Different 3’ End Regions Strongly Influence the Level of Gene Expression in Plant Cells

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We have investigated the functional role of a 3’ end region on the expression of a reporter gene in plant cells. In stably transformed plants, expression of the reporter gene without a plant gene 3’ end is variable and depends on the fortuitous presence of polyadenylation signals in the downstream sequences. When the reporter gene is flanked by pBR322 DNA, 3’-processing and polyadenylation occurs at (a) cryptic site(s) within these vector sequences. Using a transient gene expression system, we present a deletion analysis of the 3’ end of the octopine synthase gene showing that the most proximal polyadenylation signal per se is not sufficient to ensure expression but that a downstream (G)T-rich sequence is also required. Optimal expression of the fusion gene requires more than 98 base pairs and at most 142 base pairs downstream from the most distal polyadenylation site. We analyzed the expression of chimeric genes with 3’ end sequences originating from different plant genes. In the transient expression assay, all constructs direct similar neomycin phosphotransferase II activities. However, in stably transformed tissue, the gene constructs displayed characteristic expression levels which varied as much as 60-fold. This result suggests a role for 3’ end sequences in post-transcriptional processes such as efficiency of 3’-processing and/or mRNA stability.

INTRODUCTION

In eukaryotic cells, biogenesis of a functional mRNA requires complex post-transcriptional modifications including 5’-capping, intron splicing, and 3’ end-processing. In animal cells, formation of most mRNA 3’ ends involves endonucleolytic cleavage of larger precursors followed by the addition of up to 250 adenylate residues [the poly(A) tract] (for review, see Birnstiel, Busslinger, and Strub, 1985; Proudfoot and Whitelaw, 1987). Extensive deletion analysis and site-specific mutagenesis have defined two cis-acting elements that are both essential for accurate and efficient 3’ end formation: the highly conserved AAUAAA, located 10 nucleotides to 30 nucleotides upstream from the poly(A) addition site (Proudfoot and Brownlee, 1976; Fitzgerald and Shenk, 1981; Montell et al., 1983; Wickens and Stephenson, 1984) and the less conserved (G)T-rich sequences immediately 3’ to the cleavage site (Gil and Proudfoot, 1984; Woychik et al., 1984; Hart, McDevitt, and Nevins, 1985; McLauchlan et al., 1985; McDevitt et al., 1986; Gil and Proudfoot, 1987). In addition to these sequences, the 3’-untranslated region can also contain regulatory sequences that affect the mRNA stability. For example, a family of mammalian mRNAs encoding growth regulatory functions contains a conserved AT-rich element in their 3’-noncoding region that mediates the selective degradation of these mRNAs (Caput et al., 1986; Shaw and Kamen, 1986; Brewer and Ross, 1988). Similarly, sequences in the trailer of the Drosophila hsp70 mRNA are important in directing its turnover (Simcox et al., 1985; Petersen and Lindquist, 1988). Together, these data indicate that 3’ sequences can play an important role in the establishment of mRNA prevalence levels in animal cells.

In contrast, little information is available about the sequences governing 3’-processing and polyadenylation of plant pre-mRNAs. Compilation analyses indicate that an AATAAA-like sequence is conserved among nuclear plant gene 3’-untranslated sequences, implying a common function for this sequence in both plant and animal cells (Joshi, 1987; Elliston and Messing, 1988). Although a majority of plant genes do not contain a perfect AATAAA signal, this plant polyadenylation signal rarely deviates from the animal sequence in more than one position, most of the bias being restricted to position 6. Recently, Hunt et al. (1987) reported that tobacco cells do not recognize the polyadenylation signals of a human and two animal virus genes as animal cells do. Apparently, there must be significant differences regarding either the sequence requirements for 3’-processing and polyadenylation or the utilization of these processing signals in animal and plant cells.

In this paper we have investigated the role of a 3’ end...
importance of 3′-processing signals for efficient expression of a gene

To evaluate the influence of 3′ end sequences on gene expression in plant cells, chimeric genes were constructed, based on the neomycin phosphotransferase II (nptII) coding sequence (Herrera-Estrella et al., 1983) fused to the cauliflower mosaic virus 35S promoter (Odell, Nagy, and Chua, 1985). The plasmids pHBSOCS and pHBSD contain a P35S-nptII chimeric gene with and without the 3′ end of the octopine synthase gene (3′ ocs) (De Greve et al., 1982), respectively. Both plasmids also contain a hygromycin resistance gene under the control of the nopaline synthase promoter as an independent selection marker (van den Elzen et al., 1985). Using the pGV3850 Agrobacterium transfer system (Zambryski et al., 1983), these chimeric genes were introduced into the tobacco genome. Figure 1 shows the structure of the two T-DNAs after integration in the plant genome.

Hygromycin-resistant calli were selected and, after regeneration of plants, a neomycin phosphotransferase II (nptII) enzymatic assay was performed on leaf extracts of independent transformants. Transformed plants containing the pHBSOCS T-DNA (the HBSOCS plants) show a 12-fold higher amount of NPTII activity than that observed in the HBSD plants (data not shown). For each construct, six transformants were assayed and, in all cases, expression levels varied as much as 60-fold among the various constructs. We conclude that posttranscriptional processes, associated with 3′ end formation, are important in determining mRNA prevalences.

RESULTS

Importance of 3′-Processing Signals for Efficient Expression of a Gene

To determine whether gene expression can be detected when this pBR322 sequence is removed, the same nptII chimeric genes were introduced into plant cells via the pGV1503 T-DNA vector (see Methods). After transfer and integration of the T-DNA, the nptII stop codon of the chimeric gene without 3′ end is located 360 bp from the right T-DNA border, as illustrated in Figure 1. Hygromycin-resistant calli were selected, and, after plant regeneration, leaf tissue was assayed for NPTII activity. As expected, the NPTII activity detected in GVOCS plants was very similar to the activity found in the HBSOCS plants (data not shown).
Influence of 3' End Regions on Gene Expression

Figure 2. RNA Gel Blot Analysis of Transcripts from the P35S-\textit{nptll} Gene with and without the 3' ocs Fragment in Stably Transformed Plants and after Transient Expression.

Hybridizations were carried out using the \textit{nptll} coding region as a probe. Blots containing total RNA (A, B) and poly(A)$^+$ RNA (B) were simultaneously reacted with a probe representing the \(\beta\)-subunit mitochondrial ATP synthase gene as an internal standard; the hybridizing transcript is 2.1 kb in length.

(A) RNA gel blot containing total, poly(A)$^+$, and poly(A)$^-$ RNA from leaf tissue transformed with the T-DNA of the plasmids pHBSOCS and pHBSD as indicated above the individual lanes. The \textit{nptll} transcripts are 1.1 kb and 1.2 kb in length in HBSOCS and HBSD plants, respectively.

(B) RNA gel blot containing total, poly(A)$^+$, and poly(A)$^-$ RNA from leaf tissue transformed with the T-DNA of the plasmids pGVOCS and pGVD as indicated above each lane. The hybridizing \textit{nptll} transcripts are 1.1 kb long in GVOCS and GVD2 plants and 1.4 kb long in the GVD1 plant.

(C) RNA gel blot containing total and poly(A)$^+$ RNA isolated from tobacco protoplasts 1 hr (a) and 4 hr (b) after electroporation with the indicated plasmids. pHBSOCS control lane contains 5 \(\mu\)g of total RNA from an HBSOCS transgenic plant.

not shown). However, of the 10 GVD transgenic plants analyzed, seven did not contain detectable NPTII activity, whereas three plants showed variable levels of expression. To determine whether the NPTII activity in two of these GVD plants was correlated with the presence of a defined transcript, RNA gel blot analysis was performed. Figure 2B demonstrates that the transcripts hybridizing with an \textit{nptll} probe are less abundant in the GVD plants than in the GVOCS plant. More important, the \textit{nptll} transcripts are detected only in the poly(A)$^+$ fractions and have clearly different lengths. These results show that the plant DNA downstream from the \textit{nptll} gene must contain cryptic polyadenylation signals that permit mRNA 3'-processing and/or stabilization with reduced efficiency.

Because of this inherent background of gene expression associated with chimeric gene integration, we tested whether a transient gene expression assay system could be used to identify 3' sequences necessary for efficient 3'-processing and polyadenylation of the chimeric \textit{nptll} transcript. We introduced the plasmids pGemOCS and pGemD, which contain a chimeric \textit{nptll} gene with and without the 3' ocs, respectively, into tobacco protoplasts by electroporation and then assayed protoplast extracts for both NPTII activity and \textit{nptll} mRNA. Figure 2C shows that a polyadenylated \textit{nptll} transcript was detected in pGemOCS-containing protoplast extracts that was equal in length to that present in stably transformed leaf tissue. In contrast, no \textit{nptll} transcript was detected in either the total or poly(A)$^+$ RNA isolated from protoplasts electroporated with pGemD (Figure 2C). Similarly, the NPTII enzymatic activity level in pGemOCS-containing cells was at least 20-fold higher than in cells containing the pGemD plasmid (data not shown).

Together these data show that DNA sequences contained within the 3' ocs region are essential for efficient expression of the chimeric \textit{nptll} gene in stably transformed plants. When a 3' end is absent, expression of the chimeric \textit{nptll} gene is dependent on the presence of cryptic poly-
adenylation signals in the downstream DNA region. Moreover, 3′ end sequences are also required for the expression of genes within isolated tobacco protoplasts after transient expression of electroporated plasmids.

Deletion Analysis of the 3′ End of the Octopine Synthase Gene via Transient Gene Expression

To begin to localize the cis sequences present within the 3′ ocs region that are necessary for optimal nptll fusion gene expression levels, we constructed a series of 3′ deletions and then introduced these constructs into tobacco protoplasts by electroporation. As an internal control, we included a chimeric chloramphenicol acetyltransferase (cat) gene within each construct to reduce experimental errors due to differential uptake of the exogenously added DNA by the protoplasts. For each construction, we first measured a fraction of the protoplast extract for chloramphenicol acetyltransferase (CAT) activity using a quantitative “diffusion” assay (Neumann, Morency, and Russian, 1987) and then determined NPTII enzymatic levels in fractions containing identical amounts of CAT activity. Figure 3A shows schematically the intact 3′ ocs fragment present in plasmid pCNOCS and the various 3′ deletions present in plasmids pCNOCSΔ1 through pCNOCSΔ6. As illustrated in Figure 3A and shown in Figure 3C, the 3′ ocs fragment contains two polyadenylation sites at positions 178 and 152, designated the major and minor sites, respectively (Dhaese et al., 1983). As shown in Figure 3B and summarized in Figure 3C, NPTII activity levels were not affected by deletion of the distal 385 bp (pCNOCSΔ1 lane). In contrast, NPTII levels decreased fourfold upon deletion of sequences between 98 bp and 142 bp downstream from the major polyadenylation site (pCNOCSΔ2 lane) and then dropped to background levels only after deletion of the 35-bp region containing the “putative” major polyadenylation signal and both minor and major sites (compare pCNOCSΔ4 and pCND). RNA gel blot analysis showed similar reductions in relative nptll mRNA levels (data not shown).

Together, these data indicate that maximal nptll expression levels require sequences contained within a region extending 144 bp downstream from the major polyadenylation site but that the most crucial signals are found within a 35-bp fragment that contains the major polyadenylation signal and the minor and major polyadenylation sites.

Modulation of the nptll Gene Expression Levels by Different Plant 3′ Gene Sequences

In the previous experiments, we demonstrated that cryptic polyadenylation signals can be used to produce variable protein and steady-state mRNA levels. To find out whether different plant gene 3′ regions similarly can affect mRNA

Figure 3. Deletion Analysis of the 3′ ocs Region.
(A) Schematic representation of the different deletions. The plasmids containing the different 3′ ocs deletions are indicated on the left. The numbers at the endpoints of the lines define the last nucleotide that is still present in the deletion derivatives [see also (C)]. The putative minor and major polyadenylation signals, as defined by Dhaese et al. (1983), are indicated by a small and large solid box, respectively; the minor and major poly(A) addition sites are pointed out by a small and a large arrowhead, respectively.
(B) NPTII enzymatic assay of extracts from tobacco protoplasts electroporated with the plasmids indicated above the individual lanes. The different samples were standardized on CAT activity (see Methods). PK, plant kinase; NPTII, neomycin phosphotransferase II.
(C) DNA sequence of the 3′ ocs fragment in pCNOCS. Deletion endpoints are indicated below the DNA sequence. The polyadenylation signals are indicated by bold lettering, and the minor and major polyadenylation sites are indicated above the DNA sequence with a large and small arrowhead, respectively. The (G)T-rich sequence is underlined.
prevalence levels, we constructed a series of chimeric nptll genes containing different 3’ gene sequences. The various 3’ gene regions used in this study are listed in Table 1 and include DNA sequences from genes encoding (1) a 2S seed storage protein, (2) a small subunit of ribulose-1,5-bisphosphate carboxylase, (3) extensin, and (4) a chalcone synthase. These genes display a characteristic expression program showing either a marked tissue specificity—for example, the 2S and rbcS small subunit genes—or clear physiological inducibility, as in case of the chalcone synthase gene.

To assess the functionality of each 3’ gene region, we first introduced each construct into isolated tobacco protoplasts by electroporation and determined NPTII activity levels in protoplast extracts. As shown in Figure 4A, all nptll chimeric genes containing plant 3’ regions directed the production of significantly higher NPTII activity levels (lanes pGemCHS, pGemOCS, pGemSS, pGem2S, and pGemEXT) than that observed for a gene without 3’ end sequences (lane pGemD). In addition, NPTII activity levels were virtually identical for all gene constructs analyzed. These results strongly suggest that all signals required for efficient 3’-processing of the nptll transcripts are present within the cloned 3’ end sequences.

We then introduced each gene construct into the tobacco genome via the T-DNA vector pGV1503 (see Methods) and determined NPTII activity and nptll mRNA levels in leaves of stably transformed tobacco plants. To eliminate variation due to position effects, we analyzed nptll gene expression levels in at least 10 independently transformed plants for each construct. As shown in Figure 4B and summarized in Table 2, each nptll fusion gene displayed a characteristic expression level in tobacco plants. If we normalize the NPTII activity found in the GVOCs plants to one, then, on average, the NPTII activity found in GVSS plants is 3 times higher. On the other hand, GV2S, GVEXT, and GVCHS plants contain a fivefold, 10-fold, and 20-fold lower NPTII activity than that found in GVOCs plants. These results indicate that NPTII enzymatic activity levels vary as much as 60-fold, depending upon the origin of the 3’ gene sequences.

To determine whether the nptll gene expression in GVSS plants shows an organ specificity similar to that of the small subunit gene, we measured NPTII activity in callus and roots as compared with leaves. We found equally high levels of NPTII enzymatic activity in the different extracts analyzed (data not shown). Therefore, we conclude that the 3’ end of the small subunit gene does not impose a differential processing or turnover rate on the nptll transcript in a tissue-specific manner.

To determine whether the observed NPTII activity actually reflects the steady-state mRNA content, total RNA was isolated from two representative transgenic plants for each construction. The RNA gel blot represented in Figure 4C indicates that, for each construct, the nptll transcript length corresponds to that predicted on the basis of the available transcriptional start and polyadenylation sites. This result confirms that the polyadenylation sites of the original genes are utilized. More important, Figure 4C also demonstrates that the steady-state mRNA content is different for the various constructs corresponding well with the NPTII enzymatic activity levels. Therefore, we can conclude that the NPTII activity as measured in at least 10 plants for each construct is a mere reflection of the mRNA content and that no unexpected translational effects are involved in establishing the NPTII activity.

**DISCUSSION**

**Functional 3’ End Regions Are Necessary for Efficient Gene Expression**

This report focuses on the functional roles of 3’ gene sequences for expression of a chimeric gene in tobacco cells.

In a first experiment, we demonstrate that the expression level of an nptll reporter gene with the 3’ end sequences of the octopine synthase gene is uniformly high, whereas expression of the same gene without 3’ end sequences depends on the presence of cryptic polyade-

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**Table 1. Description of the Cloned 3’ End Regions**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Origin</th>
<th>Length of the Noncoding Segment Relative to the Poly(A) Addition Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octopine Synthase</td>
<td>Agrobacterium tumefaciens</td>
<td>320</td>
</tr>
<tr>
<td>2S Seed Protein</td>
<td>Arabidopsis thaliana</td>
<td>±650 ND, ND</td>
</tr>
<tr>
<td>Small Subunit of rbcS</td>
<td>Arabidopsis thaliana</td>
<td>±600 ND, ND</td>
</tr>
<tr>
<td>Extensin</td>
<td>Daucus carota</td>
<td>735b 450 106</td>
</tr>
<tr>
<td>Chalcone Synthase</td>
<td>Antirrhinum majus</td>
<td>287 170 113</td>
</tr>
</tbody>
</table>

* ND, not determined.

b This 3’ fragment contains a 185-bp intron.
nylation signals in the downstream DNA. These fortuitous polyadenylation events result in variable, usually low expression levels of the reporter gene. For example, if the \textit{ntpII} gene is followed by pBR322 DNA, \textit{ntpII} mRNA production and NPTII activity is approximately 12-fold lower than the same \textit{ntpII} gene with the 3' ocs fragment. Since this \textit{ntpII} transcript is polyadenylated and has a defined length, this low-level expression is due to 3'-processing and polyadenylation events centered around a fortuitous polyadenylation signal within the pBR sequences. In fact, an AATAAT signal followed by two sequence motifs similar to the YGTGTTYY consensus are present within the pBR322 region corresponding to the 3' terminus of the transcript. Also, in animal systems, polyadenylation of extended transcripts at three distinct sites in pBR322 has been reported, and one of them is located in the region characterized in this study (Zhang, Denome, and Cole, 1986; Kessler et al., 1987). Polyadenylation at these cryptic sites was found to be inefficient, consistent with our results.

The fact that the same cryptic polyadenylation signal in pBR322 is used in plant and animal cells suggests not only that the structural requirements for polyadenylation are highly flexible, but also that the sequences responsible for 3' end-processing are similar in plants and animals.

The Polyadenylation Signal and a YGTGTTYY-like Sequence Constitute Important Sequence Elements in the 3' ocs Region

We show that, after electroporation of the P35S-\textit{ntpII}-3' ocs fusion gene, a polyadenylated transcript is produced

### Table 2. NPTII Activities in Plants Transformed with Chimeric \textit{ntpII} Genes Containing Different Plant Gene 3' Ends

<table>
<thead>
<tr>
<th>Experiment</th>
<th>GVOCS (\times 10^{-3} \text{ cpm})</th>
<th>GVSS</th>
<th>GV2S</th>
<th>GVEXT</th>
<th>GVCHS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1(8.7)</td>
<td>3</td>
<td>1/5</td>
<td>1/4</td>
<td>1/15</td>
</tr>
<tr>
<td>2</td>
<td>1(18.2)</td>
<td>3</td>
<td>1/5</td>
<td>ND*</td>
<td>1/15</td>
</tr>
<tr>
<td>3</td>
<td>1(17.8)</td>
<td>3</td>
<td>1/5</td>
<td>1/16</td>
<td>1/20</td>
</tr>
<tr>
<td>4</td>
<td>1(11.0)</td>
<td>5</td>
<td>1/7</td>
<td>1/10</td>
<td>1/15</td>
</tr>
<tr>
<td>5</td>
<td>1(13.0)</td>
<td>1</td>
<td>1/12</td>
<td>1/18</td>
<td>1/23</td>
</tr>
<tr>
<td>6</td>
<td>1(5.8)</td>
<td>5</td>
<td>1/4</td>
<td>1/9</td>
<td>1/30</td>
</tr>
<tr>
<td>7</td>
<td>1(3.1)</td>
<td>2</td>
<td>1/2</td>
<td>1/6</td>
<td>1/30</td>
</tr>
<tr>
<td>8</td>
<td>1(1.6)</td>
<td>3</td>
<td>1/2</td>
<td>1/6</td>
<td>1/30</td>
</tr>
<tr>
<td>Average</td>
<td>1</td>
<td>3</td>
<td>1/5</td>
<td>1/10</td>
<td>1/22</td>
</tr>
</tbody>
</table>

*ND*, not determined.

Identical amounts of protein were loaded in each experiment. Quantification of the NPTII activity was performed by counting the phosphorylated kanamycin bound onto the P81 paper (see Methods). The absolute counts per minute (cpm) value from the GVOCS plants, which is indicated in parentheses, has been standardized to 1 in all experiments. The counts per minute values of the other constructions are expressed relative to this standardized GVOCS value.
equal in length to the nptll mRNA present in stably transformed tissue. In contrast, no nptll transcript can be detected, and NPTII enzymatic activity levels are at least 20-fold lower after electroporation of the P35S-nptll fusion gene without functional 3’ end sequences. Therefore, this transient expression system provides a reliable assay for the characterization of the 3’ ocs sequences that are required for efficient expression of the gene.

The octopine synthase 3’ region contains a major and a minor polyadenylation site, and the corresponding polyadenylation signals are accordingly called the major and minor polyadenylation signals (De Greve et al., 1982; Dhaese et al., 1983). Analysis of plasmids containing progressive deletions in the 3’ ocs region indicates that the NPTII activity decreases stepwise.

When the region between 98 bp and 142 bp downstream from the major polyadenylation site is deleted, NPTII activity is reduced by a factor of 4. Since this region contains a 1-base variation of the consensus signal YGTGTTYY, it is likely that this sequence is important for efficient processing of the transcript. A majority of the mammalian genes contain the YGTGTTYY sequence at approximately 30 bp downstream from the polyadenylation signal, and disruption of this spatial relationship reduces the efficiency of 3’-processing. YGTGTTYY-like sequences also occur in the 3’ region of plant genes, but apparently not at a fixed position relative to the polyadenylation signal (Joshi, 1987).

In the 3’ ocs region, the YGTGTTYY-like motif is situated some 140 bp downstream from the major polyadenylation signal, whereas, for the potato proteinase inhibitor gene, a similar sequence, situated 46 bp downstream from the polyadenylation signal, has been shown to be important for 3’ end formation (An et al., 1989).

After deletion of the 35-bp region containing the major polyadenylation signal and the major and minor polyadenylation sites, the NPTII activity decreases to background levels. Therefore, this 35-bp region must contain sequences that are required for maturation of the nptll transcripts. Moreover, we can conclude that a polyadenylation signal, as such, is not sufficient to ensure expression, a fact that also holds true for animal genes.

Expression Level of an nptll Reporter Gene is Strongly Modulated by Different 3’ Ends

We demonstrate that the expression level of nptll chimeric genes in transgenic tobacco plants varies as much as 50-fold, depending on the origin of the 3’ end. The different 3’ ends included in this comparative study were derived from genes with entirely different expression characteristics. The genes encoding extensin and chalcone synthase are both inducible by environmental stimuli. The octopine synthase gene is believed to be constitutive, whereas the 2S seed storage protein and small subunit genes are expressed primarily in seeds and leaves, respectively.

Although none of the 3’ ends originates from tobacco genes, the resulting chimeric genes were all equally well expressed in a tobacco protoplast transient expression system, indicating that all signals required for proper 3’ end formation are present. This result contrasts with the NPTII activity found in stably transformed leaf tissue. Here, we observed pronounced different expression levels of the various fusion genes. Expression of the nptll fusion gene with the 3’ end of a ribulose-1,5-bisphosphate carboxylase small subunit gene results in approximately 3 times more NPTII enzymatic activity than that with the 3’ end of octopine synthase. This latter gene is expressed 5 and 10 times higher than the fusion with the 3’ end of the 2S and extensin genes, respectively. Expression of the nptll gene containing the 3’ end of the chalcone synthase gene is uniformly low, on average 60 times less than the nptll gene with the 3’ end of the small subunit gene. The steady-state mRNA levels correspond well with the results obtained from the NPTII assays, thus excluding unexpected translational differences between the chimeric mRNAs. The difference in steady-state mRNA levels suggests that post-transcriptional events such as efficiency of 3’-processing and polyadenylation or transcript stability differ for each 3’ end used. It is not unlikely that the observed difference would, at least in some cases, be due to a different stability of the transcript. The small subunit gene is expressed mainly in chloroplast-containing tissues where its mRNA constitutes a major fraction (1% to 2%) of the poly(A)+ RNA. Its gene product is a subunit of the chloroplast enzyme ribulose-1,5-bisphosphate carboxylase, which accounts for up to 50% of the total organellar protein. The obvious requirement for large amounts of the protein can be met not only by providing the gene with a strong promoter, which is in fact the case, but also by rendering its transcript stable. In a similar way, the low-level expression of the fusion gene with the 3’ end of the chalcone synthase could be explained. Since this enzyme disappears quickly after induction by external stimuli such as UV illumination (Lipphardt et al., 1988), it is attractive to speculate that its mRNA might be unstable. Rapid turnover of these mRNAs would indeed facilitate their rapid removal in response to environmental stimuli. It will now be interesting to find out whether the different mRNA prevalences are due to differential processing, transport, or turnover of the transcripts.

METHODS

Restriction Analysis and Cloning Techniques

Restriction endonucleases, calf intestine phosphatase, the large fragment of DNA polymerase I, Bal31, phage T4 ligase, and phage T4 polynucleotide kinase were purchased from Boehringer (Mannheim, Federal Republic of Germany), Bethesda Research Labo-
ratories (N.V. Gibco, Belgium), New England BioLabs, or Pharmacia (Upsala, Sweden) and were used according to the suppliers' instructions. All recombinant DNA techniques were performed according to Maniatis, Fritsch, and Sambrook (1982).

**Plasmids**

The basic construction pGemD was obtained by fusing the coding sequence of neomycin phosphotransferase II (nptII) (Herrera-Estrella et al., 1983) to the cauliflower mosaic virus 35S promoter (Odell, Nagy, and Chua, 1985) in the Gemini vector pGem2 (Promega Biotec). In the polynucleotide 18 bp downstream from the nptII stop codon, fragments containing the 3' end sequences of the octopine synthase gene, the Arabidopsis 2S-1 gene, the Arabidopsis rbcS3 small subunit gene, the Daucus carota extensin gene, and the Antirrhinum majus chalcone synthase gene were cloned. The resulting plasmids were called pGemOCS, pGem2S, pGemSS, pGemEXT, and pGemCHS, respectively. A description of the cloned 3' end sequences is presented in Table 1. These nptII fusion genes were subcloned in pGV1503, which is derived from pGV1500 (Deblaere et al., 1987) by insertion of the hygromycin resistance gene, yielding the plasmids pGV OCS, pGV2S, pGVSS, pGVEXT, and pGVCHS, respectively.

The nptII fusion genes from pGemD and pGemOCS were also subcloned into the phBS plasmid, which is similar to pGV1503 but lacks T-DNA borders, yielding phBSOCS and phBSOCS.

The deletion mutants were generated by Bal31 reaction on the 3' ocs fragment in pGemOCS, starting from the EcoRI site in the polynucleotide downstream from the 3' end sequence. After treatment with the Klenow enzyme, molecules were self-ligated and transformed in Escherichia coli MC1061 (Casadaban and Cohen, 1980). The various deletion mutants were characterized by DNA sequencing (Maxam and Gilbert, 1980). A chloramphenicol acetyltransferase gene under control of the T32' promoter and 3' ocs regions was subcloned in pGemD, in pGemOCS, and in the different deletion plasmids, yielding the plasmids pCND, pCN OCSa1, pCN OCSa2, pCN OCSa3, pCN OCSa4, pCN OCSa5, and pCN OCSa6, respectively. In addition, a 705-bp fragment from pAGV828 (Herrera-Estrella et al., 1983) containing the 3' end sequences of the ocs gene was subcloned in pCND, yielding pCNOCS.

**Conjugation and Plant Transformation**

All pGV1503- and phBS-derived plasmid constructions were mobilized to Agrobacterium C58ClRifR by triparental mating (Van Haute et al., 1983). pGV1503-derived plasmids were mobilized to Agrobacterium C58ClRifR by coinintegration into the resident pGV2260 virulence plasmid (Deblaere et al., 1985). The pHBS-derived plasmids were introduced into the Agrobacterium strain C58ClRifR containing the plasmid pGV850 (Zambryski et al., 1983). DNA gel blot analysis on total Agrobacterium DNA was performed to confirm the structure and the integrity of the coinTEGRATES (Dhaese et al., 1983).

Nicotiana tabacum var. SR1 was transformed via the leaf disc infection method or via co-cultivation of regenerating protoplasts (De Block et al., 1987). All transformed plant lines were selected in medium containing 50 μg/mL hygromycin.

**NPTII Assay**

NPTII activity was determined by in situ detection of NPTII activity in nondenaturing polyacrylamide gels (Van den Broeck et al., 1985). The samples were standardized on either total protein content or on the CAT activity levels (see CAT Assays). The protein concentration was determined by the method of Bradford (1976) using the kit supplied by Bio-Rad.

Quantification of the NPTII activity was performed as follows: the phosphorylated kanamycin spots were cut out of the P81 blot together with a control piece isolated from a location below the signals to estimate background levels of radioactive activity. Radioactivity in each piece of P81 paper was determined using nonaqueous scintillation counting (5 min) in the 32P channel of a scintillation counter. The counts per minute values obtained were between 500 cpm and 100,000 cpm. Because the absolute amount of 32P counts differed significantly in independent experiments due to experimental variables, the counts per minute values obtained from the different constructs were expressed relative to the counts per minute values of the GVOCS plants.

**CAT Assay**

CAT activity was determined using a modified version of the "diffusion CAT assay protocol" (Neumann, Morency, and Russian, 1987). At 20-min intervals, the samples were counted for 45 sec in the 32P channel of a liquid scintillation counter. Three to five measurements were routinely used to plot counts per minute values versus time for the different reactions. CAT activity was determined by calculating the reaction rate, and these values were used to standardize the amount of crude cell extracts to be analyzed for NPTII activity. A linear correlation was found between the reaction rate and CAT concentration if final counts per minute values were not exceeding ±40,000.

**Electroporation Conditions**

Generally, 1 to 2 × 10^6 protoplasts were electroporated with 10 μg of plasmid DNA using a homemade 200-μF capacitor at 200 V across a 0.6-cm path (Dekeyser et al., 1989). For the CAT and NPTII assays, the protoplasts were incubated for 48 hr at 28°C. For RNA preparations, the protoplasts were incubated only for 1 hr or 4 hr.

**RNA Analysis**

Total RNA was extracted from transformed N. tabacum leaves and from electroporated protoplasts essentially as described by Jones, Dunsmuir, and Bedbrook (1985). Poly(A) RNA was purified by oligo(dT)-cellulose affinity chromatography according to Slatyer (1984). RNA was electrophoresed in a 5.5% agarose/formaldehyde gel and transferred to nylon filters (Hybond-N; Amersham Corp.). The blotting and hybridization were performed as recommended by Amersham Corp. The coding sequence of the nptII gene and of the β-subunit gene of the mitochondrial ATP synthase (Boutry and Chua, 1985) were cloned in antisense orientation in an SP6
vector and transcribed into a riboprobe according to Melton et al. (1984).

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