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Expression of tandem gene fusions in transgenic tobacco plants

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ABSTRACT

We have studied the expression of four sets of tandem gene fusions in transgenic tobacco plants. This was to determine if the problem of between-transformant variability in expression of introduced genes could be overcome by using a linked reference gene as a co-ordinately expressed control. Tandem gene fusions containing identical 5' flanking regions (SSU301-ocs with either SSU301-cat or SSU301-SSU911) were not co-ordinately expressed in the transgenic tobacco plants whereas the tandem gene fusions containing similar but not identical 5' flanking regions (SSU301-ocs with SSU911-cat or SSU911-SSU301) were co-ordinately expressed. The lack of co-ordinate expression of some of the tandem gene fusions appears to be partially explained by absence of the corresponding genomic DNA segments in the transgenic plants.

INTRODUCTION

Variability in the expression of an introduced gene in transgenic plants is a major technical problem in the quantitative analysis of plant genes. This has led to several strategies being tested which might reduce the between-transformant variability (1). We have demonstrated that buffering an introduced petunia rbcS gene with relatively large amounts of natural flanking DNA does not reduce the variability in expression of the gene in transformed tobacco plants (Dean et al adjoining paper). This result suggests that, if the adjacent genomic DNA causes the variability in expression observed between transgenic plants, the "position effects" must act over relatively long distances (10kb) in the chromosome. A prediction arising from this observation is that two closely linked genes introduced into plants may be affected by "position effects" in a similar way and their expression may vary co-ordinately in transgenic plants. Consequently, it may be possible to use a

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linked reference gene as an internal standard when comparing different gene fusions in transgenic plants.

In some previous experiments where the expression of two linked genes was studied in transgenic plants there was no evidence for co-ordinate expression (2,3). In these experiments the two linked genes were of different origins. In one example, the expression of a chlorophyll a/b binding protein / octopine synthase (Cab-ocs) fusion was compared to that of a linked nopaline synthase (nos) gene (2). Cab gene expression is normally light and developmentally regulated (4), whereas nos expression appears to be constitutive (5). These two genes may be affected in a different manner by the surrounding plant DNA. Also, in most of the reported experiments addressing this issue, one of the gene fusions has been used as the selectable marker in the transformation experiments. This could preferentially affect the expression of only one of the linked genes being assayed.

We describe in this paper experiments in which we have studied the expression of four sets of tandem gene fusions. The gene fusions contained either identical or very similar rbcS 5' and 3' flanking regions and none of the gene fusions were used as selectable markers. We observe that the expression of two sets of adjacent gene fusions vary co-ordinately, whereas the expression of the other two do not vary co-ordinately. We discuss these data with respect to the possibility of using a linked reference gene system for the analysis of plant gene expression in transgenic plants.

METHODS

General methods

All general manipulations of DNA and RNA were carried out essentially as in Maniatis et al (6). Outlines of the cloning strategies used are given in the figure legends. Further details of any of the constructions are available upon request. Oligonucleotides were synthesized on an Applied Biosystems Model 381A DNA synthesizer and purified as described in Dean et al (7). The autoradiographs from the primer extension gels were scanned using an LKB 2202 scanning densitometer. A standard dilution series was used to correct for the non-linearity of the response

of the X-ray film. These numbers were then used to calculate correlation coefficient values using the Macintosh Cricket Graph program.

Generation and analysis of transgenic plants

Mobilization of clones into the *Agrobacterium* strain LBA 4404/pAL 4404 (8), co-cultivation of the *Agrobacterium* strains with tobacco protoplasts and selection of kanamycin resistant plants were carried out as described in van den Elzen et al (9). The kanamycin resistant calli were screened for hygromycin resistance by subculture on MS media containing 2mg/l NAA, 0.5mg/l BAP and 20mg/l hygromycin (Calbiochem).

RNA was isolated from the tobacco leaves as described in Dean et al (10) and analyzed using the primer extension protocol described in Dean et al (7). The oligonucleotides 301T and 911T have been described previously (7). The oligonucleotides used to analyze the cat and ocs RNAs respectively were : 5' TGTTCTTTACGATGCCATTGGG 3' ; 5' AGATCACCTGCAAGAGTAAGAG 3'.

Genomic DNA was isolated from the tobacco plants as described in Dunsmuir et al (11) and analyzed using genomic Southern blots and RNA probes as described by Jones et al (12). The number of independent T-DNA insertions into each plant was assessed by analysis of the genetic segregation of kanamycin resistant seedlings. Seeds derived from self crosses were surface-sterilized and germinated on moist filter paper. The seedlings were excised at the hypocotyl and transplanted to MS plates containing 3% sucrose and 100mg/l kanamycin. The seedlings were classified as kanamycin resistant if they rooted.

RESULTS

Construction of the Agrobacterium binary Ti plasmid vector containing the SSU301-ocs reference gene flanked by two selectable markers

The kanamycin-resistance conferring *Agrobacterium* binary Ti plasmid vector pAGS135 described in Dean et al, (adjoining paper) was modified to contain a second plant selectable marker, hygromycin phosphotransferase. A 1.7kb PvuII fragment from pAGS120 (13) which carries a nos-hygromycin phosphotransferase (HPTII) gene fusion (13) was cloned into the HpaI site located

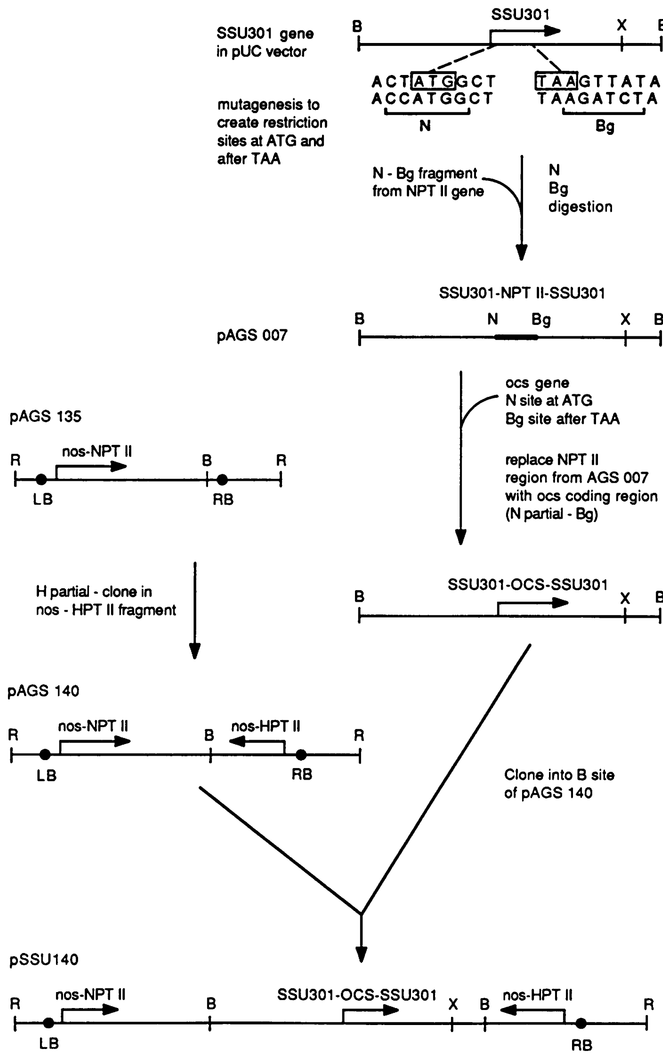


Fig. 1. Construction of the Agrobacterium binary vector pSSU140 containing both nos-NPTII and nos-HPTII selectable markers and the SSU301-ocs fusion. The plasmid pSSU301 carries the SSU301 gene (15) with 5kb of 5' and 1.5kb of 3' flanking DNA sequences. The direction of the arrow shows the direction of transcription. Site-directed mutagenesis was used to create an NcoI site at the ATG codon and a BglII site immediately after the TAA codon. An NcoI site 3.5kb 5' to the transcription start site of SSU301 was removed using Klenow treatment. The coding region of the SSU301 gene was replaced with a BglII-NcoI fragment (carrying the promoter and 5' end of the NPTII gene) from Tn5, nucleotides

1515-2111 (25). The ocs coding region from plasmid pJJ6710 (17) contains an NcoI site at the ATG codon and a BglII site was introduced immediately after the TGA codon. The ocs coding region (NcoI partial-BglII) was cloned into pAGS007 replacing the NPTII fragment.

The PvuII fragment from pAGS120 (13) was cloned into the Agrobacterium binary vector pAGS135 (Dean et al, adjoining paper) which had been partially digested with HpaI, to give the plasmid pAGS140. The SSU301-ocs fusion was cloned into pAGS140 at the BamHI site. The resulting plasmid, pSSU140 contains a unique XhoI site 800bp 3' to the ocs coding region.

E=EcoRI; N=NcoI; Bg=BglII; B=BamHI; H=HpaI; X=XhoI; LB and RB= left and right borders of T-DNA; nos-NPTII= nopaline synthase- neomycin phosphotransferase II gene fusion; nos-HPTII= nopaline synthase-hygromycin phosphotransferase II gene fusion.

260bp inside the right border of the T-DNA in pAGS135 (14). The resulting plasmid, pAGS140, confers both kanamycin and hygromycin resistance to plant cells. DNA introduced into the BamHI site of pAGS140 is therefore flanked by two selectable markers (Fig.1). Selection or screening for both drug resistance markers, in the transformation experiments, increases the probability of complete transfer of the sequences between the nos-NPTII and nos-HPTII markers, which in some of the constructions is 22kb long.

A fusion between the 5' and 3' regions of the most abundantly expressed petunia gene, encoding the small subunit of ribulose bisphosphate carboxylase (rbcS), SSU301, (15) and the coding region from the Agrobacterium tumefaciens T-DNA gene, octopine synthase (ocs, 16) was constructed. This fusion was used as the reference gene fusion in the subsequent experiments. In order to create the SSU301-ocs fusion the SSU301 gene was modified by site-directed mutagenesis to create an NcoI site at the translation initiation codon and a BglII site immediately downstream of the translation termination (TAA) codon. For ease of use, the 5' and 3' regions of the SSU301 gene were combined to form an expression cassette (pAGS007) which contained 5kb of SSU301 DNA 5' to the translation initiation codon, 1.5kb of SSU301 DNA 3' to the translation termination codon and a 600bp NcoI-BglII fragment from the neomycin phosphotransferase II (NPTII) gene (Fig.1).

The ocs coding region carried on the plasmid pJJ6710 (17) contains an NcoI site at the translation initiation codon. A

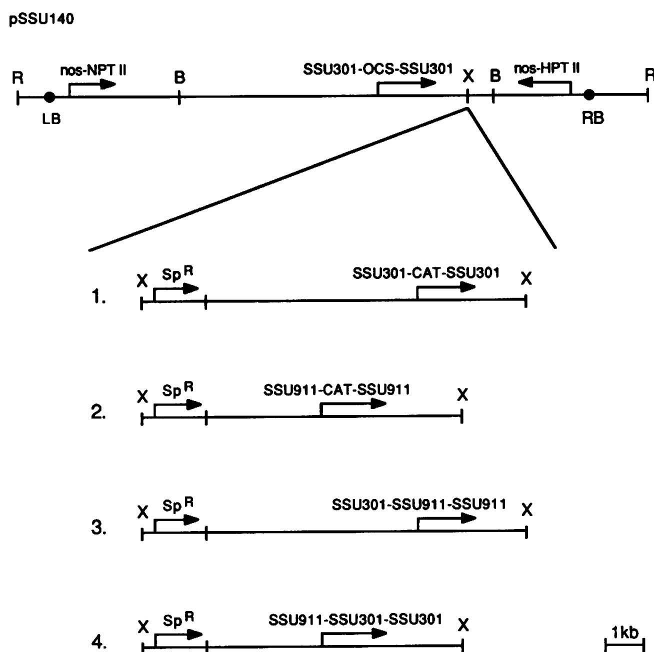


Fig. 2. Summary of the construction of the four tandem gene fusions. Site-directed mutagenesis was used to create NcoI and BglII restriction sites at the ATG and TAA codons respectively of SSU911 and the CAT coding region (chloramphenicol acetyltransferase, 19). The coding regions of SSU301 and SSU911 were replaced with the CAT coding region (NcoI partial, BglII) to give the SSU301-cat and SSU911-cat fusions (1 and 2). The two exchange fusions (3 and 4) were constructed by exchanging the sequences of SSU301 and SSU911 at NcoI sites located at the ATG codons. The gene fusions, 1-4 were modified using linkers to have an XhoI site at each end and to include a a bacterial spectinomycin resistance gene, a 1.5kb HindIII-PvuII fragment from pDPT274 (20). The fusions were then cloned into the XhoI site of pSSU140. SP^R= bacterial spectinomycin resistance gene. Other symbols are as in Fig.1.

BglII site was introduced immediately downstream of the translation termination (TGA) codon by site-directed mutagenesis. The ocs coding region was then used to replace the NPTII fragment in pAGS007 (Fig.1). The SSU301-ocs fusion was cloned into the pAGS140 binary Ti plasmid vector at the BamHI site (Fig.1). The resulting vector, pSSU140, contains a unique XhoI restriction site approximately 600bp downstream of the SSU301 polyadenylation sites (18) (Fig.1).

Construction of the four tandem gene fusions

Four gene fusions were cloned individually into the XhoI site of the pSSU140 binary vector (Fig.2). The SSU301-cat (chloramphenicol acetyltransferase, (19)) fusion and the SSU911-cat fusion (SSU911 is the most weakly expressed petunia rbcS gene, (10)) were constructed as described for the SSU301-ocs fusion above. The SSU301-cat fusion contains 3' sequences from the SSU301 gene and the SSU911-cat fusion contains 3' sequences from the SSU911 gene. The other two fusions cloned downstream of the SSU301-ocs fusion, in pSSU140, were "ATG exchange fusions" between the SSU301 and SSU911 rbcS genes. The exchange point between the two genes was at an NcoI site, which was introduced by site-directed mutagenesis, at each translation initiation codon. The four sets of tandem fusions are shown in Fig.2.

One consequence of the construction of sets of tandem gene fusions was the formation of large regions of directly repeated DNA. In bacteria, recombination between the sequences within the large direct repeats would result, at some frequency, in the loss of DNA between the repeats. In order to select for the maintenance of the large direct repeats during passage through E.coli and A.tumefaciens, a 1.5kb HindIII-PvuII fragment from pDPT274 (20) carrying a bacterial spectinomycin gene was inserted between the two gene fusions (Fig.2). Spectinomycin resistance was selected during all the bacterial stages until the stage of co-cultivation with plant protoplasts. Genomic Southern blot analysis of the introduced DNA in the transgenic tobacco plants was also carried out to confirm the presence of the introduced DNA.

Expression of the tandem gene fusions SSU301-ocs and SSU301-cat

Expression of the various fusions was quantitated by primer extension analysis on RNA extracted from the transformed plants at a developmental stage shown to be optimal for rbcS expression (Dean et al adjoining paper). Sixteen transgenic tobacco plants containing the tandem gene fusions SSU301-ocs and SSU301-cat were assayed and the results are shown in Fig.3. The oligonucleotides designed to be complementary to the ocs and cat RNA do not anneal to RNA isolated from untransformed tobacco (Fig.3). Since the expression of the SSU301-ocs fusion is on average 8 fold lower

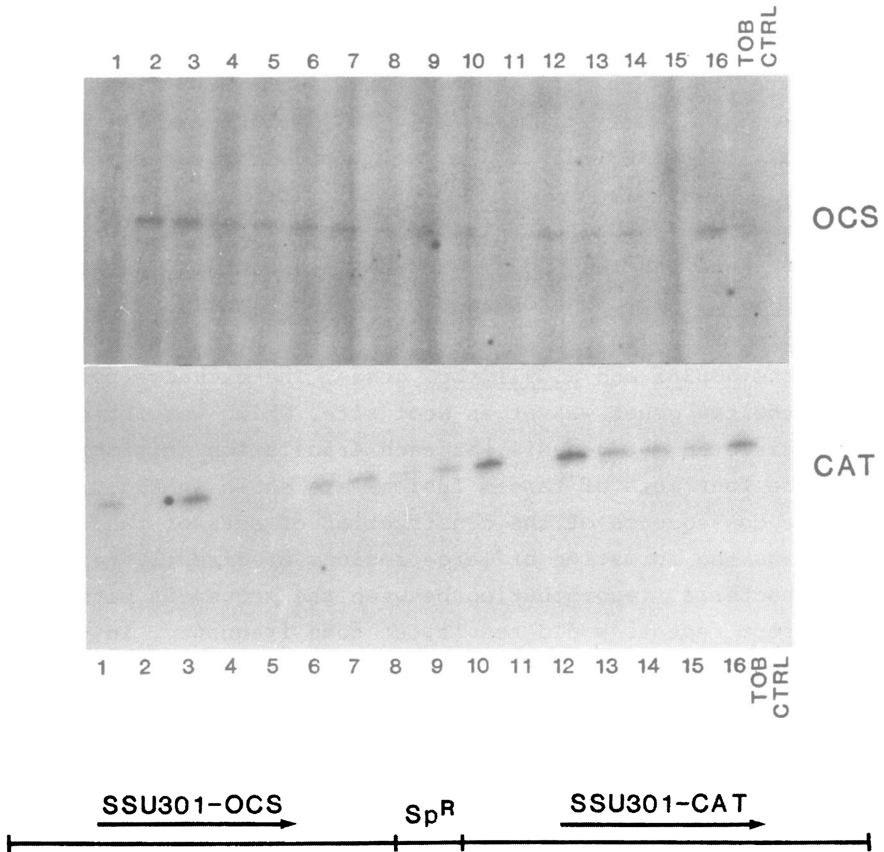
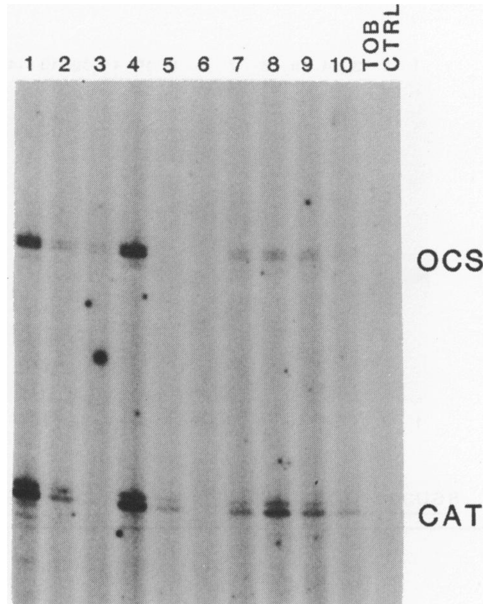


Fig. 3. Primer extension analysis of RNA isolated from transgenic tobacco plants carrying two SSU301 gene fusions in tandem. The ocs (upper panel) and cat (lower panel) primer extended fragments are shown for 16 transgenic tobacco plants. An untransformed tobacco control was included. The tandem gene fusion contained in the transformed plants is shown at the base of the figure.

than that of the SSU301-cat fusion (21), the exposures of the autoradiographs shown in Fig.3 have been selected to show approximately the same ocs and cat signal. Both the SSU301-ocs and SSU301-cat fusions show variable expression between individual transgenic plants. In the first tobacco plant shown in Fig. 3 there is no primer extended fragment corresponding to ocs RNA but the predicted 117bp primer extended fragment from the cat oligonucleotide is present. In contrast, in the second plant



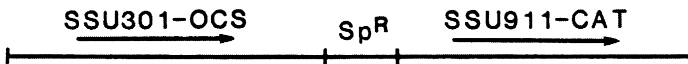


Fig. 4. Primer extension analysis of RNA isolated from transgenic tobacco plants carrying an SSU301-ocs fusion and an SSU911-cat fusion in tandem. The primer extended fragments for the ocs and cat fusions in 14 independently transformed plants are indicated. The tandem gene fusion contained in the transformed plants is shown at the base of the figure.

shown in Fig. 3 the predicted 114bp ocs primer extended fragment is present but there is no fragment corresponding to cat RNA. A comparison of the primer extended fragments corresponding to the ocs and cat RNA in all 16 transgenic tobacco plants clearly shows that there is no co-ordination between the expression of the two SSU301 gene fusions. The correlation coefficient between the ocs and cat RNA levels was calculated to be 0.23.

Expression of the tandem gene fusions SSU301-ocs and SSU911-cat

The primer extension analysis of the 10 transgenic tobacco plants carrying the SSU301-ocs and SSU911-cat tandem gene fusions

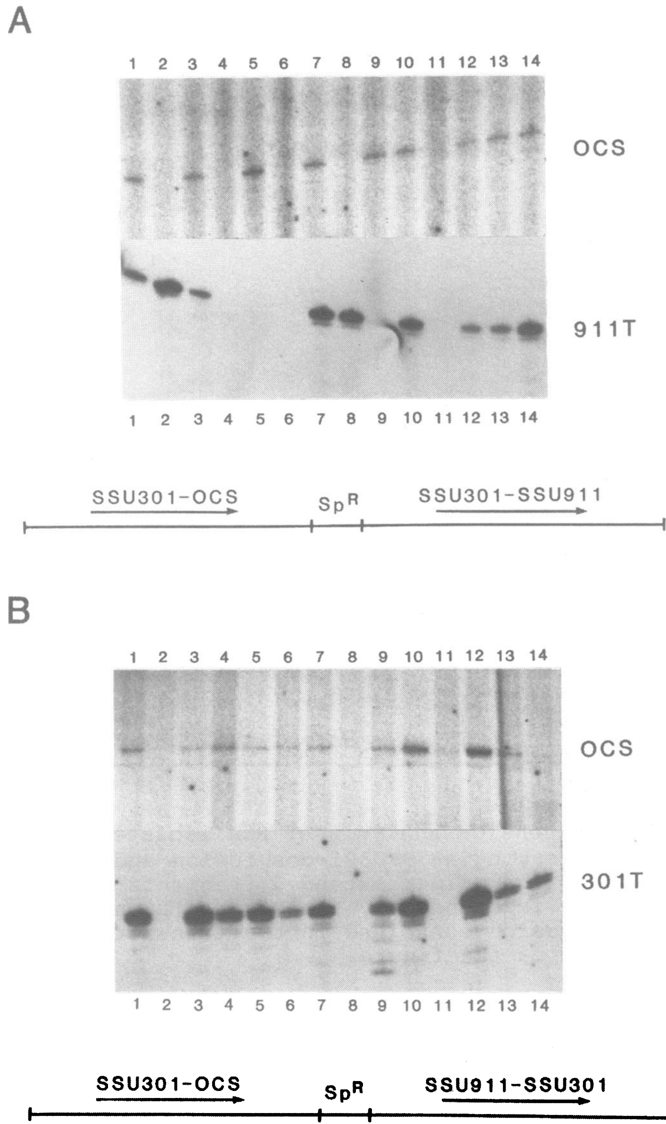


Fig. 5. Primer extension analysis of RNA isolated from 14 transgenic tobacco plants carrying fusion and hybrid petunia rbcS genes in tandem. The upper panel in sections A and B show the primer extended fragments for the SSU301-ocs fusion. The lower panel shows the primer extended fragments for the exchange fusions: A. the SSU301-SSU911 fusion using the oligonucleotide 911T; B. the SSU911-SSU301 fusion using the oligonucleotide 301T. The tandem gene fusion contained in the transformed plants is shown at the base of each section.

is shown in Fig.4. The primer extended fragment from the SSU911-cat fusion is only 85bp compared to 114bp for the SSU301-ocs fusion. Both the SSU301-ocs and SSU911-cat fusions show variable expression between individual transgenic plants, however there is a good degree of co-ordination between the expression of the two different gene fusions in the transgenic plants. Those plants showing high levels of ocs RNA also show high levels of cat RNA and vice versa. The correlation coefficient between the ocs and cat RNA levels is 0.9. These results are clearly very different from the results with the two SSU301 gene fusions in tandem (Fig.3).

Expression of SSU301-ocs and hybrid rbcS genes in tandem

Figures 3 and 4 give contrasting data as to whether two rbcS gene fusions in tandem are co-ordinately expressed in independently transformed plants. The tandem gene fusions containing the SSU301 5' and 3' flanking regions did not show co-ordinate expression whereas, the tandem gene fusions containing 5' and 3' flanking regions derived from different rbcS genes did show co-ordinate expression. We have therefore examined the expression of two additional sets of tandem gene fusions. These constructions contained the SSU301-ocs reference gene fusion linked with reciprocal ATG exchange fusions between the SSU301 and SSU911 rbcS genes (Fig.2). The two gene fusions were assayed in the tobacco plants using primer extension, and the 301T and 911T oligonucleotides (7) were used to assay the hybrid gene expression (Fig.5).

Primer extension analysis on RNA of the plants containing the SSU301-ocs fusion linked to the SSU301-SSU911 hybrid (Fig.5A) shows there is no co-ordination between the expression of the these two linked gene fusions (the correlation coefficient between the two RNA levels is 0.05). However, there is co-ordination between the expression of the SSU301-ocs fusion and the SSU911-SSU301 hybrid in the transgenic plants (Fig.5B), the correlation coefficient between these RNA levels is 0.78.

In summary, the tandem gene fusions containing identical 5' flanking regions (SSU301-ocs with SSU301-cat or SSU301-ocs with SSU301-SSU911) were not co-ordinately expressed. Conversely, the tandem gene fusions containing different 5' sequences (SSU301-ocs

Table 1. Summary of the hygromycin sensitivity, RNA and DNA analysis and kanamycin resistance segregation of selected transgenic tobacco plants carrying the indicated tandem gene fusions.

plant, SSU301-ocs plus:	hyg^s	RNA		DNA		progeny kan^r:kan^s (approx. locus#)
		ocs	cat/hybrid	ocs	cat/hybrid	
SSU301-cat no.2	+	+	-	+	-	80:20 (1)
3		+	+	+	+	91:9 (2)
11	+					
12		+	+	+	+	72:28 (1)
16	+					
SSU301/911 no.2		-	+	-	+	53:24 (1)
5		+	-	+	-	79:21 (1)
6	+					
8		-	+	-	+	65:35 (1)
9		+	-	+	-	73:26 (1)
11	+					
SSU911/301 no.1		+	+			97:3 (3)
2	+	-	-	-	-	0:100 (0)
4		+	+	+	+	98:2 (3)
10		+	+	+	+	
12		+	+			74:26 (1)

with SSU911-cat or SSU301-ocs with SSU911-SSU301 were co-ordinately expressed.

DNA analysis of the transgenic tobacco plants

In many of the transgenic tobacco plants there was detectable RNA from only one of the tandem fusions. Deletion of part of the insert or incomplete transfer of the T-DNA could account for this. Incomplete transfer was assessed by constructing the tandem gene fusions so that they were flanked by two different selectable markers. The original transformed calli which had been selected using kanamycin resistance were screened for hygromycin resistance. The kanamycin resistant calli that were scored as hygromycin sensitive are shown in Table 1. Only 20% of the kanamycin resistant calli lacked the hygromycin marker. This indicated that at least one complete T-DNA transfer had occurred in the majority of the transformed calli.

Jones et al (12) have extensively investigated whether structure of introduced DNA accounts for the between-transformant variability in transformed plants. They concluded that the lack of co-ordinate expression of two linked genes (a nopaline synthase gene and a chlorophyll a/b binding protein promoter -ocs

fusion) in transformed petunia plants was not a consequence of incomplete transfer or structure of the introduced DNA. This conclusion was subsequently confirmed by Odell et al (22). We checked the genomic DNA of several of the transgenic tobacco plants for the presence of the individual gene fusions, concentrating on those showing expression of one but not the other gene fusion. Digests of genomic DNA that give rise to both internal fragments and border fragments of the introduced DNA were hybridized with probes specific to either the ocs or cat coding regions, or 3' tail probes specific to the introduced rbcS hybrid genes. The presence or absence of hybridizing bands in the DNA isolated from the different plants was scored and the data is shown in Table 1.

The results in Table 1 show that, for those plants examined, the lack of expression of one of the gene fusions can be explained by the absence of the corresponding DNA. Also included in Table 1 are the segregation ratios of kanamycin resistance in seedlings from self-pollinated plants. These ratios show that the majority of the plants contain introduced DNA that segregates as a single locus. Two of the plants examined, SSU911/301 nos. 1 and 12, however, appear to contain two or three loci. Comparison of the expression levels of the fusions in the tobacco plants containing the tandem fusion SSU301-ocs and SSU911/SSU301 (which show a good degree of co-ordination in their expression) shows that there is no correlation between expression levels of the different fusions and the number of copies of the introduced DNA. For example, plant no.4 in Fig. 5B (which has 3 segrgating loci) shows moderate levels of expression of both fusions, whereas plants no. 10 and 12 (where the introduced DNA segregates as one locus) show a high level of expression of both fusions.

DISCUSSION

We have introduced four sets of tandem gene fusions into tobacco (Fig.2) to determine if the problem of between-transformant variability could be overcome by using a linked reference gene as a co-ordinately expressed control. In two sets of tandem gene fusions each fusion contained identical

5' flanking regions from the petunia *rbcS* gene *SSU301*. In the other two sets the fusions contained similar, but not identical, 5' flanking regions from two different petunia *rbcS* genes, *SSU301* and *SSU911*.

We reasoned that tandem gene fusions with identical 5' and 3' flanking regions may be influenced by the "position effects" in a similar way and therefore their expression would vary co-ordinately in the transgenic plants. Contrary to expectations, the expression of the two tandem gene fusions containing the same 5' regions did not vary co-ordinately in the transgenic plants whereas the tandem gene fusions containing different 5' regions did vary co-ordinately. This result is partially explained after analysis of the introduced DNA. In each plant examined, lack of expression of one of the gene fusions was correlated with the absence of the corresponding DNA fragment. The lack of expression of one of the gene fusions and loss of corresponding DNA occurred predominantly in the tandem fusions which contained the repeated 5kb of DNA containing the *SSU301* 5' region (namely in the tandem gene fusions *SSU301*-ocs with *SSU301*-cat or *SSU301*-ocs with *SSU301*-*SSU911*). One simple interpretation of the data is that recombination occurred between the directly repeated sequences (for *SSU301*-ocs with *SSU301*-cat either the 5kb of 5' repeated sequences or the 800bp of 3' repeated sequences) which led to loss of the sequences positioned between the direct repeats. Recombination may have occurred during the transfer of DNA from *Agrobacterium* to the plant nucleus or during integration of the DNA into the plant chromosome. From studies on recombination in bacteria one would predict a higher frequency of recombination between the longer (5kb) 5' repeated sequences than between the shorter (800bp) 3' repeated sequences. If this were the case the upstream *SSU301*-ocs fusion would be lost at a higher frequency than the downstream fusion. Since both gene fusions are lost with approximately equal frequency this recombination model is clearly an oversimplification.

The lack of co-ordinate expression of the gene fusions containing the identical 5' regions (*SSU301*-ocs with *SSU301*-cat or *SSU301*-ocs with *SSU301*-*SSU911*) cannot solely be explained by loss of DNA encoding the different gene fusions. In 9 of the 16

transgenic tobacco plants containing the SSU301-ocs and SSU301-cat fusions, RNA corresponding to both gene fusions was present. The correlation coefficient between the ocs and cat RNA levels was calculated for the plants showing expression of both ocs and cat fusions. Unexpectedly, the value was 0.42 showing that even in the plants which show expression of both fusions their expression is not co-ordinated. Since we have not done a complete genetic analysis on the T-DNA integration pattern and locus number for every transformed plant, it is possible that many of the plants contain multiple T-DNA insertions at different positions in the genome and there is loss of one, or other, of the gene fusions at the different loci. This could explain the lack of co-ordination of expression of the SSU301-ocs and SSU301-cat fusions in those plants where both fusions are expressed.

The tandem gene fusions that contain similar but not identical 5' flanking regions show a good degree of co-ordination in their expression. In one of these sets (SSU301-ocs and SSU911-SSU301) there is a direct repeat of 800bp in the 3' flanking region of the genes. Whatever mechanism causes the lack of co-ordination between the tandem gene fusions containing the 5' regions of SSU301 may be partially operating in this case. This may explain why this set of tandem gene fusions shows slightly less co-ordination in expression (the correlation coefficient is 0.78) than the set containing the SSU301-ocs and SSU911-cat fusions (the correlation coefficient is 0.9).

The conclusions from these data suggest that it may be possible to set up a reference gene system for use in the analysis of plant gene expression in transgenic plants. However, before one can use a reference gene system, extensive experiments must be carried out to establish that the levels of expression of the particular gene, or gene fusion, being investigated do indeed vary co-ordinately with a linked reference gene (ideally with a correlation coefficient of at least 0.9). It may be possible to use a reference gene system where the tandem fusions contain identical 5' flanking regions (23) provided that the length of the repeated DNA sequences is kept to a minimum.

An alternative to using a set of tandem gene fusions as a

reference gene system is to use a pair of divergently transcribed genes. Two naturally occurring pairs of divergently transcribed genes, T-DNA genes (24) and petunia Cab genes (17) have also been used to give a high degree of co-ordinate expression when introduced into plants as gene fusions. We need to test if an artificially created pair of divergently transcribed genes (eg. SSU301-ocs and SSU301-cat fusions in an inverted orientation) also give a high degree of co-ordinate expression in transgenic plants.

A concern when using two closely linked gene fusions in experiments addressing gene expression is that one gene will influence the expression of the other. It is therefore important to introduce the different gene fusions individually into transgenic plants and ensure that their expression is unaffected by the linked gene. We have introduced individually into tobacco plants all but one of the gene fusions described in this paper. The different fusions gave the same average level of RNA irrespective of whether they were introduced into tobacco plants individually or in tandem (data not shown). This indicates that there were no cis-acting effects of promoter or enhancer elements from the strongly expressed SSU301 gene, upon the SSU911 fusions.

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