (1.3 ng per gram fresh weight) was obtained in tests of over 60 separate control tumors. Protein extracted from test tissues was also separated electrophoretically, and blots of these separations revealed immunoreactive peptides of 28, 26, and 14 kD (Fig. 6A). The relative intensities of these bands varied, and in some instances an immunoreactive peptide was detected that migrated very close to the position of phaseolin from bean tissues (Fig. 6B). The occurrence of the smaller immunoreactive polypeptides indicated that specific cleavage or degradation of phaseolin was taking place in the transformed sunflower tissue. Confirmation that sunflower tissue can degrade phaseolin was obtained by incubation of authentic phaseolin with extracts of A66 control tissues; protein blots of electrophoretic separations of these extracts revealed an immunoreactive peptide of 14 kD, but little or no intact phaseolin (Fig. 6C).

Because phaseolin mRNA quantities were much lower in tumors incited by plasmids containing the genomic construction than those incited by plasmids containing the OCS-phaseolin fusion construction, it was technically more difficult to obtain immunoprecipitable products from translation experiments in vitro. Nevertheless, we have observed trace amounts of an immunoprecipitable polypeptide in such experiments that migrated with a 45-kD phaseolin polypeptide made by authentic phaseolin mRNA (data not shown).

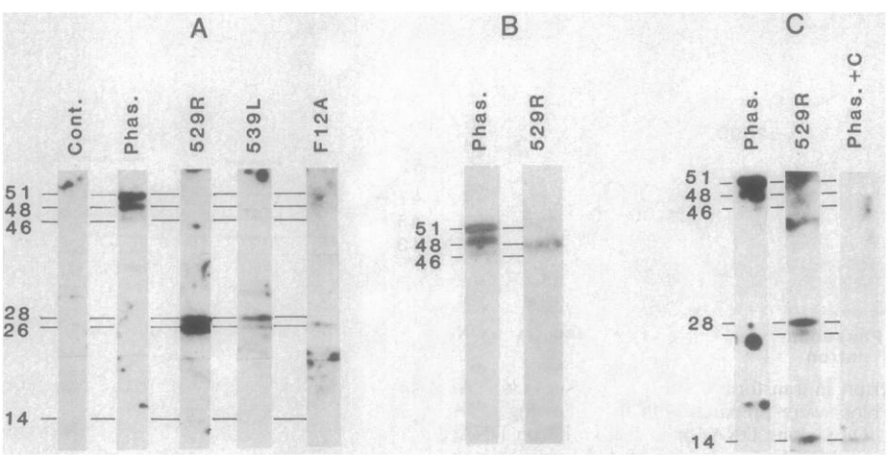

Fig. 6. Immunological detection of phaseolin protein in sunflower tumors after electrophoresis and transfer to nitrocellulose membranes (*39*). (A) *Cont.*, 75 μg of A66 control tissue protein; *Phas.*, 10 ng of purified phaseolin; *529R*, 75 μg of 529R genomic tissue protein; *539L*, 75 μg of 539L genomic tissue protein; *F12A*, 75 μg of F12A fusion tissue protein. (B) Detection of undegraded phaseolin in transformed tissue: *Phas.*, 10 ng of phaseolin; *529R*, 75 μg of 529R genomic tissue protein. (C) Phaseolin degradation during extraction; *Phas.*, 10 ng of phaseolin; *Phas. + C.*, 10 ng of phaseolin was incubated (15 minutes at 25°C) with 75 μg of protein extracted (*26*) from A66 control tissue; *529R*, 75 μg of 529R genomic tissue protein from tumor tissue. Molecular weights were calculated from the mobilities of protein standards included on the same gel.

Discussion

Our results show that fusion of a eukaryotic gene to a T-DNA gene results in the transcription and translation of the inserted DNA sequences in transformed tissues. It was of special interest that the intervening sequences present in the phaseolin gene were excised to yield a functional mRNA. Several lines of evidence support this conclusion. (i) The synthesis of the major phaseolin fragments (26 to 28 kD) in transformed sunflower tissues (Fig. 6) requires that at least introns 1, 2, and 3 be excised, since each contains translational termination signals (*14*). (ii) No sequences homologous to intron 4 were present in the 1850-base OCS-phaseolin transcript (Fig. 4). (iii) Translation in vitro of poly(A) RNA isolated from transformed tissues led to the production of immunoprecipitable phaseolin of the size predicted for a full-length fusion molecule (Fig. 5).

The possibility that phaseolin mRNA–specific splicing enzymes are encoded in introns is excluded by the small sizes of the introns and their lack of open reading frames (*14*). Although we cannot rule out the possibility of nonprotein splicing (*23*) of phaseolin mRNA, our data reveal that if RNA splicing proteins are involved, both the RNA signal sequences and the protein recognition sites are highly conserved between widely divergent plant families. Such conservation suggests that specific splicing mechanisms do not

play a major role in the developmental regulation of phaseolin in bean.

Expression of prokaryotic genes that do not contain introns after fusion to T-DNA promoters has been reported (*9–11*). Consequently, it was to be expected that fusion of the phaseolin coding sequence to the OCS promoter would result in expression at the RNA level. However, the possibility remained that promoter sequences within the T-DNA are especially adapted to expression in tissues transformed with Ti plasmid. Therefore, our evidence that transcription from the phaseolin promoter can occur in undifferentiated sunflower tissues carrying the genomic construction (Fig. 7) is especially important. The level of transcription from the phaseolin promoter was significantly lower than from the OCS promoter, and this may reflect the fact that the phaseolin gene is normally under developmental regulation. Indeed, it is interesting that transcription occurs at all in the undifferentiated sunflower tissue. Additional research is required to determine the extent to which this expression reflects the undifferentiated state of the tissue or the particular region of the T-DNA into which the gene was inserted. It will be informative to follow phaseolin transcription during differentiation of transformed tissues.

In addition to potential difficulties in obtaining transcriptional expression, previous failures to detect protein expression after insertion of DNA sequences into T-DNA (*8, 9*) suggested that problems may also exist in translation mechanisms. However, our data show that not only were poly(A) RNA's of the expected size transcribed from the engineered genes, but also that they were competent templates for translation in vitro to yield the predicted polypeptides. Phaseolin peptides were shown to be susceptible to degradation in the alien cellular environment, a finding that might have been anticipated in light of proteolysis observed in bacterial cells containing protein products coded by foreign DNA sequences (*24*). The discovery that most of the phaseolin synthesized in sunflower cells was cleaved to peptides that reacted to phaseolin antibody (Fig. 6) led to control experiments that confirmed the ability of sunflower tumor extracts to actively degrade this seed protein. Such activity requires further elucidation, and the possibility of degradation is evidently an important consideration for all attempts to express "foreign" proteins through genetic engineering.

In bean cotyledons, phaseolin is sequestered in membrane-protected protein bodies (*25*), and the presence of the amino-terminal hydrophobic region (*14*) is probably essential to its deposition within these sites. Deletion of much of the putative signal sequence in the fusion construction probably prevents sequestering of the OCS-phaseolin protein products. This could explain our inability to detect this protein by ELISA techniques in sunflower tissue transformed by fusion constructions despite the apparently greater abundance of fusion mRNA in such tissues than of mRNA transcribed from the genomic constructions. Nevertheless, the confirmation provided here that eukaryotic genes can be expressed to yield detectable levels of proteins in alien plant cells will stimulate further experiments in which gene regions having strong transcriptional promoter activity are combined with regions coding for proteins capable of enhancing crop characteristics.

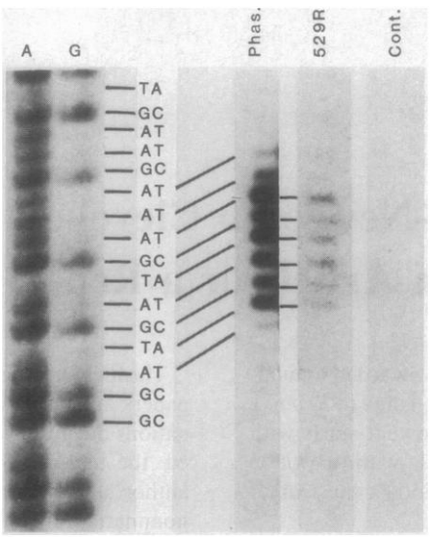

Fig. 7. S1 nuclease mapping of 5′ termini of phaseolin mRNA isolated from bean cotyledons and transformed sunflower tissue. A 550-bp end-labeled fragment spanning the phaseolin promoter region was reassociated with RNA and treated with S1 nuclease; protected fragments were sized on a DNA sequencing gel (*40*). Sequencing lanes for A and G reactions are shown for reference, and the sequence of the cap region is shown on the right. *Phas.*, the protected fragments resulting from annealing with 10 ng of *Phaseolus* cotyledon total RNA (containing about 100 pg of phaseolin mRNA). *529R*, the protected fragments resulting from annealing with about 1 μg of 529R genomic tissue poly(A) RNA. The fragments protected by 529R RNA, while less intense, are in agreement with the authentic phaseolin mRNA and with the expected cap sites found from sequencing a full length cDNA clone (*14*). Lines drawn between the sequence and the S1 mapping lanes show positions of the capped nucleotides after a correction of 1.5 bp resulting from excess protection of the capping group (*41*). *Cont.*, 1 μg of A66 control tissue poly(A) RNA resulted in no protection.

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from this derivative plasmid was treated with Sma I and ligated to synthetic Hind III linkers.
34. The plasmid pKSKB3.8 was the result of ligating a 4.1-kbp Cla I–Bam HI fragment bearing the entire phaseolin gene from AG-pPVPh3.8, and a 1.9-kbp Cla I–Bam HI fragment from pKS4 containing the NPTII gene of Tn5, into Cla I–cleaved pBR322.
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39. Protein samples isolated from tumor tissues were separated on 15 percent acrylamide gels and transferred to nitrocellulose. Membranes were incubated with polyclonal phaseolin antiserum and the antigen-antibody complex was visualized autoradiographically after incubation with ^{125}I-labeled protein A [H. Towbin, T. Staehelin, J. Gordon, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350 (1979)].
40. A 1600-bp Eco RI fragment containing the 5′ region of the phaseolin gene was labeled at the 5′ end. After cleavage with Nco I, a 550-bp fragment labeled at one end extending about 400 bp in the 5′ direction from the phaseolin cap site was isolated. Samples of this probe (about 100,000 count/min) were reassociated with the various RNA's under conditions that favor the rapid and preferential formation of RNA · DNA duplexes. RNA and probe were reassociated at 45°C for 12 hours in 3.0*M* sodium trichloroacetate, 50 m*M* PIPES (*p*H 7.0), 5 m*M* EDTA, and 5 m*M* EGTA. Y. H. Chien and N. Davidson [*Nucleic Acids Res.* **5**, 1627 (1978)] have shown that RNA · DNA duplexes are more stable than DNA · DNA duplexes in concentrated trichloroacetate solutions, and this system offers a significant increase in sensitivity over formamide-containing systems with no apparent change in specificity (M. G. Murray, N. P. Jarvis, W. F. Thompson, J. L. Slightom, in preparation). After reassociation, samples were treated with S1 nuclease (*22*) and analyzed on 6 percent acrylamide–7*M* urea sequencing gels [A. Garoff and W. Ansorge, *Anal. Biochem.* **115**, 450 (1981)].
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42. We thank Duncan Talbot, Mark Yeazel, and Robert McLeester for preparing antibodies to phaseolin protein; Steven Nelson for in vitro translations, and Marie Peterson and Dorothy Nesbit for technical assistance. N.M. was supported by a grant from Agrigenetics Research Corporation.

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