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Expression of Dicistronic Transcriptional Units in Transgenic Tobacco

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We investigated whether the two cistrons of a dicistronic mRNA can be translated in plants to yield both gene products. The coding sequences of various reporter genes were combined in dicistronic units, and their expression was analyzed in stably transformed tobacco plants at the RNA and protein levels. The presence of an upstream cistron resulted in all cases in a drastically reduced expression of the downstream cistron. The translational efficiency of the gene located downstream in the dicistronic units was 500- to 1,500-fold lower than that in a monocistronic control; a 500-fold lower value was obtained with a dicistronic unit in which both cistrons were separated by 30 nucleotides, whereas a 1,500-fold lower value was obtained with a dicistronic unit in which the stop codon of the upstream cistron and the start codon of the downstream cistron overlapped. As a strategy to select indirectly for transformants with enhanced levels of expression of a gene which is by itself nonselectable, the gene of interest can be cloned upstream from a selectable marker in a dicistronic configuration. This strategy can be used provided that the amount of dicistronic mRNA is high. If, on the other hand, the expression of the dicistronic unit is too low, selection of the downstream cistron will primarily give clones with rearranged dicistronic units.

The most consistent model to describe initiation of translation in eucaryotes is the modified scanning model (27), which states that 40S ribosomal subunits bind at or near the 5′ cap structure of the mRNA and migrate downstream along the mRNA until the first AUG codon is reached. If this AUG codon occurs in a favorable context for initiation, the 40S ribosomal subunits are halted, the 60S subunits associate with them, and translation is started; if the context around the first AUG codon is less favorable, some or most of the 40S subunits proceed to the next AUG. The favorable contexts for initiation, as established by nucleotide sequence analysis and site-directed mutagenesis, are AACAAUGGC for plant mRNAs (23, 25, 36, 45) and (GCC)GCC$^{A}_{G}$CCAUGG for vertebrate mRNAs (29–31). The scanning model is in accordance with the finding that nucleus-encoded genes in eucaryotes are normally organized in monocistronic transcriptional units.

The concept of reinitiation has been included in the scanning model to account for the observation that an upstream AUG codon, even in an optimal context, still allows initiation of translation at a downstream AUG codon provided that a termination codon in-frame with the upstream AUG codon precedes the downstream AUG codon. Indeed, in several reports it has been demonstrated that in mammalian cells a "minicistron" inserted upstream from the main cistron does not abolish translation of the main cistron (22, 28, 35). After translation of the minicistron, reinitiation of translation seems to occur at the start codon of the main cistron on the same mRNA. Similar processes have been described in barley protoplasts (16). Kozak (32) showed that the efficiency of reinitiation increases when the distance between a minicistron and a downstream reporter gene increases.

Although these studies with minicistrons gave insights into the mechanism of translation initiation, they did not answer the question of whether several longer polypeptides can be translated from a polycistronic mRNA. It is conceivable that a "normal-sized" cistron affects translation of a downstream cistron in a different way than a minicistron encoding a small peptide (32, 33). In several studies, artificially engineered transcriptional units, comprising several normal-sized cistrons, were introduced into mammalian cells. Peabody and Berg (41) found that the downstream cistron of a dicistronic unit is expressed with an efficiency of 5 to 20% relative to a monocistronic construct, if translation beginning at the upstream cistron is terminated before or immediately after the start codon of the downstream cistron. Kaufman et al. (26) found that the translational efficiency of the downstream open reading frame (ORF) in a dicistronic construct is only 0.3 to 2.5% that in a monocistronic construct, while the efficiency of translation of the third ORF in a tricistronic construct is only slightly reduced relative to that of the second ORF in a dicistronic construct. The Epstein-Barr virus nuclear antigen 2 mRNA is an example of a viral mRNA from which two proteins can be translated from nonoverlapping reading frames (53).

In plants, translation of several proteins from nonoverlapping ORFs of a polycistronic mRNA is likely to occur during cauliflower mosaic virus (CaMV) infection. The CaMV genome contains six closely packed genes for which no individual mRNAs have been found in infected cells. The six ORFs are probably translated from the abundant polycistronic 35S transcript by a reinitiation mechanism (13; K. Sieg and B. Gronenborn, Abstr. NATO Adv. Stud. Inst. 1982, p. 154). Analysis of transcripts containing different combinations of two adjacent CaMV ORFs by in vitro translation showed that the expression of the downstream ORF relative to the first ORF varied from less than 1 to 20% (17).

Until now, polycistronic mRNAs in eucaryotes have been studied almost exclusively in virus-infected cells, in transient expression systems, or by in vitro translation. In this work, we analyzed the expression of dicistronic transcrip-

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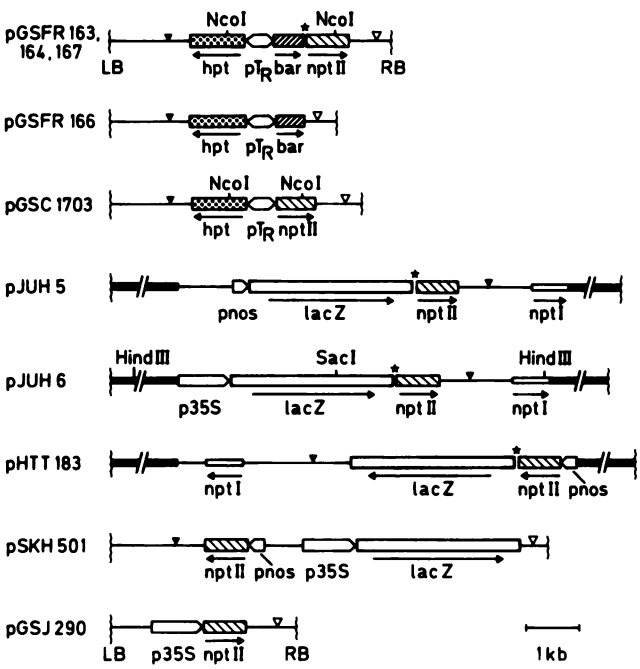

FIG. 1. T-DNA structures of the dicistronic constructs and the monocistronic controls. The *nptII* (18), *lacZ* (46), *bar* (9), and *hpt* (49) genes are indicated by different symbols. The genes were placed under the control of the dual T-DNA T_R promoter (pT_R) (52), the *nos* promoter (pnos) (19), or the CaMV 35S promoter (p35S) (40). The pJUH5, pJUH6, and pHTT183 vectors are represented as they are recombined into the T-DNA of pGV3850. Symbols and abbreviations: ■, pBR322 and T-DNA sequences derived from the pGV3850 vector; LB and RB, left and right T-DNA borders, respectively; nptI, bacterial Km^r marker of Tn*903*, which is included in these vectors to select the *A. tumefaciens* exconjugants; ▼ and ▽, polyadenylation sites in the 3′ untranslated region of the octopine synthase gene (10) and T-DNA gene 7 (51), respectively; *, intercistronic regions from the dicistronic units (TGATCTCACGCG TTCTAGGATCCGGCCAAGCTTATG for pGSFR163; TGATC TCACGCGTATG for pGSFR164; ATGA for pGSFR167; TAATAATAACCGCGCAGGGGGCATCATG for pJUH5 and pJUH6; and TGAGCGGGACTCTGGGGTTCGATAAGCTTCATG for pHTT183); kb, kilobases.

tional units stably integrated into the genome of tobacco plants. Moreover, since all the data presently known about polycistronic mRNAs in plants are derived from studies of the CaMV 35S transcript, this report provides a more general picture of the ability of the plant translational machinery to synthesize several polypeptides from a polycistronic mRNA. We analyzed various dicistronic constructs and showed that the downstream cistron can be translated, but at a very low efficiency.

MATERIALS AND METHODS

Plasmid constructions. The plasmids described in Results and depicted in Fig. 1 were constructed by standard techniques (38).

Mobilization of plasmids from *Escherichia coli* to *Agrobacterium tumefaciens*. Plasmids were constructed and maintained in *E. coli* MC1061 (4). Mobilizations to *A. tumefaciens* were done via a triparental cross with *E. coli* GJ23 (50) or HB101(pRK2013) (15) as a helper. Plasmids pGSFR163, pGSFR164, pGSFR166, pGSFR167, and pGSC1703 are binary vectors based on the stability and replication functions of plasmid pVS1 (8). They were mobilized to *A. tumefaciens* C58C1 Rif^r(pGV2260) (7).

Other plasmids were mobilized to *A. tumefaciens* C58C1 Rif^r harboring the virulence plasmid pGV2260 (in the case of pSKH501 and pGSJ290) or pGV3850 (54) (for pJUH5, pJUH6, and pHTT183). They were stabilized by homologous recombination through the pBR322 sequences into the Ti plasmid. The DNA segments to be transferred to the plant cells (T-DNAs) of these cointegrates were verified by Southern blot analysis of total bacterial DNA.

Leaf disk transformation and protoplast cocultivation. *Nicotiana tabacum* cv. Petit Havana SR1 (37) was transformed by the leaf disk infection method or by cocultivation of regenerating protoplasts as described by De Block et al. (9). Plants transformed with the T-DNAs of plasmids pGSFR163, pGSFR164, etc., are referred to as GSFR163 plants, GSFR164 plants, etc.

Extraction of soluble proteins and RNA from protoplasts. Protoplasts from transgenic tobacco plants were isolated as described by Denecke et al. (12) and cultured in the dark at 24°C. After 24 h the protoplasts from each individual plant (approximately 5×10^6) were concentrated in 400 μl of culture medium. From this concentrated protoplast suspension, 40 μl was used to determine phosphinothricin acetyltransferase (PAT) activity, 80 μl was used to determine neomycin phosphotransferase II (NPTII) activity, and 280 μl was frozen in liquid nitrogen and stored at −70°C for subsequent RNA extraction. Extraction of soluble proteins from the protoplasts for the enzymatic assays was done as described by Denecke et al. (12), except that the extraction buffer for PAT assays was 50 mM Tris hydrochloride (pH 7.2)–2 mM disodium EDTA–0.15 mg of phenylmethylsulfonyl fluoride per ml–0.3 mg of bovine serum albumin per ml–0.3 mg of dithiothreitol per ml, and that for NPTII assays was 50 mM Tris hydrochloride (pH 6.8)–0.15 mg of leupeptin per ml–0.15 mg of phenylmethylsulfonyl fluoride per ml–1% β-mercaptoethanol.

Plant DNA preparations and Southern analysis. Total plant DNA was prepared from tobacco leaves as described by Dellaporta et al. (11). Digested DNA was separated in 0.8% agarose gels. Transfer to nylon membranes and hybridizations were carried out in accordance with manufacturer protocols (Amersham International, Amersham, United Kingdom). Probes were prepared with a random primed labeling kit (Boehringer GmbH, Mannheim, Federal Republic of Germany) or by nick translation (38).

Plant RNA preparations and Northern (RNA) analysis. Total RNA was isolated from leaves as described by Jones et al. (24). Poly(A)$^+$ RNA was prepared by oligo(dT)-cellulose column chromatography. Total RNA from protoplasts was isolated as described by Denecke et al. (12). Denaturing formaldehyde-agarose gel electrophoresis, transfer to nylon membranes, and hybridizations were performed in accordance with Amersham protocols. The *bar*, *nptII*, and *hpt* genes were cloned into SP6 vectors, and SP6 polymerase was used to synthesize ^{32}P-labeled riboprobes complementary to the *bar*, *nptII*, and *hpt* mRNAs.

Enzymatic assays. NPTII activity was determined by the in situ detection method on nondenaturing polyacrylamide gels (48). For some experiments, the P81 filters were treated with proteinase K (44), resulting in the partial removal of the plant kinase bands. PAT activity was determined by thin-layer chromatography as described by De Block et al. (9). β-Galactosidase assays in gel and in solution were done as described by Teeri et al. (46). For all enzymatic assays, samples were standardized against total soluble protein

content by the Bradford assay (Bio-Rad Laboratories, Richmond, Calif.).

RESULTS

Dicistronic constructs. To test whether plants are able to translate both gene products from a dicistronic transcriptional unit, we constructed a set of vectors with marker genes in monocistronic or dicistronic configurations. The constructions are depicted in Fig. 1.

Plasmids pGSFR163, pGSFR164, and pGSFR167 contain the bialaphos resistance (*bar*) gene, encoding PAT, followed by the *nptII* gene, encoding NPTII, as a dicistronic unit fused to the T_R2' promoter. The distances between the two genes in these constructs are 30 base pairs (bp) in pGSFR163 and 10 bp in pGSF164, whereas in pGSFR167, the stop codon of *bar* and the start codon of *nptII* overlap in an ATGA configuration. A hygromycin phosphotransferase gene (*hpt*) under the control of the T_R1' promoter is present in these constructs as a selection marker independent of the dicistronic unit. The control plasmids pGSFR166 and pGSC1703 contain the *bar* gene and the *nptII* gene, respectively, attached to the T_R2' promoter as monocistronic units.

Plasmids pJUH5 and pJUH6 contain the *lacZ* gene, encoding β-galactosidase, followed by the *nptII* gene in a dicistronic unit. Both genes are separated by a 22-bp intercistronic region. This unit is fused to the nopaline synthase (*nos*) promoter in pJUH5 and to the CaMV 35S promoter in pJUH6. In pHTT183, an *nptII-lacZ* dicistronic unit, in which both genes are separated by 27 bp, is under the control of the *nos* promoter. pSKH501 and pGSJ290 are monocistronic control plasmids with the *lacZ* gene and the *nptII* gene, respectively, attached to the 35S promoter.

The chimeric genes were cloned between T-DNA borders or in a pBR322 derivative which can be cointegrated between the T-DNA borders of the nononcogenic Ti plasmid pGV3850 (54). The plasmids were mobilized to the appropriate *A. tumefaciens* strains (see Materials and Methods) to allow the T-DNA to be stably integrated in the tobacco genome.

Plant transformation and selections. *N. tabacum* SR1 was transformed by leaf disk infection or cocultivation of regenerating protoplasts with the different *A. tumefaciens* strains. The T-DNAs of plasmids pGSFR163, pGSFR164, and pGSFR167 contain an *hpt* marker and the two marker genes of the dicistronic unit, *bar* and *nptII*. In cocultivation experiments, selection on hygromycin (25 mg/liter) and on phosphinothricin (1 mg/liter) gave a normal frequency of resistant calli (10% of the regenerating calli). Selection on kanamycin (50 mg/liter), however, gave a 100-fold-lower frequency of resistant calli than that obtained in a control experiment with the *A. tumefaciens* strain containing the monocistronic *nptII* transcriptional unit (pGSC1703). In a different set of experiments, tobacco leaf disks were cocultivated with *A. tumefaciens* strains containing pGV3850::pJUH5, pGV3850::pJUH6, pGV3850::pHTT183, pGV2260::pGSJ290, or pGV2260::pSKH501. Transformed calli and shoots were selected on medium supplemented with 25 or 100 mg of kanamycin per liter. Both selections gave similar results. Leaf disks infected with agrobacteria containing the *lacZ-nptII* dicistronic transcriptional units (pJUH5 and pJUH6) produced a much lower amount of Km^r shoots than did leaf disks infected with the other agrobacteria. This effect was more pronounced for pJUH5 than for pJUH6. However, it is not possible to calculate frequencies with the leaf disk infection procedure.

Expression of the downstream gene of the dicistronic unit. GSFR163, GSFR164, and GSFR167 plants, obtained by selection on hygromycin, were assayed for the expression of the downstream *nptII* gene. NPTII activity was detected in all the tissues tested, i.e., roots, leaves, and calli. To quantitate the level of NPTII produced by the dicistronic constructs, we compared NPTII activity in extracts of GSFR163, GSFR164, and GSFR167 plants with NPTII activity in serially diluted GSC1703 extracts. NPTII expression directed by the three dicistronic transcriptional units was 3,000- to 7,500-fold lower than that directed by the monocistronic control (Fig. 2A and B).

To exclude nonrepresentative expression levels in an individually chosen plant, we analyzed at least four plants for each construct, but no more than a two- to threefold difference in NPTII expression was observed.

The T_R2' promoter is stimulated by wounding and exhibits a marked tissue specificity (34, 46). The NPTII level in plants transformed with the dicistronic constructs is expected to follow the same pattern. Indeed, NPTII activity in wounded GSFR163, GSFR164, and GSFR167 leaves was 10 to 20 times higher than that in intact leaves, similar to the stimulation observed in GSC1703 plants. Also, NPTII activity expressed from the dicistronic units was low in leaves, high in calli, and highest in roots, corresponding to the distribution of NPTII activity observed in plants transformed with pGSC1703 (data not shown).

Figure 2B also demonstrates that the NPTII enzyme detected in GSFR163, GSFR164, and GSFR167 plants is not a degradation product of a PAT-NPTII fusion protein. Such a fusion protein could arise by ribosomal frameshifting when the *bar* and *nptII* coding sequences are in different reading frames (pGSFR164 and pGSFR167) or by translational readthrough over the *bar* stop codon in pGSFR163. In plants transformed with a construct in which the *bar* and *nptII* coding sequences were fused in-frame (pGSFRKm210; G. Angenon et al., manuscript in preparation), a degradation product of the PAT-NPTII fusion protein, migrating at the same position as the authentic NPTII enzyme, was indeed produced in low amounts. However, no fusion protein was detected in the GSFR163, GSFR164, or GSFR167 plants (Fig. 2B).

In a different series of experiments, leaves of JUH5 and JUH6 plants selected on kanamycin were assayed for the expression of the downstream *nptII* gene. JUH6 plants, which have the dicistronic unit under the control of the strong CaMV 35S promoter, all showed NPTII activity. By comparing extracts from JUH6 plants with serially diluted extracts from GSJ290 plants, we found that NPTII expression from the dicistronic unit was 1,000 to 2,000 times lower than that from the monocistronic control (Fig. 2C). In JUH5 plants, which have the dicistronic unit under the control of the weaker *nos* promoter, no NPTII activity could be detected, although these plants were selected on kanamycin. Similarly, HTT183 plants, which contain an *nptII-lacZ* dicistronic unit under the control of the *nos* promoter, did not show any detectable expression of the downstream *lacZ* gene.

These data allow us to conclude that with all our constructs the amount of gene product of the downstream cistron correlates in qualitative and quantitative ways with the expression pattern of the promoter driving the dicistronic unit.

DNA and RNA blot analyses. To verify that the T-DNA constructs were intact in the transgenic plants and that the two marker enzymes were translated from the dicistronic

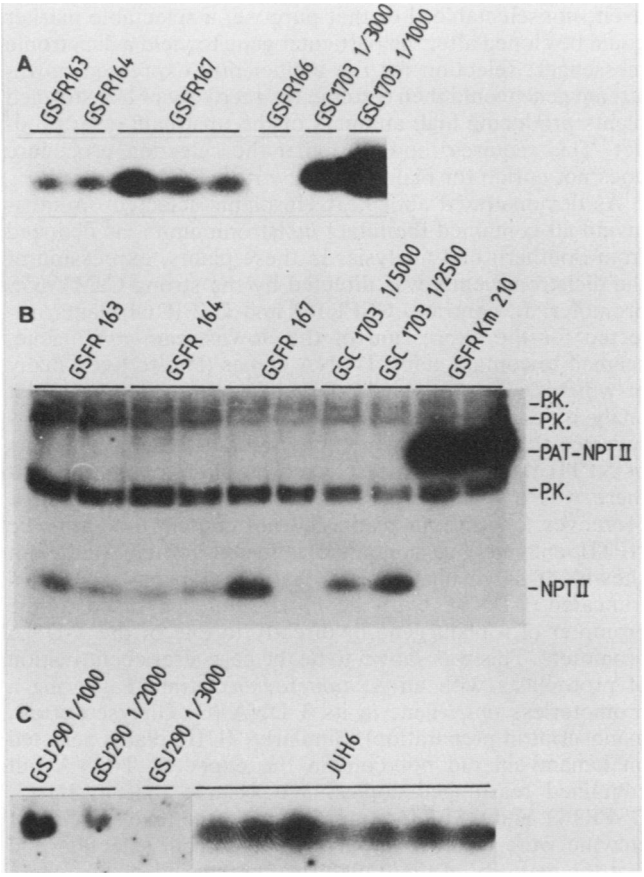

FIG. 2. (A) NPTII activity in root extracts from plants transformed with the *bar-nptII* dicistronic constructs and from control plants. Lanes 3 to 5 contain three independent pGSFR167 transformants. The total protein content of the samples loaded in lanes 1 and 2 was 50 μg, and that in lanes 3 to 6 was 100 μg; in lanes 7 and 8, 0.033 and 0.1 μg of total protein from a GSC1703 extract were loaded (representing 1/3,000 and 1/1,000 of the amount in lanes 3 to 6). To ensure a quantitative comparison, we diluted GSC1703 extracts with an extract from nontransformed SR1 roots. (B) NPTII activity in wounded leaves from plants transformed with the *bar-nptII* dicistronic constructs and from control plants. Two independent transformants were analyzed for each construct. The total soluble protein contents of the samples were 500 μg in lanes 1 to 6, 0.1 and 0.2 μg of a GSC1703 extract, diluted with an extract from nontransformed SR1 leaves, in lanes 7 and 8, respectively, and 1 μg of a GSFRKm210 extract, diluted with an SR1 extract, in lanes 9 and 10. The degradation product of the PAT-NPTII fusion protein produced in the GSFRKm210 plants could only be detected after a prolonged exposure of the filter. The P81 filter was treated with proteinase K to partially remove the nonspecific bands representing plant kinase (P.K.) activities. (C) NPTII activity in leaf extracts of seven independent JUH6 plants and in serially diluted extracts from a GSJ290 plant. The dilutions were made with extracts from nontransformed SR1 plants. The total protein content of the samples was 133 μg.

mRNAs, we examined the plants by Southern and Northern blot analyses.

Total DNA from GSFR163, GSFR164, and GSFR167 plants selected on hygromycin was digested with *Nco*I, which cuts in the *hpt* sequence and the *nptII* sequence of the T-DNA (Fig. 1). The DNA blot was probed with a *Hin*dIII-*Nco*I *nptII* fragment. As expected, the only hybridizing band was the 2-kilobase *Nco*I internal T-DNA fragment (Fig. 3A).

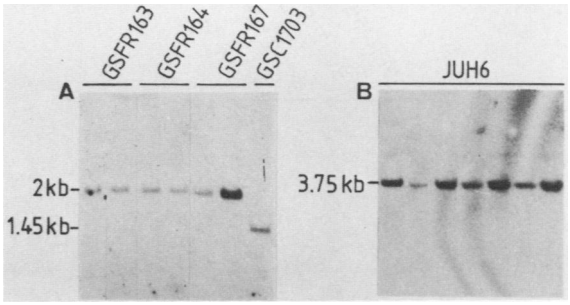

FIG. 3. Genomic characterization of the T-DNA structure in two independent GSFR163 (lanes 1 and 2), GSFR164 (lanes 3 and 4), and GSFR167 (lanes 5 and 6) transformants and a GSC1703 transgenic plant (lane 7). DNA (10 μg) was digested with *Nco*I. The blot was hybridized with an *nptII* probe extending from the 5′ end of the *nptII* gene to the *Nco*I site. (B) Genomic characterization of the T-DNA structure in seven independent JUH6 plants. DNA (5 μg) was digested with *Sac*I and *Hin*dIII. The blot was hybridized with an *nptII* probe. The sizes of the bands in kilobases (kb) are indicated to the left of each panel.

The pJUH6 construct does not contain a selectable marker apart from the dicistronic unit. Consequently, JUH6 plants were obtained by selection of the downstream gene of the dicistronic unit (*nptII*). Therefore, it was very important to exclude the possibility that NPTII expression in the JUH6 plants derived from a T-DNA copy truncated in front of the *nptII* gene and activated by insertion downstream of a plant promoter. Total DNA from JUH6 plants was cut with *Sac*I and *Hin*dIII (Fig. 1). Blots probed with an *nptII* fragment showed that only the 3,746-bp *Sac*I-*Hin*dIII internal T-DNA fragment hybridized (Fig. 3B). Blots probed with a *lacZ* fragment showed the same 3,746-bp fragment and a second 6,411-bp fragment extending from the *Sac*I site in *lacZ* to the *Hin*dIII site in the pBR sequences of the pGV3850 Ti plasmid, in which pJUH6 is cointegrated (data not shown). These results proved that the dicistronic unit is correctly inserted in the genome of these transgenic plants.

Northern analysis showed that the only transcript that could be detected with an *nptII* probe in GSFR163 and GSFR164 plants was 2.1 kilobases long, the expected size for the dicistronic mRNAs (Fig. 4). Even after prolonged exposure, no smaller mRNA species could be detected.

Translational efficiency of the downstream gene. To calculate the relative translational efficiency of the downstream gene of the *bar-nptII* dicistronic units, the amounts of NPTII enzyme in GSFR163, GSFR164, GSFR167, and GSC1703 transgenic plants were compared with the steady-state levels of the respective *nptII*-specific mRNAs. The PAT levels and the *bar*-specific mRNA levels were also determined. Protoplasts prepared from the respective transgenic plants served as the source of material for these experiments. The same batch of protoplasts was used for the preparation of RNA as well as for the measurement of NPTII and PAT activities (see Materials and Methods). The results of these experiments are shown in Fig. 5.

The level of NPTII activity in protoplasts prepared from the plants transformed with the *bar-nptII* dicistronic constructs was approximately 5,000-fold lower than that in protoplasts from GSC1703 plants (Fig. 5A), a result which is in accordance with the values found in root and leaf tissues (Fig. 2). However, the *bar-nptII* dicistronic mRNA levels were also approximately 5- to 15-fold lower than the *nptII* monocistronic mRNA level (Fig. 5C). Therefore, the relative

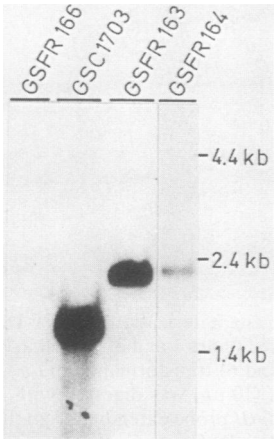

FIG. 4. Northern blot hybridization of poly(A)$^+$ RNA isolated from leaves of transgenic plants. The blot was probed with an *nptII* riboprobe. On the right, the sizes of RNA markers are indicated in kilobases (kb).

translational efficiency of the downstream gene was estimated by comparing the NPTII activity with the *nptII*-specific mRNA level in each individual plant (Table 1). The translational efficiencies of the *nptII* gene were about 500-, 1,000-, and 1,500-fold lower in the dicistronic constructs pGSFR163, pGSFR164, and pGSFR167, respectively, than in the monocistronic reference construct pGSC1703. Interestingly, a positive correlation was found between the translational efficiency of the downstream gene and the length of the intercistronic region in these three constructs.

The levels of the *bar*-, *bar-nptII*-, and *hpt*-specific mRNAs were also compared. The *bar-nptII* dicistronic unit and the *hpt* gene are transcribed from the closely linked T_R1' and T_R2' promoters, which are thought to be coordinately regulated (46, 51). Accordingly, the dicistronic and *hpt* transcripts were found to be present in about the same ratios in all plants, except for GSFR163-3 (Fig. 5D). Moreover, the steady-state levels of the dicistronic transcripts were 20- to 50-fold lower than those of the *bar* monocistronic transcript (Fig. 5D), although the promoter-gene fusion was identical in the pGSFR166 and dicistronic constructs. The reduced steady-state levels of the dicistronic mRNAs correlated very well with the PAT activities, which were also 20- to 50-fold lower in the GSFR163, GSFR164, and GSFR167 transgenic plants than in the GSFR166 plants (Fig. 5B). These reduced PAT levels were also observed in extracts from roots and leaves (data not shown).

We also compared the expression of the *lacZ* gene in plants transformed with either the *lacZ-nptII* dicistronic construct pJUH6 or the monocistronic control construct pSKH501. The *lacZ* activity was determined by a β-galactosidase assay of leaf extracts (46). The average β-galactosidase activity for seven JUH6 plants was 93 U/mg of total soluble protein, whereas it was 170 U/mg for six SKH501 plants. These results show that the expression of the upstream gene of the *lacZ-nptII* dicistronic construct was also reduced in comparison with that of the monocistronic control, albeit not to the same extent as with the *bar-nptII* dicistronic units.

Direct selection of the downstream gene of the dicistronic transcriptional unit. A possible application of dicistronic transcriptional units is the development of a selection strategy for enhanced expression of a gene product which is, by itself, nonselectable. For that purpose, a selectable marker could be cloned after the particular gene to yield a dicistronic messenger; selection for the inefficiently expressed downstream gene would then result in the recovery of transformed plants producing high amounts of the upstream gene product. This requires, however, that the selection procedure does not enrich for plants with aberrant T-DNA copies.

As demonstrated above, JUH6 plants selected on kanamycin all contained the intact dicistronic units, as deduced from Southern blot analysis. In these plants, expression of the dicistronic units was directed by the strong CaMV 35S promoter. In contrast, GSFR163 and GSFR164 plants, selected for the expression of the downstream *nptII* gene, seemed to contain many T-DNA copies (two to five), many of which were rearranged, as deduced from Southern blot analysis (data not shown). NPTII assays of these plants revealed that most of them expressed much higher amounts of NPTII than did plants selected on hygromycin and that there was a much larger variation in NPTII expression. Moreover, one in four plants did not contain the authentic NPTII enzyme but contained a fusion protein (data not shown). This result suggests that these plants contained truncated T-DNAs with the *nptII* sequence fused to the promoter of a plant gene or directly to one of the T-DNA promoters. This was shown to be the case after cocultivation of protoplasts with an *A. tumefaciens* strain harboring a promoterless *nptII* gene in its T-DNA (G. Gheysen et al., manuscript in preparation). Similarly, JUH5 plants selected on kanamycin did not contain the expected T-DNA but contained rearranged copies. We assume that in JUH5, GSFR163, and GSFR164 transformants, the levels of NPTII enzyme were too low to survive kanamycin selection and that the majority of the transformants that did survive were those which contained rearranged T-DNAs.

DISCUSSION

Low-efficiency expression of the downstream gene of the dicistronic unit. We have investigated whether two proteins can be translated from dicistronic mRNAs in plants and how efficiently the downstream gene is expressed. This is the first report on the translational efficiency of dicistronic transcriptional units stably integrated into the plant genome.

We have analyzed plants stably transformed with a set of dicistronic T-DNA constructs with different promoters, different marker genes, and different intercistronic regions. The downstream gene of these various dicistronic units is expressed at a drastically reduced level, and its translational efficiency is 500- to 1,500-fold lower than that in a monocistronic control.

Southern blot analysis showed that the T-DNAs are intact in the transgenic plants, and Northern blot analysis confirmed that the only transcripts that can be detected with a probe specific for the downstream gene have the sizes expected for the dicistronic mRNAs. We cannot completely exclude the possibility that the downstream gene product is translated from a smaller transcript of very low abundance and generated from a cryptic promoter or derived from the original dicistronic mRNA by splicing. However, this possibility is very unlikely, for the following reasons. Firstly, the gene product corresponding to the downstream cistron (NPTII), retains the well-characterized expression pattern of the promoter to which the dicistronic units are fused with all our constructs. This result argues strongly against the use of a cryptic promoter. In GSFR163, GSFR164, and GSFR167 plants, the observed tissue specificity and stimulation by

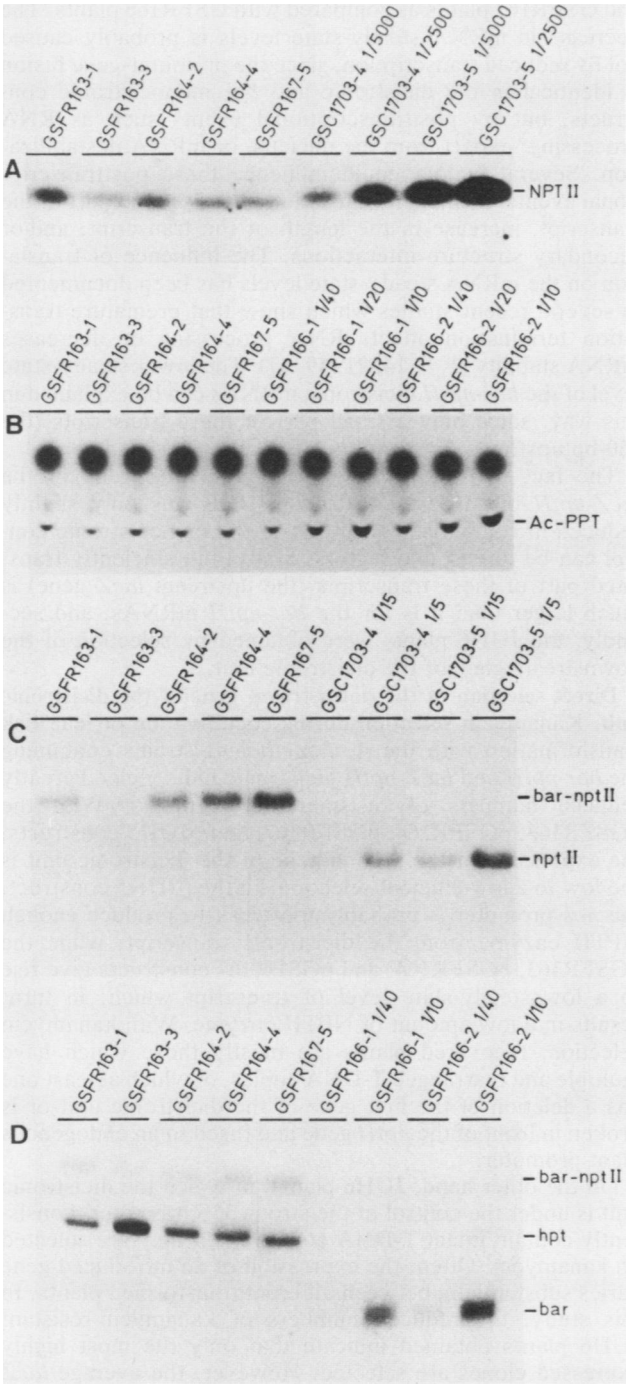

FIG. 5. Measurement of NPTII activity (A), PAT activity (B), *nptII*-specific mRNA levels (C), and *bar*- and *hpt*-specific mRNA levels (D) in protoplasts prepared from GSFR163, GSFR164, GSFR167, GSC1703, and GSFR166 plants. (A) NPTII assay. The total soluble protein content of the samples in lanes 1 to 5 was 300 μg. In lanes 6 to 9, the indicated dilutions of GSC1703 extracts were loaded; dilutions were made with an extract of protoplasts from nontransformed SR1 plants. (B) PAT assay. The samples in lanes 6 to 11 were diluted as indicated with an extract of SR1 protoplasts. Ac-PPT, Acetylated phosphinothricin. (C) Northern blot hybridization with an *nptII*-specific riboprobe. In lanes 1 to 5, 12 μg of total RNA was loaded. The samples in lanes 6 to 9 contained total RNA from GSC1703 protoplasts diluted with total RNA from SR1 protoplasts. (D) Northern blot hybridization with *bar*- and *hpt*-specific riboprobes. In lanes 1 to 5, 12 μg of total RNA was loaded. The samples in lanes 6 to 9 contained total RNA from GSFR166 protoplasts diluted with total RNA from SR1 protoplasts.

wounding are as expected for a T_R2'-driven transcript. In JUH5 and JUH6 plants, there is a correlation between NPTII activity and promoter strength: NPTII activity derived from an intact T-DNA copy can be detected in JUH6 plants, which contain a *lacZ-nptII* dicistronic unit under the control of the 35S promoter, but not in JUH5 plants, which contain the same dicistronic unit under the control of the *nos* promoter. The *nos* promoter has been shown to be at least one order of magnitude weaker than the 35S promoter (43). Secondly, a spliced derivative of the dicistronic mRNA is unlikely to be the transcript from which NPTII is translated. Since the *bar* coding sequence contains multiple AUG codons, only a precise excision of the *bar* gene from the *bar-nptII* dicistronic transcripts would generate an mRNA in which the first AUG codon is the *nptII* initiator codon. In view of the different intercistronic regions in constructs pGSFR163, pGSFR164, and pGSFR167, such a precise splicing event is even less probable. Similarly, in JUH6 plants, the precise excision of the *lacZ* coding sequence, which contains as many as 55 AUG codons, would be required to generate an easily translatable transcript.

From the experiments described here it is not possible to distinguish between the two mechanisms which could be responsible for the expression of the downstream cistron: reinitiation by ribosomes which have translated the upstream gene or direct internal initiation. Most of the evidence in the literature favors reinitiation and not direct internal binding of ribosomes to explain the synthesis of the downstream gene product (for a review, see reference 33). Only picornaviruses could represent an exception: a region several hundreds of nucleotides long, derived from the leaders of some of these viruses, allows internal binding of ribosomes (20, 42).

The translational efficiency of the downstream gene which we observed (approximately 0.1%) is lower than that found in most other studies dealing with polycistronic mRNAs (1 to 20%). Only Kaufmann et al. (26) have observed translational efficiencies of the second gene of 1% or less. In this respect,

TABLE 1. Relative translational efficiencies of the downstream gene of the *bar-nptII* dicistronic units

Plant	Level of:		Relative translational efficiency[c]
	NPTII[a]	*nptII*-specific mRNA[b]	
GSFR163-1	1/3,000	1/6	1/500
GSFR163-3	1/6,000	1/15	1/400
GSFR164-2	1/4,000	1/5	1/800
GSFR164-4	1/5,000	1/4	1/1,250
GSFR167-5	1/5,000	1/3	1/1,660
GSC1703-4	1	1	1
GSC1703-5	2.2	2.2	1

[a] NPTII levels, relative to the level in plant GSC1703-4, were estimated from the NPTII assay shown in Fig. 5A.

[b] *nptII*-specific mRNA levels, relative to the level in plant GSC1703-4, were estimated from the Northern blot analyses shown in Fig. 5C; the values are averages of two independent RNA blots.

[c] Ratio between the relative NPTII level and the relative mRNA level. Average translational efficiencies for GSFR163-1 and GSFR163-3, for GSFR164-2 and GSFR164-4, for GSFR167-5, and for GSC1703-4 and GSC1703-5 were 1/450, 1/975, 1/1,660, and 1, respectively.

any of at least four factors might affect the level of expression of the downstream gene.

Firstly, the length of the upstream cistron is likely to have a strong influence on the translational efficiency of the downstream gene. Constructs which have an upstream minicistron allow reinitiation efficiencies of between 10 and 100% (16, 22, 28, 32, 35). In contrast, constructs with a normal-sized upstream cistron exhibit lower translational efficiencies for the downstream cistron (2, 17, 26, 41). One of the reasons why the length of the upstream cistron could affect translation of the downstream cistron is that initiation factors might remain associated with the 40S ribosomal subunit upon initiation of translation and be gradually released during elongation. Consequently, these factors would still be present after translation of a minicistron but not after translation of a normal-sized cistron, resulting in a poor reinitiation in the latter case (32, 33).

Another important factor is the length of the intercistronic region. Kozak (32) has observed that in constructs with upstream minicistrons, the reinitiation frequency is about 5% when the stop codon of the minicistron and the start codon of the downstream cistron overlap in an ATGA configuration, whereas reinitiation frequencies are 7 to 10, 20, and 50 to 100% with intercistronic regions of 2 to 11, 45, and 79 bp, respectively. Although the values we found with the *bar-nptII* constructs were much lower, the same tendency was noted: the translational efficiency of the downstream gene was higher for constructs with a longer intercistronic region. However, we do not exclude the possibility that the difference in translational efficiency is caused by the nucleotide sequence rather than by the length of the intercistronic region.

A third factor is the assay system used for studying polycistronic transcripts. It is clear that viral, in vitro translation, and transient expression systems do not always faithfully represent the situation in stably transformed cells. For instance, during in vitro translation in reticulocyte lysates, in addition to the full-length translation product, incomplete polypeptides are produced in considerable amounts, arising from both premature termination and initiation at internal sites (3, 6). Transient expression in carrot cells or translation in vitro of dicistronic constructs containing two adjacent CaMV ORFs (2, 17) yielded higher values for the translational efficiency of the downstream gene than that which we found in transgenic plants. Viruses, on the other hand, may contain specific translational signals in the mRNA or may alter the translational machinery of the host to enhance initiation at downstream cistrons. Such could be the case for CaMV. Evidence that several proteins are translated from the CaMV 35S transcript by a reinitiation mechanism is derived from deletion mutagenesis studies (13; Sieg and Gronenborn, Abstr. NATO Adv. Stud. Inst. 1982). However, a reinitiation mechanism with an efficiency of about 0.1%, like that which we found in tobacco plants, cannot be responsible for what is observed in CaMV-infected cells. Probably a virus- or host-encoded factor is responsible for enhancing translation of the downstream genes (17).

Finally, the plant and mammalian translational machineries could have different potentials for reinitiation.

Abundance of the dicistronic and monocistronic mRNAs. The steady-state level of the *bar-nptII* dicistronic transcripts is reduced severalfold in comparison with those of both the *bar* and the *nptII* monocistronic transcripts (20- to 50-fold and 5- to 15-fold, respectively). This result correlates with a similar reduction in PAT activity in GSFR163, GSFR164, and GSFR167 plants as compared with GSFR166 plants. The decrease in mRNA steady-state levels is probably caused not by reduced transcription, since the promoter-gene fusion is identical in the dicistronic and *bar* monocistronic constructs, but by posttranscriptional events such as RNA processing, export from the nucleus, or mRNA destabilization. Several factors could influence these posttranscriptional events: inefficient translation of the second part of the transcript, increase in the length of the transcript, and/or secondary structure interactions. The influence of translation on the mRNA steady-state levels has been documented in several recent studies which show that premature translation termination affects RNA processing or decreases mRNA stability (1, 5, 14, 21, 39, 47). The lower steady-state level of the *bar-nptII* dicistronic mRNAs can be explained in this way, since only a small part of these transcripts (the 550-bp upstream *bar* gene) is efficiently translated.

The fact that the expression of the *lacZ* gene in the *lacZ-nptII* dicistronic construct pJUH6 was only slightly reduced in comparison with that in the monocistronic control can be due to two factors: firstly, the efficiently translated part of these transcripts (the upstream *lacZ* gene) is much larger than it is for the *bar-nptII* mRNAs, and secondly, the JUH6 plants were obtained by selection of the downstream gene of the dicistronic unit.

Direct selection of the downstream gene of the dicistronic unit. Kanamycin selection during cocultivation or leaf disk transformation with the *A. tumefaciens* strains containing the *bar-nptII* and *lacZ-nptII* dicistronic units yielded greatly reduced numbers of resistant transformants. With the pGSFR163, pGSFR164, pGSFR167, and pJUH5 constructs, the expression of the second gene of the dicistronic unit is too low to allow efficient selection. In the pJUH5 construct, the *nos* promoter is probably too weak to produce enough NPTII enzyme from the dicistronic transcript, while the pGSFR163, pGSFR164, and pGSFR167 constructs give rise to a low steady-state level of transcript which, in turn, results in a low amount of NPTII enzyme. With kanamycin selection, recovered plants are mostly those which have multiple and rearranged T-DNA copies, of which at least one has a deletion of the first gene of the dicistronic unit or is broken in front of the *nptII* gene and fused to an endogenous plant promoter.

On the other hand, JUH6 plants, in which the dicistronic unit is under the control of the strong 35S promoter, consistently contain intact T-DNA copies when they are selected on kanamycin. Often, the expression of an introduced gene varies substantially between different transformed plants. In this study, the reduced numbers of kanamycin-resistant JUH6 plants obtained indicate that only the most highly expressed clones are selected. However, the average *lacZ* activity in these plants is still lower than that in the SKH501 plants, probably because of the negative effects of the dicistronic gene configuration on the mRNA steady-state level, as discussed above.

In conclusion, our results show that the downstream gene of a dicistronic transcriptional unit stably integrated in the plant genome is expressed with a lower efficiency than might have been assumed from previous studies. The inefficient translation of the downstream cistron agrees with the predictions of the scanning model for translation initiation and demonstrates the disadvantages of polycistronic gene configurations in eucaryotic organisms. On the other hand, dicistronic transcriptional units are potentially useful as a tool to select transgenic plants with enhanced levels of

expression of a nonselectable gene, provided that a high steady-state level of the dicistronic mRNA is obtained.

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LITERATURE CITED

1. **Baserga, S. J., and E. J. Benz, Jr.** 1988. Nonsense mutations in the human β-globin gene affect mRNA metabolism. Proc. Natl. Acad. Sci. USA **85**:2056–2060.
2. **Baughman, G., and S. H. Howell.** 1988. Cauliflower mosaic virus 35S RNA leader region inhibits translation of downstream genes. Virology **167**:125–135.
3. **Brierley, I., M. E. G. Boursnell, M. M. Binns, B. Bilimoria, V. C. Blok, T. D. K. Brown, and S. C. Inglis.** 1987. An efficient ribosomal frame-shifting signal in the polymerase-encoding region of the coronavirus IBV. EMBO J. **6**:3779–3785.
4. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
5. **Daar, I. O., and L. E. Maquat.** 1988. Premature translation termination mediates triosephosphate isomerase mRNA degradation. Mol. Cell. Biol. **8**:802–813.
6. **Dasso, M. C., and R. J. Jackson.** 1989. On the fidelity of mRNA translation in the nuclease-treated rabbit reticulocyte lysate system. Nucleic Acids Res. **17**:3129–3144.
7. **Deblaere, R., B. Bytebier, H. De Greve, F. Deboeck, J. Schell, M. Van Montagu, and J. Leemans.** 1985. Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene transfer to plants. Nucleic Acids Res. **13**:4777–4788.
8. **Deblaere, R., A. Reynaerts, H. Höfte, J.-P. Hernalsteens, J. Leemans, and M. Van Montagu.** 1987. Vectors for cloning in plant cells. Methods Enzymol. **153**:277–292.
9. **De Block, M., J. Botterman, M. Vandewiele, J. Dockx, C. Thoen, V. Gosselé, R. Movva, C. Thompson, M. Van Montagu, and J. Leemans.** 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. EMBO J. **6**:2513–2518.
10. **De Greve, H., P. Dhaese, J. Seurinck, M. Lemmers, M. Van Montagu, and J. Schell.** 1982. Nucleotide sequence and transcript map of the *Agrobacterium tumefaciens* Ti plasmid-encoded octopine synthase gene. J. Mol. Appl. Genet. **1**:499–512.
11. **Dellaporta, S. L., J. Wood, and J. B. Hicks.** 1983. A plant DNA minipreparation: version II. Plant Mol. Biol. Reporter **1**:19–21.
12. **Denecke, J. V. Gosselé, J. Botterman, and M. Cornelissen.** 1989. Quantitative analysis of transiently expressed genes in plant cells. Methods Mol. Cell. Biol. **1**:19–27.
13. **Dixon, L. K., and T. Hohn.** 1984. Initiation of translation of the cauliflower mosaic virus genome from a polycistronic mRNA: evidence from deletion mutagenesis. EMBO J. **3**:2731–2736.
14. **Eccles, S. J., M. A. Vidal, C. J. Wrighton, F. G. Grosveld, and J. F. Burke.** 1989. Rapid detection of ultraviolet-induced reversion of an amber mutation in mouse L cells. Proc. Natl. Acad. Sci. USA **86**:1297–1301.
15. **Figurski, D. H., and D. R. Helinski.** 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648–1652.
16. **French, R., M. Janda, and P. Ahlquist.** 1986. Bacterial gene inserted in an engineered RNA virus: efficient expression in monocotyledonous plant cells. Science **231**:1294–1297.
17. **Gordon, K., P. Pfeiffer, J. Fütterer, and T. Hohn.** 1988. *In vitro* expression of cauliflower mosaic virus genes. EMBO J. **7**: 309–317.
18. **Herrera-Estrella, L., M. De Block, E. Messens, J.-P. Hernalsteens, M. Van Montagu, and J. Schell.** 1983. Chimeric genes as dominant selectable markers in plant cells. EMBO J. **2**:987–995.
19. **Herrera-Estrella, L., A. Depicker, M. Van Montagu, and J. Schell.** 1983. Expression of chimeric genes transferred into plant cells using a Ti-plasmid-derived vector. Nature (London) **303**: 209–213.
20. **Jang, S. K., H.-G. Kräusslich, M. J. H. Nicklin, G. M. Duke, A. C. Palmenberg, and E. Wimmer.** 1988. A segment of the 5′ nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. J. Virol. **62**:2636–2643.
21. **Jofuku, K. D., R. D. Schipper, and R. B. Goldberg.** 1989. A frameshift mutation prevents Kunitz trypsin inhibitor mRNA accumulation in soybean embryos. Plant Cell **1**:427–435.
22. **Johansen, H., D. Schümperli, and M. Rosenberg.** 1984. Affecting gene expression by altering the length and sequence of the 5′ leader. Proc. Natl. Acad. Sci. USA **81**:7698–7702.
23. **Jones, J. D. G., C. Dean, D. Gidoni, D. Gilbert, D. Bond-Nutter, R. Lee, J. Bedbrook, and P. Dunsmuir.** 1988. Expression of bacterial chitinase protein in tobacco leaves using two photosynthetic gene promoters. Mol. Gen. Genet. **212**:536–542.
24. **Jones, J. D. G., P. Dunsmuir, and J. Bedbrook.** 1985. High level expression of introduced chimeric genes in regenerated transformed plants. EMBO J. **4**:2411–2418.
25. **Joshi, C. P.** 1987. An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res. **15**:6643–6653.
26. **Kaufman, R., P. Murtha, and M. Davies.** 1987. Translational efficiency of polycistronic mRNAs and their utilization to express heterologous genes in mammalian cells. EMBO J. **6**: 187–193.
27. **Kozak, M.** 1981. Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res. **9**:5233–5252.
28. **Kozak, M.** 1984. Selection of initiation sites by eucaryotic ribosomes: effect of inserting AUG triplets upstream from the coding sequence for preproinsulin. Nucleic Acids Res. **12**: 3873–3893.
29. **Kozak, M.** 1986. Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. Cell **44**:283–292.
30. **Kozak, M.** 1987. At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. J. Mol. Biol. **196**:947–950.
31. **Kozak, M.** 1987. An analysis of 5′-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. **15**:8125–8148.
32. **Kozak, M.** 1987. Effects of intercistronic length on the efficiency of reinitiation by eucaryotic ribosomes. Mol. Cell. Biol. **7**: 3438–3445.
33. **Kozak, M.** 1989. The scanning model for translation: an update. J. Cell Biol. **108**:229–241.
34. **Langridge, W. H. R., K. J. Fitzgerald, C. Koncz, J. Schell, and A. A. Szalay.** 1989. Dual promoter of *Agrobacterium tumefaciens* mannopine synthase genes is regulated by plant growth hormones. Proc. Natl. Acad. Sci. USA **86**:3219–3223.
35. **Liu, C.-C., C. C. Simonsen, and A. D. Levinson.** 1984. Initiation of translation at internal AUG codons in mammalian cells. Nature (London) **309**:82–85.
36. **Lütcke, H. A., K. C. Chow, F. S. Mickel, K. A. Moss, H. F. Kern, and G. A. Scheele.** 1987. Selection of AUG initiation codons differs in plants and animals. EMBO J. **6**:43–48.
37. **Maliga, P., A. Sz.-Breznovits, and L. Márton.** 1973. Streptomycin-resistant plants from callus culture of haploid tobacco. Nature (London) New Biol. **244**:29–30.
38. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
39. **Nilsson, G., J. G. Belasco, S. N. Cohen, and A. von Gabain.** 1987. Effect of premature termination of translation on mRNA stability depends on the site of ribosome release. Proc. Natl. Acad. Sci. USA **84**:4890–4894.

40. **Odell, J. T., F. Nagy, and N.-H. Chua.** 1985. Identification of DNA sequences required for the activity of the cauliflower mosaic virus 35S promoter. Nature (London) **313**:810–812.
41. **Peabody, D., and P. Berg.** 1986. Termination-reinitiation occurs in the translation of mammalian cell mRNAs. Mol. Cell. Biol. **6**:2695–2703.
42. **Pelletier, J., and N. Sonenberg.** 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature (London) **334**:320–325.
43. **Sanders, P. R., J. A. Winter, A. R. Barnason, S. G. Rogers, and R. T. Fraley.** 1987. Comparison of cauliflower mosaic virus 35S and nopaline synthase promoters in transgenic plants. Nucleic Acids Res. **15**:1543–1558.
44. **Schreier, P. H., E. A. Seftor, J. Schell, and H. J. Bohnert.** 1985. The use of nuclear-encoded sequences to direct the light-regulated synthesis and transport of a foreign protein into plant chloroplasts. EMBO J. **4**:25–32.
45. **Taylor, J. L., J. D. G. Jones, S. Sandler, G. M. Mueller, J. Bedbrook, and P. Dunsmuir.** 1987. Optimizing the expression of chimeric genes in plant cells. Mol. Gen. Genet. **210**:572–577.
46. **Teeri, T. H., H. Lehväslaiho, M. Franck, J. Uotila, P. Heino, E. T. Palva, M. Van Montagu, and L. Herrera-Estrella.** 1989. Gene fusions to *lacZ* reveal new expression patterns of chimaeric genes in transgenic plants. EMBO J. **8**:343–350.
47. **Urlaub, G., P. J. Mitchell, C. J. Ciudad, and L. A. Chasin.** 1989. Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. Mol. Cell. Biol. **9**:2868–2880.
48. **Van den Broeck, G., M. P. Timko, A. P. Kausch, A. R. Cashmore, M. Van Montagu, and L. Herrera-Estrella.** 1985. Targeting of a foreign protein to chloroplasts by fusion to the transit peptide of ribulose 1,5-bisphosphate carboxylase. Nature (London) **313**:358–363.
49. **Van den Elzen, P. J. M., J. Townsend, K. Y. Lee, and J. R. Bedbrook.** 1985. A chimaeric hygromycin resistance gene as a selectable marker in plant cells. Plant Mol. Biol. **5**:299–302.
50. **Van Haute, E., H. Joos, M. Maes, G. Warren, M. Van Montagu, and J. Schell.** 1983. Intergeneric transfer and exchange recombination of restriction fragments cloned in pBR322: a novel strategy for the reversed genetics of Ti plasmids of *Agrobacterium tumefaciens*. EMBO J. **2**:411–418.
51. **Velten, J., and J. Schell.** 1985. Selection-expression plasmid vectors for use in genetic transformation of higher plants. Nucleic Acids Res. **13**:6981–6998.
52. **Velten, J., L. Velten, R. Hain, and J. Schell.** 1984. Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*. EMBO J. **3**:2723–2730.
53. **Wang, F., L. Petti, D. Braun, S. Seung, and E. Kieff.** 1987. A bicistronic Epstein-Barr virus mRNA encodes two nuclear proteins in latently infected, growth-transformed lymphocytes. J. Virol. **61**:945–954.
54. **Zambryski, P., H. Joos, C. Genetello, J. Leemans, M. Van Montagu, and J. Schell.** 1983. Ti plasmid vector for the introduction of DNA into plant cells without alteration of their normal regeneration capacity. EMBO J. **2**:2143–2150.