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High level expression of introduced chimaeric genes in regenerated transformed plants

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Promoter DNA sequences from a petunia chlorophyll *a/b* binding protein gene were fused to octopine synthase DNA sequences and the resulting chimaeric genes were introduced into petunia and tobacco cells. Populations of transformed regenerated petunia plants containing the chimaeric genes were examined so that the expression of any particular construction could be compared between independent transformants. Substantial variation was observed between transformants in the level of chimaeric gene expression. In general, transcriptional fusions in which a linker sequence interrupted the 5'-untranslated region gave rise to less chimaeric mRNA accumulation than a translational fusion. In the most actively expressing transformants the amount of mRNA from the introduced chimaeric genes was half that of the endogenous wild-type gene. Transcription initiated at the same place in the chimaeric and endogenous genes. Construction of the translational *cab/ocs* fusion caused three amino acid changes in the octopine synthase protein and functional octopine synthase enzyme was absent from plants in which mRNA for the chimaeric gene was abundantly expressed.

Key words: Agrobacterium/chlorophyll *a/b* binding protein gene/ octopine synthase/position effect/transformed plants

Introduction

It is now possible to use the plant pathogenic bacterium *Agrobacterium tumefaciens* (Kahl and Schell, 1982; Ream and Gordon, 1982; Bevan and Chilton, 1982) to introduce foreign genes into plant cells and then to investigate their expression. In early studies (Leemans *et al.*, 1981) DNA sequences of interest were inserted into the T-DNA and introduction of these sequences into plant cells was selected for using the hormone-independent growth phenotype conferred by the linked T-DNA functions. Investigations into the expression of the introduced gene were usually carried out on non-clonal callus tissue in which only a fraction of the cells may have been transformed (van Slogteren *et al.*, 1983). Several studies exploited the nopaline synthase (*nos*) promoter to express foreign genes in plants (Herrera-Estrella *et al.*, 1983a), and particular attention has now been given to constructing chimaeric genes which will confer drug resistance and thus provide a selectable marker other than hormone autotrophy. The most useful marker has proved to be kanamycin resistance (Herrera-Estrella *et al.*, 1983b; Bevan *et al.*, 1983; Fraley *et al.*, 1983) conferred by a *nos*/neomycin phosphotransferase (*nos/kan*) fusion although other selections, (e.g., hygromycin resistance) (van den Elzen *et al.*, 1985) are becoming available.

Several studies have focused on the expression of introdu photosynthetic genes such as the gene which encodes the sn subunit protein (SSU) of ribulose bisphosphate carboxylase. these experiments either the entire gene was introduced (Bro *et al.*, 1984) or the promoter was fused to an assayable g (Herrera-Estrella *et al.*, 1984). The construction was introd ed either into callus which was artificially greened with cytoki (Broglie *et al.*, 1984) or with a partially disarmed T-DNA wh confers, in addition to the gene of interest, a cytokinin synth but not an auxin synthesis capacity on the transformed pl material (Herrera-Estrella *et al.*, 1984). Such experiments h only been reported on the expression of pea SSU genes in petu or tobacco.

We report here on our investigations into the expression introduced chimaeric genes in a population of transformed pla in which most individuals are independent, clonal transforma The experiment addresses the issue of variability in the expr sion of a particular construct in independent transformants at whole plant level. We used a promoter from a well-expres leaf gene (a chlorophyll *a/b* binding protein, or *Cab*, gene) fr petunia var. Mitchell (Dunsmuir *et al.*, 1983) which had b fused to an assayable marker, octopine synthase (*ocs*), and troduced the fusion into petunia or tobacco using a kanamy resistance marker. This enabled us to test in both an homolog and a heterologous system whether the mRNA of a chima gene which used the promoter of an abundant leaf mRNA co accumulate to a level similar to that of the corresponding en genous leaf mRNA. Both transcriptional and translation fusi to octopine synthase were made and this enabled us to investig the influence of sequences in the 5'-untranslated region of mRNA on total mRNA accumulation. A second class of c struction tested the influence of sequences 3' to the presur octopine synthase polyadenylation site on mRNA accumulati Nuclease protection experiments enabled us to compare transcriptional initiation sites of the introduced chimaeric endogenous wild-type genes.

Results

Construction of Cab/ocs fusions

The gene fusion experiments used pCab221, a plasmid subcl ed from Cab phage 22 (Dunsmuir *et al.*, 1983; Dunsmuir, 19 which carries two divergently transcribed *Cab* genes, *22L* a *22R*. The gene with a *Hind*III site in the coding region (2 is known to be an expressed gene firstly because its seque corresponds exactly to that of a cDNA clone (pCab 146) secondly because in very high-stringency Southern hybridisati this is the only genomic sequence which hybridises to the pC 146 probe. Figure 1 summarizes the construction of deleti at the 5' end of the *Cab 22R* gene using *Bal*31 deletions fr the *Hind*III site. *Bam*HI linkers were placed at three locati within 60 bp 3' to the 5' end of the *Cab 22R* mRNA (map by David Gidoni, unpublished data) and ~1.1 kb away fr a *Pst*I site which was used to clone the resulting fragments i

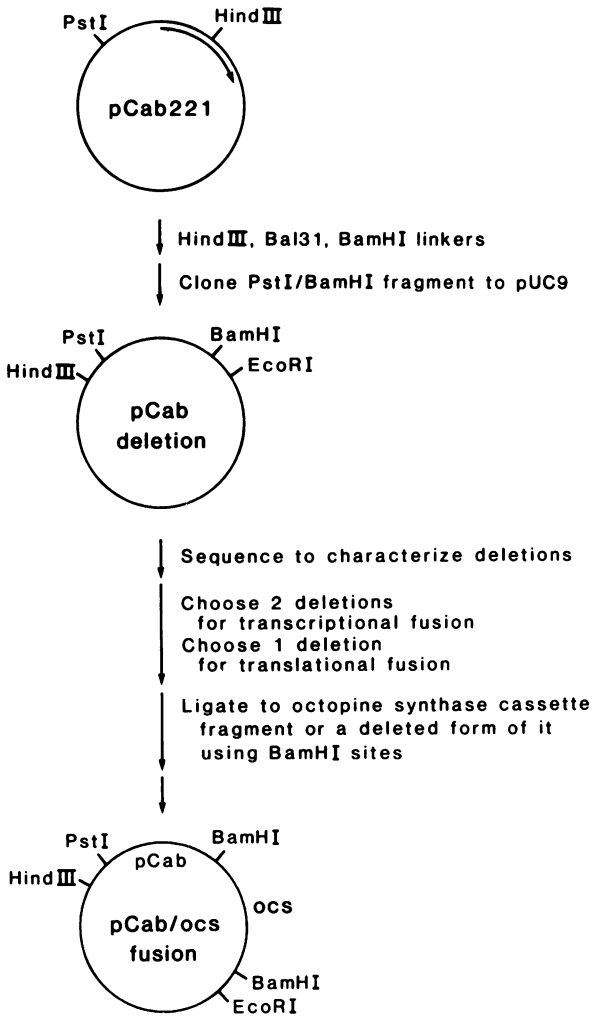

Fig. 1. Construction of *Cab/ocs* fusions. The direction of transcription of *Cab 22R* in pCab221 is indicated by an arrow. Plasmid DNA was linearized at the unique *Hind*III site, ligated to *Bam*HI linkers and deleted fragments were cloned as ~1.1-kb *Pst*I/*Bam*HI fragments into pUC9. We identified three deletions, two of which attached a *Bam*HI linker within the presumed 5′-untranslated region and one of which attached such a site six bases 3′ to the translational start site. The first two of these were ligated directly to an *ocs* cassette fragment (Herrera-Estrella *et al.*, 1983a) to create two transcriptional fusions, 511 and 521, which could then be cloned into pMUC9 as *Pst*I/*Sma*I fragments. This cassette fragment places an *ocs* coding sequence lacking any promoter but containing 3′ sequence to an *Rsa*I site 12 bp 3′ to the polyadenylation site between the convenient restriction sites *Bam*HI and *Hind*III. A translational fusion was made by constructing deletions of the *ocs* cassette fragment. The cassette fragment was cloned as a *Bam*HI fragment from pAGV40 (Herrera-Estrella *et al.*, 1983a) into pUC9 and an orientation was chosen with the 5′ end towards the *Pst*I site. After *Pst*I digestion, *Bal*31 treatment and *Bam*HI linker attachment, *Hind*III/*Bam*HI fragments were cloned into mp9 and sequenced. Two deletions placed a *Bam*HI linker 9 and 11 bp from the initiation codon. One of these was ligated (after *Bam*HI digestion and filling in with DNA polymerase large fragment and dNTPs) to the *Bam*HI-cut, filled-in deletion of the *Cab 22R* gene which had placed a *Bam*HI site in the translated sequence. After construction of these fusions the structure at the fusion junction was confirmed by DNA sequencing. The translational fusion construction was called no. 503 and was cloned as a *Hind*III fragment into pMUC9 in the same orientation as the two transcriptional fusions.

pUC9. These were ligated either to the *Bam*HI site of an octopine synthase cassette fragment (Herrera-Estrella *et al.*, 1983a) to produce two different transcriptional fusions or to a deleted form of the cassette to produce a translational fusion (Figures 1 and 2A).

The sequences at the 5′ end of the *Cab 22R* gene, the octopine synthase gene and the chimaeric genes which were introduced to plants are shown in Figure 2A. The two transcriptional fusions retain 34 and 17 bp of Cab 5′-untranslated sequence, respectively. The leader sequences of these chimaeric genes are GC-rich at the *Cab/ocs* junction as a result of the introduction of a *Bam*HI linker and are thus dramatically different from the AT-rich leader sequences of the endogenous *Cab* gene itself or of the translational fusion. However, in the translational fusion amino acids 3, 4 and 5 of the *ocs* protein are mutagenized.

The *ocs* cassette fragment differs from the *ocs* sequence at an *Rsa*I site which is 12 bp 3′ to the polyadenylation site (Herrera-Estrella *et al.*, 1983a; de Greve *et al.*, 1982). To test whether sequences beyond this region are important for effective processing of the primary transcript into mRNA, we carried out additional constructions using a *Pvu*II site 15 bp past the translational termination codon to place an extra 500 bp of wild-type *ocs* 3′ sequence in the translational fusion. These two 3′ structures are shown in Figure 2B.

Construction of pGV3850 kanR

The modified Ti plasmid pGV3850 kanR was constructed to introduce the chimaeric genes into plants. Zambryski *et al.* (1983) have described the construction of a plasmid pGV3850 in which the internal *Hind*III fragments of the T-DNA of the C58 nopaline Ti plasmid have been replaced by pBR322 DNA. This strain is carbenicillin (ampicillin) resistant and carries no hormone perturbation genes or any other selectable marker for transformation of plant cells. However, it does carry the nopaline synthase gene which can be used to screen for transformants. Figure 3A outlines the introduction into GV3850 of a *nos/kan* fusion gene using a marker exchange technique (Ruvkun and Ausubel, 1981). This was carried out for two reasons. Firstly, we wanted to create a strain in which a selectable marker for transforming plant cells was placed in the T-DNA of a non-oncogenic *Agrobacterium* strain. Secondly, we wanted to be able to use a selection for carbenicillin resistance in a co-integrate formation strategy to introduce the DNA of interest into T-DNA adjacent to a selectable marker for plant cell transformation (van Haute *et al.*, 1983). In this approach, a pBR322 derivative (which cannot replicate in *Agrobacterium*) is mobilised from a suitable *Escherichia coli* strain (GJ23) into a strain carrying the pGV3850 derivative, and recombination at the homologous pBR322 sequences places the clone in the T-DNA. Consequently, the *nos/kan* fusion was placed in the carbenicillin resistance gene of the pBR322 sequences of pGV3850, creating pGV3850 kanR, a carbenicillin-sensitive Ti plasmid strain which transforms plant cells to kanamycin resistance (see Figure 3A).

Introduction of fusions into pGV3850 kanR

We planned to introduce our fusions into pGV3850 kanR by mobilisation from GJ23 and a carbenicillin selection for co-integrate formation. However, in the course of deleting the pBR322 *Acc*I site in the construction of pUC9 (Vieira and Messing, 1982), the basis of mobilisation, or *bom*, site was inactivated. Hence we constructed a plasmid, pMUC9 (Mobilisable UC) which resembles pUC9 except that the *bom* site is retained (Figure 3B).

Transcription fusions 1 and 2 (Figure 2A) were cloned into pMUC9 using a *Pst*I/*Sma*I digest to create plasmids no. 511 and no. 521, respectively. The translational fusion was cloned into pMUC9 in the same orientation using a *Hind*III digest to create no. 503. The plasmid carrying a translational fusion with a long 3′ tail, no. 571, could be directly mobilised into pGV3850 kanR

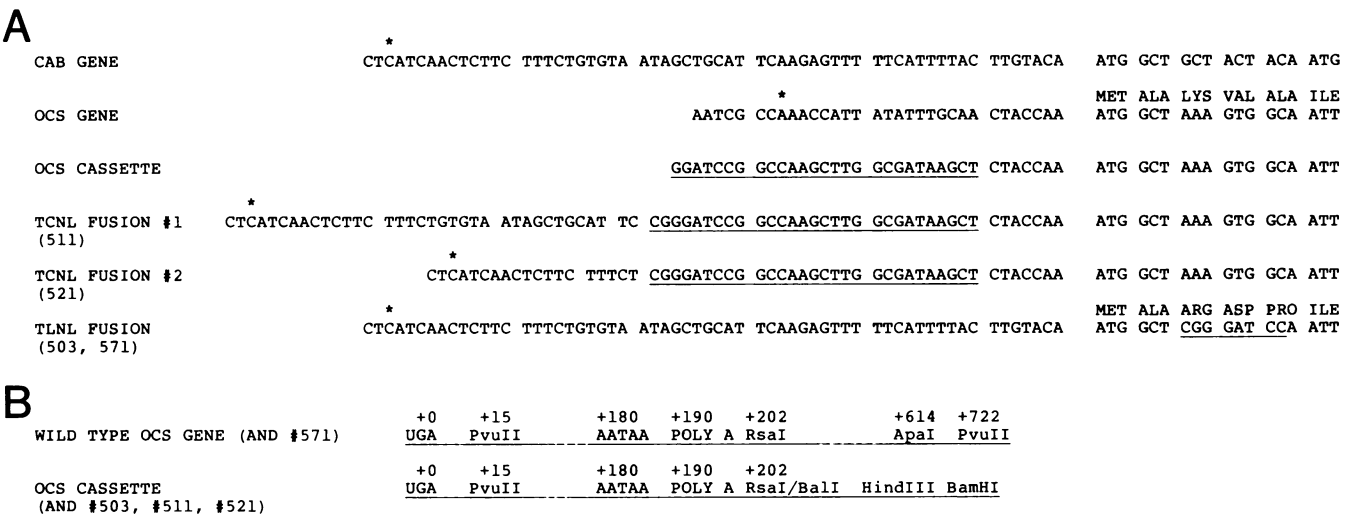

Fig. 2. (**A**) DNA sequences at the 5′ end of the *cab/ocs* fusions. The transcriptional initiation site is indicated by an asterisk. Linker sequences are underlined. The N-terminal amino acid sequences of the wild-type and mutant *ocs* proteins are shown. These sequences were confirmed by DNA sequencing the fusions. (**B**) Structure of the 3′ end of *cab/ocs* translational fusions. Sequences and restriction sites 3′ to the UGA translational termination codon are shown. The translational fusion plasmid (no. 503) was treated with *Pvu*II (which cuts 15 bp 3′ to the UGA) and *Hind*III (which cuts at a site in pUC9 past the *Pst* site used to clone in the promoter fragment). A fragment carrying the fusion was cloned into *Pvu*II/*Hind*III cut pBR322. This plasmid had a single *Pvu*II site into which could be cloned a 750-bp *Pvu*II fragment from the 3′ end of the *ocs* gene (de Greve *et al.*, 1982). An *Apa*I site 500 bp from the UG was used to screen for the correct orientation. The correct construction was designated no. 571.

since it derives directly from pBR322. These constructions were mobilised into pGV3850 kanR from GJ23 and exconjugants were selected on M9 sucrose carbenicillin (100 mg/l) plates. The resulting exconjugant *Agrobacterium* strains (containing pGV3850 kanR: 503, 511, 521 and 571) were characterized by Southern analysis to confirm the structure of the co-integrates (data not shown). Co-integrate formation always took place within the longest region of homology (between the *Pst*I and *Pvu*II sites of pBR322), and the diagram in Figure 3C illustrates the DNA fragment from the resulting co-integrate which is predicted to be transferred to plant nuclear DNA.

Plant cell transformation leading to regenerated transformed plants

The co-integrate strains were used in co-cultivation (Marton *et al.*, 1979) experiments with petunia and tobacco cells in accordance with established protocols (Fraley *et al.*, 1983) with some modifications (Townsend *et al.*, in preparation). Cefotaxime (CalBiochem, 500 μg/ml) was used to kill *Agrobacterium* since the strains used were carbenicillin-resistant. Calli which were resistant to kanamycin were kept on the antibiotic until the shoots were rooted. Individual calli were numbered 1 – 10 and where more than one plant was regenerated from the same callus each plant was designated by a letter. For example, the second petunia plant regenerated from the fifth kanR callus arising from transformation with the construction no. 511 was called 511.5B.

Chimaeric mRNA in transformed plants

After shoot regeneration and rooting of the shoots in magenta cups, 53 transformed petunia plants were transferred to perlite in 4 inch pots and incubated at 25°C in 95% humidity and a 12 h day length in a growth chamber. After 3 weeks the plants were transferred to a greenhouse. Total RNA was extracted from young leaves and meristems of individual 20 – 23 cm plants which had been photoautotrophic for 6 – 8 weeks. Care was taken to carry out extractions from material at the same stage of development. The RNA samples were electrophoresed in 1.5% agarose/formaldehyde gels and transferred to nitrocellulose filters (Seed, 1982) which were hybridised with radioactive octop synthase sequences. Figure 4A shows autoradiographs of filters after hybridisation, and the data are summarised in Ta I. We draw four conclusions from these data.

Firstly, individual plants transformed with the same constr tion vary at least 200-fold in the quantity of chimaeric mR they accumulate (as assayed by autoradiograph densitometr For example, plant 571.3A shows ~0.7% as much homologous sequence per unit weight of total RNA as does pl 571.7B. In certain shoots derived from the same transform there is a correspondence in levels of expression (e.g., 503.9 B, C or 571.9A, B, C). However, in 503.1A and 1B or 503.1 and 10B shoots from the same kanR callus show dramatica different levels of expression of the *Cab/ocs* fusion.

Analysis of DNA from 20 tranformed plants shows all but transformant (503.1A) contain 1 – 5 copies of the introduc DNA (data not shown). It is unlikely, therefore, that the obse ed differences in expression between transformants are due sol to differences in the copy number of the introduced gene. Pl 503.1A, which shows lower expression than 503.1B, conta > 10 copies of the T-DNA. This analysis also shows that pla 503.1A and 503.1B have different border fragments and thus independent transformants (data not shown).

Secondly, the translational fusion produces more chimae mRNA than either of the two transcriptional fusions. Expr sion is observed in a larger proportion of the plants transfor with the translational fusions (24/29 plants) than with transcriptional fusions (9/24). Furthermore, the highest absol levels of mRNA accumulation are observed with translatio fusions (5-fold higher than the highest in the transcriptio fusions).

A third conclusion is that there are no significant differe between the levels of expression in the 503 series and the 5 series which suggests that sequences > 12 bp 3′ to the publi ed polyadenylation site are not essential for efficient prim transcript processing.

A fourth and more tentative conclusion is that the level of

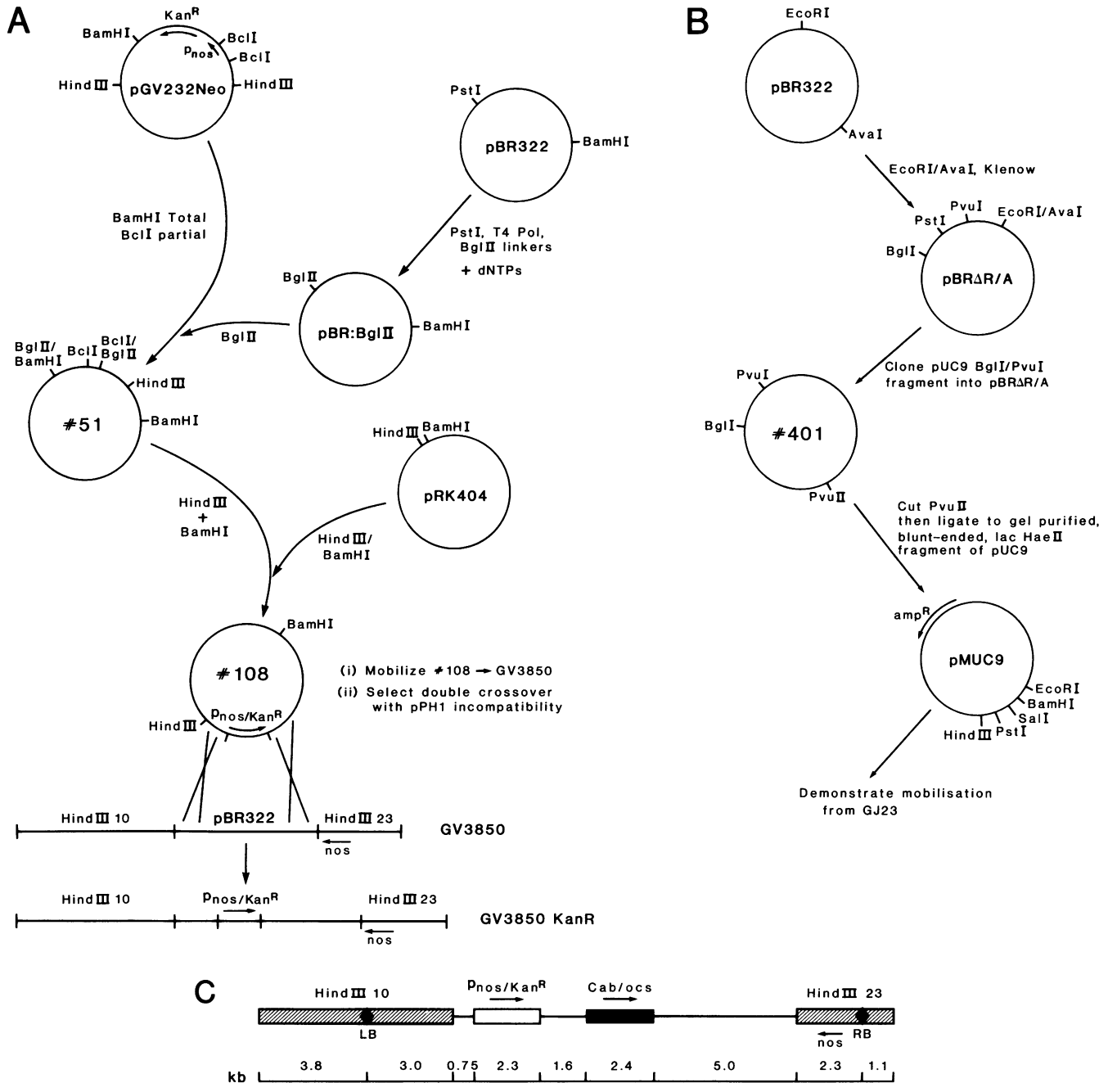

Fig. 3. (**A**) Construction of pGV3860 kanR. Firstly, a *Bgl*II linker was introduced into the *Pst*I site of pBR322. Secondly, pGV232neo [which differs from pGV23 neo (Herrera-Estrella *et al.*, 1983b) only in the presence of an *ocs* 3′ tail *Pvu*II fragment at the Tn5 *Sma*I site] was partially digested with *Bcl*I, totally digested with *Bam*HI, and then ligated with a *Bgl*II digest of the pBR322 derivative described above. Transformants were selected on LB tetracycline kanamycin plates, and a clone corresponding to no. 51 in the diagram was identified, with kanamycin resistance being presumably conferred via transcription from the β-lactamase promoter of pBR322. No. 51 was digested with *Hind*III and *Bam*HI and ligated into *Hind*III + *Bam*HI-cut pRK404 (Ditta *et al.*, 1985). The resulting plasmid was mobilised into GV3850 using pRK2013 as a helper strain (Ditta *et al.*, 1980) selecting on kanamycin (100 mg/l) M9 sucrose plates. The exconjugants were mated with HB101/pPH1 as described (Ruvkun and Ausubel, 1981), and exconjugants were selected on gentamycin kanamycin (both at 100 mg/l) M9 sucrose plates. All (8/8) exconjugants from this experiment were carbenicillin-sensitive. One was analysed by Southern hybridisation and shown to have the structure shown in this figure. Co-cultivation experiments with this strain showed that it conferred kanamycin resistance on plant cells at a frequency of 2 – 5% of input protoplasts. (**B**) Construction of pMUC9. pBR322 DNA was digested with *Eco*RI and *Ava*I and then treated with large fragment of DNA polymerase and dNTPs. After re-circularisation with T4 DNA ligase and transformation, the tetR ampS plasmid pBR E/A was recovered. The *Bgl*II/*Pvu*I fragment of this plasmid was replaced with the corresponding fragment of the β-lactamase gene of pUC9 (which lacks a *Pst*I site) to create no. 401. pUC9 was then digested with *Hae*II, the 3′ overhung and made blunt with T4 DNA polymerase and dNTPs, and the 420-bp fragment carrying the *lac* α peptide sequences was purified from a 5% acrylamide gel and ligated into the *Pvu*II-digested no. 401. Colonies on ampicillin plates were inspected for their capacity to turn X-Gal blue, and pMUC9 was chosen with an orientation of the *lac* promoter with respect to the origin of replication, which was the same as in pUC9. This plasmid was mobilised to other *E. coli* strains from GJ23 with a frequency comparable with pBR322 and at least 1000-fold better than pUC9. (**C**) Structures of co-integrate DNA carrying *Cab/ocs* fusions into plants. Numbers refer to fragment sizes in kilobases. LB and RB signify left border and right border respectively. Arrow indicates direction of transcripion.

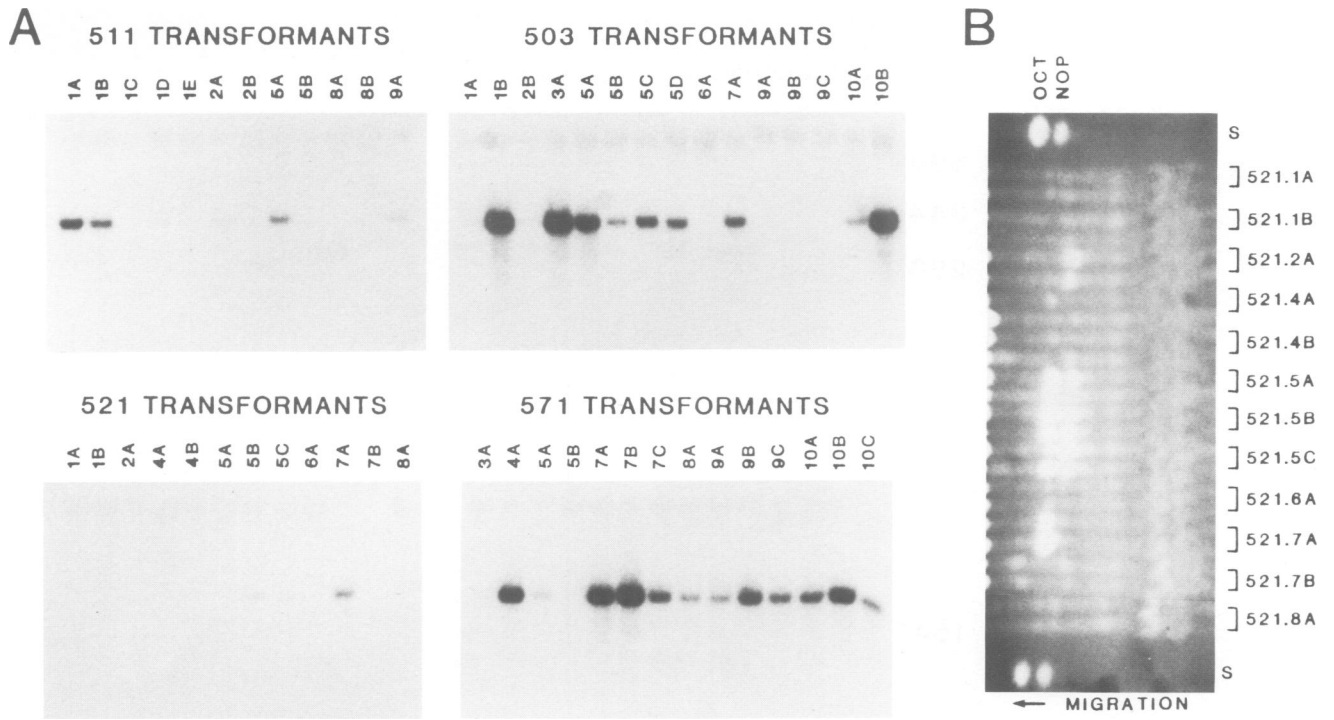

Fig. 4. (**A**) Northern blot hybridization of RNA extracted from different plants using an *ocs* probe. A fragment carrying the *ocs* cassette fragment was clon into an SP6 vector in an orientation such that SP6 polymerase produces an RNA transcript homologous to *ocs* mRNA. A probe was made and used for hybridisation at a probe concentration of 2 x 10^6 c.p.m./ml (2 x 10^8 c.p.m./μg). 10 μg aliquots of total RNA were electrophoresed in 1.5% agarose/ formaldehyde gels and blotted to nitrocellulose as described by Seed (1982). Exposure was for 4 h at −80°C with intensifying screens. (**B**) Opine synthase assays. Aliquots (200 mg) of leaf material (two from each plant) were assayed for *ocs* activity. The assays were run out by paper electrophoresis with octop and nopaline standards (outside lanes) and stained using phenanthrenequinone (Otten and Schilperoort, 1978).

Table I. Quantitation of expression of chimaeric genes in petunia by scanning autoradiographs of Northern hybridisations

Transcriptional fusions		Plant No. 1	2	5	8	9			
511 Series	A	17	0.5	5	0	3			
	B	10	0	0	0				
	C	0							
	D	0.5							
	E	0							
		Plant No. 1	2	4	5	6	7	8	
521 Series	A	0	0	0	0	0	6	0	
	B	0		0	2				
	C				2				
Translational fusions		Plant No. 1	2	3	5	6	7	9	10
503 Series	A	0.5	0.5	85	50	0	9	0	6
	B	100			6			0	95
	C				20			0	
	D				12				
		Plant No. 3	4	5	7	8	9	10	
571 Series	A	0.5	35	4	35	12	12	17	
	B			0	75		20	40	
	C				20		17	15	

Autoradiographs such as those in Figure 4A were obtained by exposure to pre-flashed film without screens at room temperature. For a particular blot autoradiographs were obtained at several exposure times to establish linearity of response. Scanning of bands was performed using an LKB scanning densitometer. The highest signal observed (in 503.1B) was called 100% and the other signals were expressed as a percentage of this number. The results were the same whether based on peak height or area.

pression of the 521 series (which retains only 17 bp of *Cab* lead sequence) appears lower than that of the 511 series (which r tains 34 bp of *Cab* leader sequence). Expression is observed 3/11 plants for the former and 6/12 for the latter, and the ma imum mRNA level observed in the 521 series is 1/3 of that the 511 series.

Opine synthase activity in transformed plants

The DNA introduced into plant cells in these experiments ca ried the *Cab/ocs* fusion, a kanamycin resistance gene and al a *nos* gene. An opine synthase assay on leaf material scree for expression of both the *nos* gene and the chimaeric *Cab/o* gene. A representative set of assays on leaves from transform petunias is shown in Figure 4B. These data demonstrate that i dependent variability occurs for the expression of both t *Cab/ocs* and *nos* genes. One plant is *nos*$^+$ *ocs*$^-$, one is *nos ocs*$^+$, three (from the same kanR callus) are *nos*$^+$ *ocs*$^+$, a seven show much lower or no synthesis of either opine. The da recorded for the petunias correlate well with the RNA blot dat Northern blot analysis of RNA from transformed plants usi *nos* probes confirms the existence of a high degree of variabili between transformants in the expression of the *nos* gene (da not shown) and also confirms that *ocs* and *nos* gene activity c exhibit independent variability. Opine synthase assays of the 5 series of petunia transformants and also of the tobacco plar transformed with both 511 and 521 constructions confirms t existence of a high degree of variation between transformar in the levels of opine synthase activity. *Ocs*$^+$ *nos*$^-$ plants we observed at a frequency of ~10% and *ocs*$^-$ *nos*$^+$ plants we observed much less frequently (2/100).

Octopine synthase activity is never observed in plar

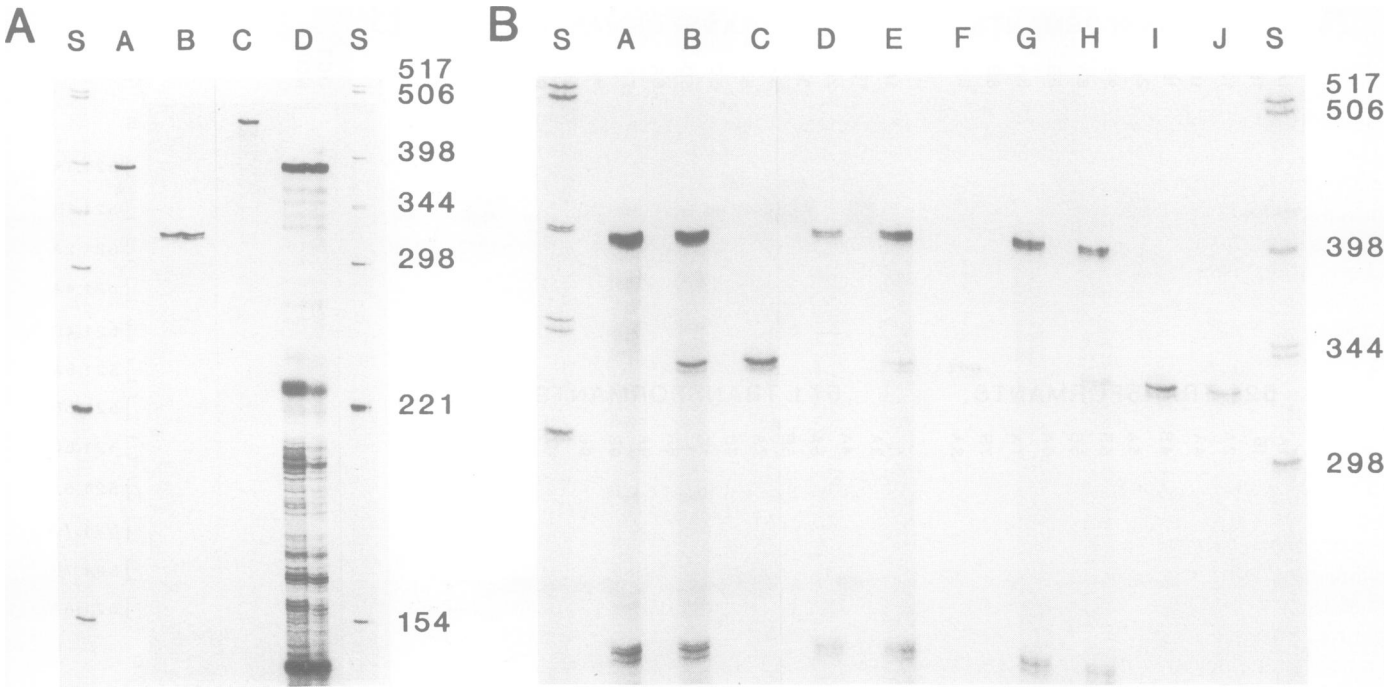

Fig. 5. Mapping the 5′ end of chimaeric and endogenous transcripts. For the *Cab* probe a 432-bp fragment extending from a *Sac*I site 333 bp 3′ to the ATG to a *Taq*I site 98 bp 5′ to the ATG was cloned into *Acc*I/*Sac*I digested pSP65 DNA. For the *Cab/ocs* probe a 369-bp fragment from an *Eco*RI site 271 bp 3′ to the ATG to the same *Taq*I site 98 bp 5′ to the ATG was cloned into *EcoRI/Acc*I cut pSP65. (**A**) **Lane S**, pBR322/*Hinf*I standards; **lane A** *Cab/ocs* probe; **lane B**, *Cab/ocs* probe protected by 50 μg tobacco 571.5 RNA; **lane C**, *Cab 22R* probe; **lane D**, *Cab 22R* probe protected by 40 μg petunia wild-type RNA. (**B**) Petunia 571.7B RNA (30 μg) protected by *Cab 22R* probe (**lane A**), *Cab/ocs* and *Cab 22R* probe in a mixed hybridisation (**lane B**) and *cab/ocs* probe (**lane C**). **Lanes D, E, F**, same experiment with 30 μg petunia 571.10B; **lanes G, H, I**, same experiment with 30 μg petunia 503.10B RNA; **lane J**, *Cab/ocs* probe hybridised to 30 μg tobacco 571.5 RNA.

transformed with the translational fusion despite the presence of abundant mRNA. In making the translational fusion three amino acid substitutions were introduced including the replacement of an alanine by a proline at position 5. We presume that this mutagenesis has inactivated the octopine synthase though we have not excluded the possibility that these mutations preclude translation.

5′ End of the mRNA from the chimaeric and endogenous genes

We have investigated whether the transcriptional initiation site is the same in the chimaeric introduced gene as in the corresponding endogenous *Cab* gene. Fragments were cloned into an SP6 vector (Zinn *et al.*, 1983) so as to produce single-stranded probes which extend beyond the presumed 5′ end of the transcripts from either an *Eco*RI site in the *ocs* coding sequence or a *Sac*I site in the *Cab* coding sequence to a *Taq*I site common to both chimaeric and parental genes. This *Taq*I site is 98 bp from the *Cab* ATG translational initiation sequence. Single-stranded probes were used in experiments involving solution hybridisation followed by RNase digestion and electrophoresis of protected fragments on denaturing gels. The results of a typical experiment are shown in Figure 5A. In this experiment mRNA from a plant which abundantly expressed a translational fusion was hybridised to a 50- to 100-fold molar excess of either *Cab* probe or *Cab/ocs* probe. In lane B a single protected band is observed when *Cab/ocs* sequences are used as a probe and the size of this band corresponds to the predicted fragment size of 331 bp. In lane D using a *Cab* probe both a large band (migrating consistent with its predicted fragment size of 394 nucleotides) and a series of smaller bands are observed. We believe the smaller bands result from hybridisations between the *Cab 22R* probe and other expressed *Cab* genes which are partially homologous to the probe. The sizes of the protected fragments correspond to transcriptional initiation at the same nucleotide (59 bp 5′ to the ATG) in both the *Cab 22R* gene and the introduced *Cab/ocs* fusion gene.

Relative levels of expression of chimaeric and endogenous genes

The solution hybridisation experiments described above are carried out at high concentrations and reach Cot values which exceed 10 times Cot1/2 for the probe lengths used. These theoretical considerations along with control experiments, in which it was shown that the signal of protected fragment was directly proportional to added plant RNA (data not shown), lead us to believe that the signals on the autoradiograph constitute a quantitative assessment of the moles of probe-homologous sequences in an RNA population. Consequently, quantitative solution hybridisation experiments were carried out on RNA from several transformants which had been shown by Northern blot analysis to make abundant chimaeric transcripts. *Cab* and *Cab/ocs* probes individually or as a mixture were hybridised to identical amounts of RNA of each transformant. Fragments protected from RNase digestion were fractionated on a denaturing gel. A typical experiment for RNA from three petunia transformants and also for a tobacco transformant is shown in Figure 5B. After autoradiogram-scanning and correction for the number of radiolabelled nucleotides per mole in the different protected fragments, a number could be deduced for the relative expression of the endogenous *Cab* promoter and that same promoter when fused to octopine synthase sequences and introduced into plant DNA using *Agrobacterium*.

In the most abundantly expressing transformant (571.7B, Figure 5B, lanes A, B, C) the amount of mRNA from the chimaeric gene is half that of the endogenous gene (which in a

diploid would be present in two copies). DNA blots suggest that the introduced DNA is present in four copies (data not shown), and chromosome counts show that this plant is diploid. The two other plants analyzed in Figure 5B (571.10B, 503.10B) each show one third as much chimaeric transcript as endogenous transcript. The most highly expressing tobacco transformant (lane J) shows a level of expression comparable with the best petunia transformants and the same 5′ end, and so there are unlikely to be specific factors in petunia for the expression of this promoter which are absent from tobacco.

Discussion

We show here that chimaeric genes can be introduced to plant cells using *Agrobacterium* and can give rise in regenerated plants to mRNA levels comparable with those derived from the corresponding endogenous gene. It is possible that even higher levels of expression could be obtained if the *Cab 22R* polyadenylation signal was used instead of the *ocs* polyadenylation signal. Selfing the 571.7B plant should lead to molar equivalence of chimaeric and *Cab 22R* transcript. The *Cab 22R* gene constitutes at least 0.1% of total mRNA so using photosynthetic promoters permits higher (in our experiments ~50-fold higher) levels of expression in plants than using opine synthase promoters (usually reported as being 0.001% of total mRNA). However, different families of photosynthetic genes may be differentially regulated both by light intensity and quality and by the stage of development of the plant, and so the absolute proportion of total mRNA which a chimaeric transcript can constitute may be affected by these factors.

To maximise expression from these promoters, it is important to minimise the extent to which the 5′-untranslated region of the chimaeric message departs from the corresponding sequence in an authentic plant gene. The effect on mRNA accumulation of differences in the 5′-untranslated sequence between the various chimaeric genes can be explained in one of several ways. Either the mRNA is destabilized by the GC-rich sequences in this region of the transcriptional fusions (increased turnover rate) or there are sequences downstream from the transcriptional start which are important for the regulation of transcriptional initiation (reduced transcription) as has been shown for globin genes (Charnay *et al.*, 1984). It could simply be that a GC-rich region after a transcriptional initiation site melts less easily and slows polymerase escape from the initiation complex. Whichever hypothesis proves to be correct, our results show that careful attention should be paid to the exact sequences at the junctions of chimaeric genes which are to be introduced into plants.

Even with the best constructions (503 and 571), there is dramatic quantitative variation between transformants in the levels of expression of the introduced gene. We have investigated the copy number of inserted DNA in 30 transformants and all but one plant contains 1 – 5 copies of the introduced DNA. This copy number difference cannot explain the >200-fold variation in expression between transformants. This level of variation has still to be satisfactorily explained. We propose as a working hypothesis that at different sites of insertion of the T-DNA the activity of the introduced DNA is influenced in *cis* by the adjacent plant DNA. However, the kanamycin selection excludes inserts in which the introduced DNA is totally inactive in callus, although it is known that very low levels of neomycin phosphotransferase activity can confer the resistant phenotype. Also, this simple model does not account for the independent variation in *nos* and *ocs* activity indicated in Figure 4B. This may be explained by short range effects extending preferentially into one end of the insertion (Figure 3C) or by a differential effect in *cis* on c constitutive and photosynthetic promoters. We also have evide for incomplete transfers as an explanation for some *nos*$^-$ plar A detailed characterization of the DNA in different transforma will be reported elsewhere.

In this work we are describing the activity in petunia of a p moter isolated from a petunia gene, and we are able to comp the activity of the introduced gene with that of the endogen gene. We show that the 571 gene can be expressed in toba to levels comparable with those in petunia. However, it is s unclear to what extent plant promoters will be fully active distantly related species, and it will be interesting to investig this with the 571 construction.

Materials and methods

DNA biochemistry

Restriction enzymes, *Bal*31 nuclease, linkers, T4 DNA polymerase, T4 D ligase and large fragment of DNA polymerase were from New England I labs and used as recommended by the manufacturer. DNA sequencing was the dideoxy technique adopting the modifications of Biggin *et al.* (1983). Plasn were purified in caesium chloride/ethidium bromide density gradients. Plas DNA was purified between steps of a construction by phenol/chloroform ext tion and ethanol precipitation.

Bacterial strains, plasmids and media

Media and antibiotics were used as described by Maniatis *et al.* (1982) or Mi (1972), except where otherwise stated. pGV3850 (Zambryski *et al.*, 1983) pPH1 (Hirsch and Beringer, 1984) have been described.

Isolation of plant RNA

Leaf tissue (2 g) was frozen in liquid nitrogen in a mortar and ground wit pestle to a fine powder. NTES buffer (4.5 ml, 0.1 M NaCl, 0.01 M Tris-H pH 7.5, 1 mM EDTA, 1% SDS) and phenol/chloroform [3 ml of a 1:1 mixt of Tris buffered (pH 8) phenol:chloroform/isoamyl alcohol (24/1)] were ad and the grinding continued until the mixture thawed. The mortar was was with 4.5 ml NTES buffer and 3 ml phenol/chloroform solution. After vortex for 5 min the solution was centrifuged at 10 000 *g* for 10 min. The aqueous ph was removed, and the nucleic acid was precipitated by the addition of 0.1 volu of 3 M NaAc, pH 6.0 and 2 volumes of ethanol. The precipitate was was with 70% ethanol and resuspended in 2.5 ml sterile water. To remove DNA low mol. wt. RNA, an equal volume of 4 M LiAc was added and the solut allowed to sit for 3 h on ice. The precipitate was pelleted at 10 000 *g* for 10 n The pellet was resuspended in 900 μl sterile water and the RNA was precipit by the addition of 100 μl 3 M NaAc, pH 6.0 and 2 ml ethanol. The pellet washed with 70% ethanol and resuspended in sterile water. The yield of R from 2 g leaf tissue was ~4 mg.

RNA probes from SP6 transcripts

SP6 RNA polymerase was used in labelling experiments and RNA probes u in Northern or solution hybridisation experiments essentially as recommen by the manufacturer (Promega Biotech). Recombinant SP6 plasmids were puri on caesium chloride gradients and linearized prior to phenol/chloroform extr tion and ethanol precipitation. The linearized DNA (0.4 μg) was incubatec a 20 μl transcription reaction with 100 μCi [^{32}P]UTP (Amersham). Routine 5 – 10 x 10^7 c.p.m. of probe were made. 1 – 2 x 10^6 c.p.m. were used in dividual solution hybridisations at 45°C overnight under paraffin oil in 30 μl quots of 80% formamide, 0.4 M NaCl, 1 mM EDTA and 10 mM Pipes, pH containing 10 – 50 μg of total RNA. Subsequent RNase digestion and anal on gels was carried out as described by Zinn *et al.* (1983).

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