Mol Gen Genet (1992) 235:389–396

MGG

Effect of T-DNA configuration on transgene expression

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Received May 13, 1992 / Accepted June 12, 1992

Summary. T-DNA vectors were constructed which carry a β-glucuronidase (*gusA*) gene fused to the promoter of the nopaline synthase (*nos*) gene and the 3′ end of the octopine synthase (*ocs*) gene. This reporter gene was cloned at different locations and orientations towards the right T-DNA border. For each construct, between 30 and 60 stably transformed calli were analysed for β-glucuronidase activity. Depending on the T-DNA configuration, distinct populations of *gusA*-expressing calli were obtained. Placing the reporter gene in the middle of the T-DNA results in relatively low expression levels and a limited inter-transformant variability. Placing the gene with its promoter next to the right border led to an increase in both the mean activity and the variability level. With this construct, some of the calli expressed the *gusA* gene at levels four to five times higher than the mean. In all these series, at least 30% of the calli contained reporter gene activities that were less than half of the mean expression level. Separating the *gusA* gene from the right T-DNA border by an additional 3′-untranslated region, derived from the *nos* gene, resulted in an increase in the mean expression to a level almost four times higher than that of constructions carrying the reporter gene in the middle of the T-DNA. Moreover, the number of transformants with extremely low activities decreased by at least 50% and this resulted in significantly lower inter-transformant variability independently of the orientation of the reporter gene on the T-DNA.

Key words: *Agrobacterium tumefaciens* – Inter-transformant variability – *Nicotiana tabacum* – Position effect – Transcription interference

Introduction

Agrobacterium-mediated plant cell transformation usually results in the stable integration of one or a few copies of a well-defined T-DNA region (reviewed in Gheysen et al. 1989). Most often, the expression pattern of the transgenes that are in the plant genome is qualitatively correct (e.g. Okamuro et al. 1986; Bogusz et al. 1990). However, the quantitative expression levels can vary widely between independent transformants (Dean et al. 1988; Gendloff et al. 1990; Peach and Velten 1991) and there is usually no positive correlation between the T-DNA copy number and the expression level of the transgenes, when limited numbers of individual transformants are compared (e.g. Odell et al. 1987; Shirsat et al. 1989; Gendloff et al. 1990; Hobbs et al. 1990).

Inter-transformant variability may be caused by several molecular and genetic phenomena: the T-DNA that enters the plant nucleus upon transformation lacks a predetermined chromatin structure and DNA modification pattern; random methylation (reviewed by Selker 1990), mutation, rearrangements (Gheysen et al. 1990) or interactions with host cell factors, occurring prior to, during or after integration might greatly influence the expression of the transgene, independently of the site of insertion (discussed in Peach and Velten 1991). On the other hand, however, inter-transformant variability is explained in many cases by position effects: transgene expression will be variably affected by the structural and functional properties of the chromatin region flanking the T-DNA integration site. Firstly, the local chromatin structure and modification pattern of the flanking DNA can determine the state of the integrated T-DNA (e.g. Coates et al. 1987; Peerbolte et al. 1986). Secondly, the higher-order chromatin structure of the region surrounding the integration site might influence gene expression. Since eukaryotic DNA seems to be organized in looped domains, which function as independent genomic units (Eissenberg and Elgin 1991), the specific loop in which the T-DNA has inserted may partially determine the DNA structure and the overall expression level of the present genes. Thirdly, transgene expression can be influenced directly by neighbouring plant regulatory sequences; it is thought that the T-DNA preferentially integrates in transcribed DNA re-

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gions since a promoterless marker gene linked to the right T-DNA border is transcriptionally activated in at least 30% of all transgenics (Koncz et al. 1989; Herman et al. 1990; Kertbundit et al. 1991). Furthermore, reconstruction experiments (Ingelbrecht et al. 1991) led to the suggestion that transcriptional activity at the site of insertion may cause a decrease in transgene expression due to transcriptional read-through. Besides transcriptional interference, plant regulatory sequences such as enhancers and silencers also may influence the activity of the introduced genes. It has been shown, for instance, that it is possible to obtain regulated expression after introducing a reporter gene driven by a constitutive promoter (Fobert et al. 1991; Goldsbrough and Bevan 1991).

These data indicate that transgene expression can vary due to dominant effects exerted by neighbouring plant sequences. If so, it is likely that this direct influence will be affected by the localization of the transgene on the T-DNA, as was previously discussed by Gidoni et al. (1988) in a study on the co-ordinated expression of two divergently coupled transgenes. These authors noticed that the degree of co-ordinated expression in stable transformants was different for two locations of the genes on the T-DNA vector. Apart from this observation, there is in plants no detailed knowledge available about the correlation between variation in transgene expression and the specific configuration of the T-DNA construct, nor about the specific nature of the influencing factors.

In this study, we have examined the influence of the T-DNA configuration on inter-transformant expression variability of a reporter gene. For this purpose, we used different constructs carrying the same chimeric *gusA* gene controlled by the promoter of the nopaline synthase gene and the 3′ end of the octopine synthase gene. We demonstrated that both the orientation of the reporter gene and its distance from the right border could significantly affect the overall pattern of β-glucuronidase (GUS) activity. Moreover we showed that insertion of an additional 3′-end region between the reporter gene and the right T-DNA border results in augmented *gusA* expression and reduced inter-transformant variability. This indicates that transcriptional interference may diminish the expression of the reporter gene in vivo in some of the transformants.

Materials and methods

Construction of T-DNA vectors. The constructed T-DNAs are shown in Fig. 1. The promoterless β-glucuronidase (*gusA*) gene (Jefferson et al. 1986) with the 3′ end of the octopine synthase (*ocs*) gene, present in the plasmid pGUS1 (Peleman et al. 1989) was fused to the promoter of the nopaline synthase (*nos*) gene (Herrera-Estrella et al. 1983). The resulting plasmid was named pNGUS. The chimeric *gusA* gene was isolated from pNGUS as a *Pvu*II fragment and cloned in both orientations in between the neomycin phosphotransferase (*nptII*) gene and the bacterial spectinomycin resistance (Sp^r) gene of the T-DNA vector pGV300 (Claes et al. 1991), resulting in the vectors pNG1 and pNG2 (Fig. 1A). In addition, these two vectors also contain the selectable hygromycin resistance (*hpt*) gene between the *nptII* gene and the left border (LB) derived from

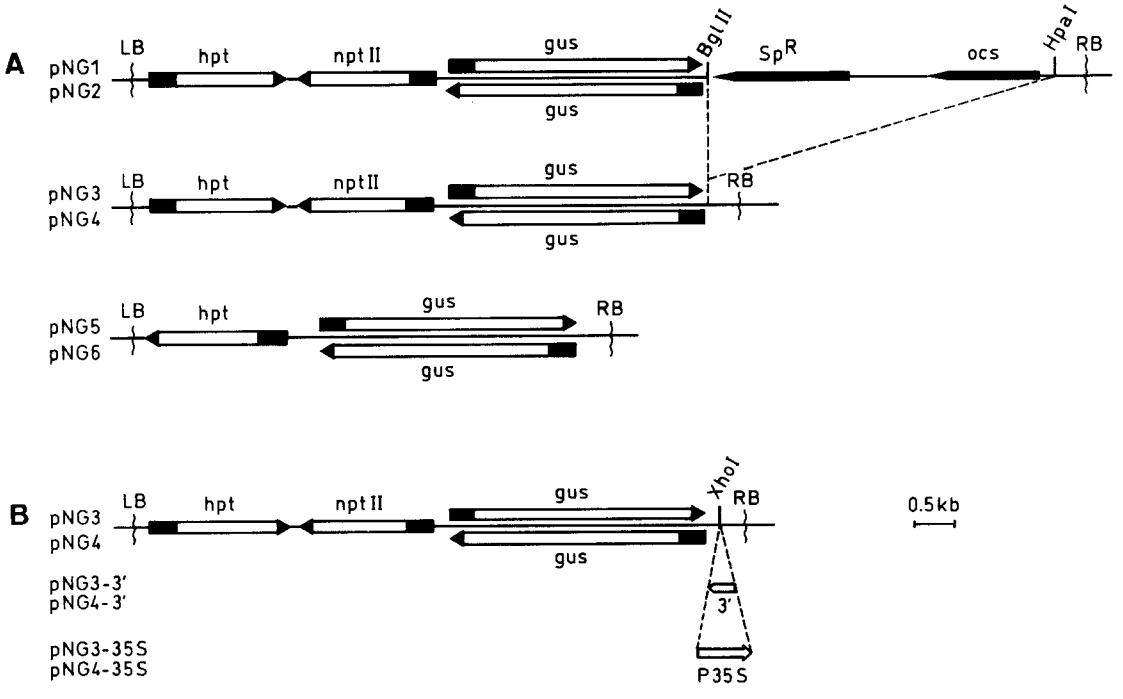

Fig. 1A, B. Schemes of the T-DNA region in the constructed vectors. **A** pNG1 to pNG6. **B** Derivatives from pNG3 and pNG4 containing 3′*nos* (3′) or P35S fragment. The cloning steps are described in the Materials and methods. *LB*, Left border; *RB*, right border; *black rectangles*, P*nos*; *black arrowheads*, 3′-end regions, 3′*nos* in case of the *hpt* gene and 3′*ocs* for the *nptII* and *gus* genes

the octopine T-region, and an octopine synthase gene between the Sp^r gene and the right octopine border (RB). The vectors pNG3 and pNG4 were created by deleting the *Bgl*II-*Hpa*I fragment containing the Sp^r and the *ocs* gene from pNG1 and pNG2 respectively. As a result the *gusA* gene comes to lie only 400 bp from the RB. In both pNG1 and pNG3, the reporter gene is oriented towards the RB, whereas in pNG2 and pNG4, its orientation is inverted. For the construction of pNG5 and pNG6, the same *gusA*-containing *Pvu*II fragment was cloned in the *Hpa*I site of pGV1503 (Ingelbrecht et al. 1989) so that the gene is 400 bp away from the right octopine T-DNA border. pGV1503 was derived from pGV1500 (Deblaere et al. 1987) by cloning a P*nos-hpt-3'nos* gene between the octopine T-DNA borders. All constructed vectors thus contain identical border sequences.

In a next step, a 250 bp fragment containing the 3'-untranslated region of *nos* (3'*nos*; Depicker et al. 1982) was cloned between the RB and the *gusA* gene of both pNG3 and pNG4, resulting in pNG3-3' and pNG4-3', respectively. The fragment was inserted such that it would terminate possible transcripts coming from the flanking plant DNA (Fig. 1 B). Similarly, a 460 bp fragment containing the cauliflower mosaic virus 35S promoter (P35S; Odell et al. 1985) was cloned oriented towards the RB, resulting in pNG3-35S and pNG4-35S. This fragment contains the basic promoter sequences and also the enhancer elements necessary for regulated transcription (Odell et al. 1988). Details of the cloning steps can be obtained upon request.

Conjugation and plant transformation. All T-DNA vectors were mobilized from *Escherichia coli* to *Agrobacterium tumefaciens* C58C1(pGV2260), (Deblaere et al. 1985). Transconjugants harbouring the correct T-DNA constructs were co-cultivated with *Nicotiana tabacum* SR1 protoplasts (Depicker et al. 1985) and regenerating calli were selected on medium containing 25 µg or 100 µg hygromycin/ml. Hygromycin-resistant calli were grown on B5 medium (Gamborg et al. 1968) supplemented with 0.2 mg benzylaminopurine/l and 0.3 mg naphthaleneacetic acid/l and 10 µg hygromycin/ml. Each construct was used at least twice in independent co-cultivation experiments and every time between 30 and 60 calli were analysed for GUS activity. Except for the number of calli lacking detectable GUS activities, the results obtained with each of the constructs were essentially the same for every transformation. Series of calli were named according to the T-DNA they contain, e.g. TNG1 series, TNG2 series.

Measurement of β-glucuronidase activity. GUS activities were determined with a colorimetric assay, initially described by Jefferson et al. (1987), but automated using a computer-directed microtitre plate reader (340-ATTC, SLT Labinstruments, Austria). The detailed protocol will be described elsewhere (Breyne et al. in preparation). Briefly, reaction mixtures were prepared directly in the wells of the microtitre plate by mixing an amount of extract corresponding to one A_{600} unit of total protein (=10 µg) with extraction buffer (Jefferson et al. 1987) and *p*-nitrophenyl-β-D-glucuronide in a total volume of 385 µl. The microtitre plates were incubated at 37° C in the reader and the kinetics of the GUS reactions were continuously recorded by following the increase in absorbance at 415 nm. The GUS activities are given in units of enzyme activity per mg of total protein (Jefferson 1987).

Transient expression analysis. All the constructed plasmids were introduced into tobacco protoplasts via electroporation (Dekeyser et al. 1989). The plasmids pGUS1 and pNGUS were included as negative and positive controls, respectively. After 48 h the protoplasts were harvested for measurement of GUS activities using 30 µg of total protein.

Data assimilation and statistical analysis. Determination of the basic statistics of location and dispersion and analysis by statistical tests was done as described by Sokal and Rohlf (1981) using a package of computer programs (Applied Biostatistics). The mean expression level is given with the standard error allowing calculation of the confidence limits. The median is the value at which the series, with the items ordered from low to high, is divided into two halves with an equal number of items having lower or higher values. The variance (VAR), which estimates the variation within a particular series, is the summation of the squared deviations of each individual item from the mean, divided by the number of items. The coefficient of variation (V) is the square root of the variance expressed as a percentage of the mean and allows comparison of the variation among several series having a different mean.

Bartlett's test and log-ANOVA were used to determine whether or not the variances of two different series were homogeneous. When they were, a *t*-test was used to test the hypothesis that two sample means are derived from populations with the same actual mean. When the variances are heterogeneous, only an approximate *t*-test is allowed. The Mann-Whitney *U*-test is a non-parametric test that allows determination of whether two samples come from populations having the same statistics of location and thus whether the items of the two samples are equally distributed.

Results

The expression of the reporter gene is influenced by its location and orientation within the T-DNA

The influence of the configuration of the *gusA* gene within the T-DNA on its expression in stable transformants was analysed using the vectors pNG1, pNG2, pNG3 and pNG4 (Fig. 1 A). These vectors carry the *gusA* gene at different positions and/or orientations with respect to the right T-DNA border. These four plasmids showed essentially the same level of GUS activity after transient expression (data not shown). The results obtained with

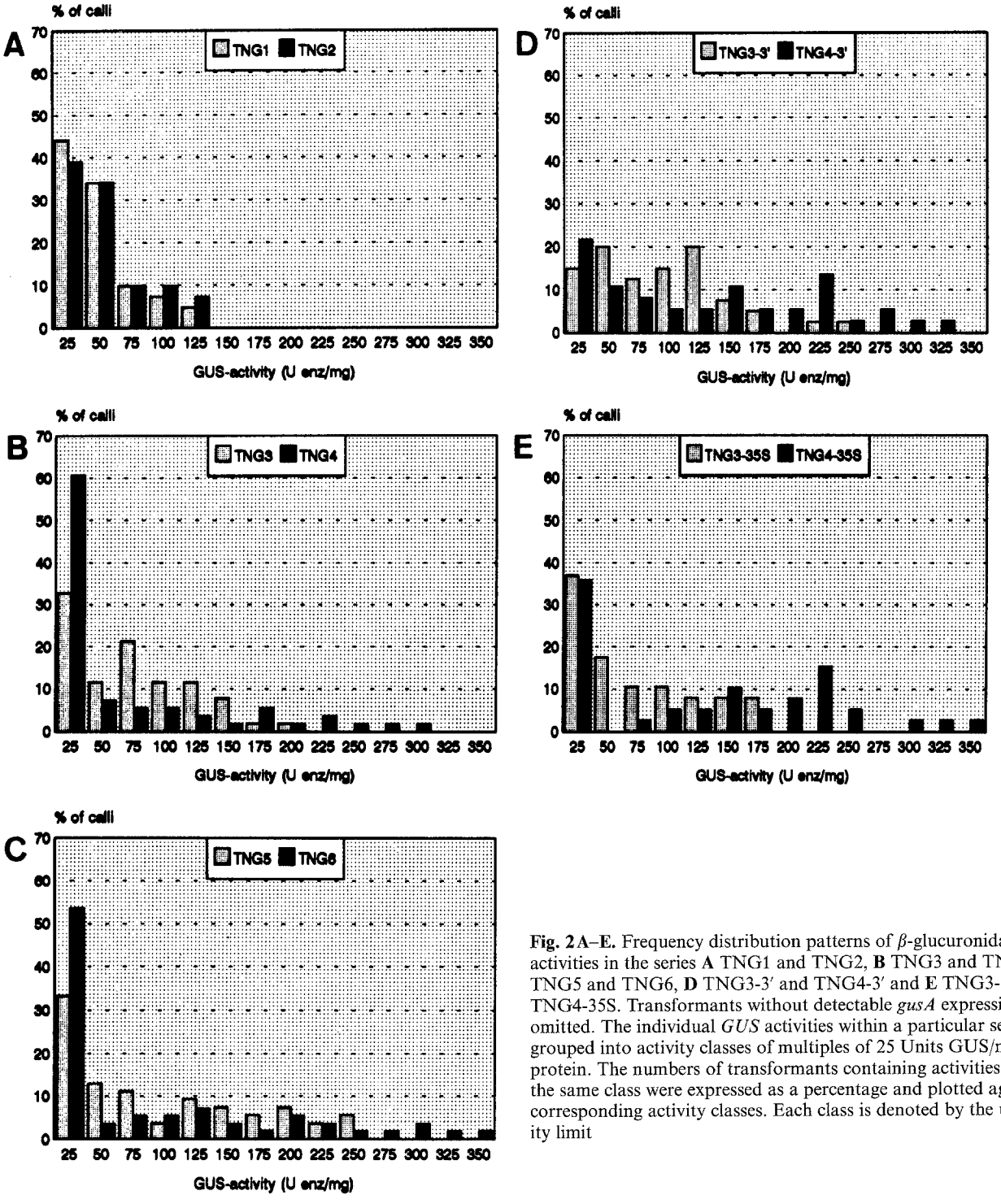

Fig. 2A–E. Frequency distribution patterns of β-glucuronidase (GUS) activities in the series **A** TNG1 and TNG2, **B** TNG3 and TNG4, **C** TNG5 and TNG6, **D** TNG3-3′ and TNG4-3′ and **E** TNG3-35S and TNG4-35S. Transformants without detectable *gusA* expression were omitted. The individual *GUS* activities within a particular series were grouped into activity classes of multiples of 25 Units GUS/mg total protein. The numbers of transformants containing activities falling into the same class were expressed as a percentage and plotted against the corresponding activity classes. Each class is denoted by the upper activity limit

transgenic calli are summarized in Tables 1 and 2 and Fig. 2A and B.

Comparison of *gusA* expression levels in the series of calli containing pNG1 or pNG2 T-DNA revealed no apparent differences. The GUS activities in the calli of both series are contained in nearly identical frequency distribution patterns (Fig. 2A) with 70 to 80% falling in the two lowest activity classes (upper limits of 25 and 50 Units GUS/mg total protein respectively). Statistical analysis (Tables 1 and 2) demonstrated that there are no significant differences between the TNG1 and TNG2 series. In contrast, the TNG3 and TNG4 calli each follow a distinct distribution pattern (Fig. 2B). Compared to the TNG3 calli, the TNG4 series has two times more calli in the lowest activity class of ≤ 25 U GUS/mg and five times more calli with GUS activities at least four times higher than the mean expression level (>200 U GUS/mg). This shows that higher expression levels can be obtained in cases where the promoter is lying next to the RB. The wide distribution of GUS

Table 1. Main characteristics and statistics of the series of *gusA*-expressing calli containing the reporter gene in different configurations within the T-DNA

	TNG1	TNG2	TNG3	TNG4	TNG5	TNG6	TNG3-3′	TNG4-3′	TNG3-35S	TNG4-35S
Sample size[a]	41	41	52	56	54	56	40	37	38	39
Mean[b]	33.9	36.3	60.6	57.6	83.3	79.6	82.1	120.3	58.2	122
	(±4.7)	(±5.1)	(±7.1)	(±10.2)	(±10.1)	(±14.2)	(±8.7)	(±14.9)	(±8.5)	(±16.6)
Median[b]	30.8	32.7	53.9	19.3	61.6	18.3	79	123	39.5	127
Lowest[b]	0.38	0.38	0.38	3.8	1.3	0.38	0.77	3.8	1.5	0.77
Highest[b]	109	116	197	300	250	485	250	308	173	335
VAR	906	1045	2602	5866	5509	11369	3060	8270	2766	10726
V	88.9	89.1	85	133	89.1	134	67.4	75.6	90.3	85.2
	(±15.8)	(±14.8)	(±12.8)	(±26.7)	(±13.8)	(±27.1)	(±10.4)	(±12.9)	(±16.8)	(±15.1)

Calli were derived from one co-cultivation experiment; independent co-cultivations resulted in series of calli displaying analogous statistics
[a] The actual numbers of calli analysed were 45 (TNG1 and TNG2), 60 (TNG3, TNG4, TNG5, TNG6), or 40 (other), but calli with undetectable GUS activities were omitted
[b] In units enzyme activity/mg total protein
VAR, variance; V, coefficient of variation (see the Materials and methods); Mean and V are given with their standard error

Table 2. Statistical comparison of the different series of calli

Series	Homogeneous variances	Equal means	Equal distributions
TNG1/TNG2	+	+	+
TNG3/TNG4	−	+	−
TNG5/TNG6	−	+	−
TNG1/TNG3	−	−	−
TNG2/TNG4	−	−	−
TNG3/TNG3-3′	−	−	−
TNG4/TNG4-3′	−	−	−
TNG3/TNG3-35S	+	+	ND
TNG4/TNG4-35S	−	−	ND

Homogeneity of variances was determined using Bartlett's test and log-ANOVA, equality of means and of distributions by *t*-test and Mann-Whitney *U*-test, respectively (see the Materials and methods)
−, Significantly different at the 95% level; +, not significantly different; ND, not determined

activities in the TNG4 series, however, also results in overall variation that is much higher than in the TNG3 series (Table 1). Although both series have an identical mean expression level, statistical analysis (Table 2) demonstrated that the *gusA* gene is differently expressed in the TNG3 versus TNG4 series.

Comparison of the data from the TNG1 and TNG2 series with those from the TNG3 and TNG4 series shows also that the location of the reporter gene on the T-DNA affects the expression pattern. Placing the gene next to the RB not only results in an elevation of the mean GUS activity by a factor about 1.5, but also in a bigger variance, because some of the calli reach higher expression levels. Nevertheless, the coefficients of variation indicate that the relative variation among the four series is only significantly higher for the TNG4 series. This demonstrates that the inter-transformant variability increases only in cases where the *gusA* gene is placed with P*nos* next to the right border. Thus, it can be concluded that both the position and the orientation of a reporter gene with respect to the right T-DNA border may influence the mean expression level, the inter-transformant variability and the number of calli with low and high GUS activities.

Analogous expression patterns are obtained with a different cointegration T-DNA vector

The T-DNA constructs pNG5 and pNG6 (Fig. 1 A) contain the same *gusA* gene at identical positions and orientations as pNG3 and pNG4 respectively, but in a cointegration vector lacking the *nptII* gene. After transient expression, these two vectors expressed the *gusA* gene at a twofold higher level than pNG3 and pNG4. The mean expression level in series of stably transformed calli was 1.33 times higher (Table 1). Nevertheless, the frequency distribution patterns and the differences between the TNG5 and TNG6 series are analogous to those observed between the TNG3 and TNG4 series. This provides further evidence that distinct *gusA* expression patterns can be obtained depending on the orientation of the gene relative to the right border of the T-DNA.

An additional 3′-end region cloned between the right T-DNA border and the reporter gene enhances gusA *expression and reduces inter-transformant variability*

The high inter-transformant variability (Table 1) and the high percentages of transformants expressing the *gusA* gene at levels 50% below the mean expression level (Fig. 2) in cases where the reporter gene is placed near the RB might be partly due to direct influences exerted from the flanking plant DNA. We therefore investigated the effect of an additional 3′-untranslated region (3′*nos*) cloned between the RB and the *gusA* gene in pNG3 and pNG4 (Fig. 1 B). The presence of 3′*nos* did not influence the *gusA* expression level after transient expression (data not shown).

For each of the constructs pNG3-3′ and pNG4-3′, 40 calli were analysed. The frequency distributions

(Fig. 2D) show that in the TNG3-3′ and TNG4-3′ series, the number of transformants with GUS activities of ≤ 25 U GUS/mg was reduced to 15% or 20% respectively. Instead, more calli express the *gusA* gene at intermediate levels (50 to 200 U/mg) whereas some even reached higher activity classes than TNG3 or TNG4 calli. As a result, the mean expression levels in the TNG3-3′ and TNG4-3′ series are 1.33- and 2-fold higher than in the corresponding series without 3′*nos* (Table 1), which represents a significant increase (Table 2).

Both the TNG3-3′ and TNG4-3′ series also have a comparable variation coefficient of approximately 70%. This is respectively 20 and 60% less than in the series involving their counterparts without 3′*nos*. These results indicate that due to 3′*nos* the inter-transformant variability can be significantly reduced, independently of the orientation of the reporter gene on the T-DNA. Moreover its presence can lead to a general enhancement of the mean expression, which is especially pronounced in cases where the promoter of the gene is next to the RB, as is the case in pNG4-3′.

An inserted promoter element does not reduce inter-transformant variability

In the last two vectors, pNG3-35S and pNG4-35S (Fig. 1B), the 3′*nos* region was substituted by the 35S promoter. Transient expression analysis revealed that pNG3-35S had the same level of GUS activity as pNG3 and pNG4, whereas pNG4-35S had an expression level that was approximately three times higher (data not shown). This is probably due to the proximity of P*nos* to regulatory sequences within the P35S fragment. Tables 1 and 2 and Fig. 2E summarize the results obtained with calli containing pNG3-35S or pNG4-35S T-DNA. It is clear that TNG3-35S calli express the reporter gene in an analogous way to TNG3 calli. Both series show comparable distribution patterns and statistics, their variances are homogeneous and their means are equal at $P<0.05$. On the contrary, the TNG4-35S series deviates significantly from the TNG4 series. The mean expression is two times higher and the number of calli expressing low levels has dropped to about 35%. Although the variance is very large, the high mean expression results in a decrease of the relative variation of 50%. In summary, we can conclude that the P35S fragment affects the GUS activity depending on the orientation of the reporter gene both relative to the nearest flanking plant DNA and the 35S promoter.

Discussion

The configuration of the transgene on the T-DNA influences its expression pattern in transgenic populations

From our results it can be concluded that both the location and the orientation of the reporter gene with respect to the right T-DNA border can affect the reporter gene expression. Depending on the T-DNA configuration, at least three distinct frequency distribution patterns can be obtained, which statistically represent populations of *gusA*-expressing transformants. These populations differ in the mean expression, the level of inter-transformant variability and the percentage of transformants containing extremely low versus high activities. The data obtained indicate that use of an appropriate T-DNA construct allows enrichment for a specific subclass of transformants. With the promoter of the transgene located next to the right T-DNA border, a series of transformants can be generated in which the inter-transformant variability is very high but with a number of individuals expressing the transgene at levels far above the mean. When one wishes to obtain a series of transformants in which the expression variation is lower, the transgene promoter should be located away from the border; in this case, however, only a few transformants will express the transgene at very high levels. The effect of the T-DNA context on reporter gene expression was previously discussed by Gidoni et al. (1988) in a study on the co-ordinated expression of two genes. Two different constructs carrying the same set of genes were compared and the degree of co-ordinate expression of the genes was shown to be influenced by their location within the T-DNA (Gidoni et al. 1988). Here we show detailed results indicating that this configuration effect might be more general and can affect the overall expression pattern of a transgene.

The differences in expression pattern were obtained with a reporter gene driven by the nopaline synthase promoter. When the same *gusA* gene driven by a 1 kb P35S fragment was cloned in a T-DNA in both orientations towards the right border, the differences obtained between the two series of transgenic calli were less pronounced (X. Danthinne, personal communication). This could be due to the nature of the promoter or the length of the promoter fragment. Nevertheless, our data imply that depending on the specific T-DNA construction and chimeric gene used, transgenic populations can be obtained with different GUS expression characteristics.

Transcription regulation signals can affect the transgene expression pattern

The inter-transformant variability levels obtained range between 90 and 130%, and at least one-third of the transformants contained GUS activity levels that were less than half of the mean. This is in good agreement with results obtained in other studies (summarized in Peach and Velten 1991). The highest level of variation was found with the constructions having the promoter of the reporter gene near the right border, which led to the suggestion that transgene activity is directly influenced by the neighbouring plant DNA and largely depends on the site of integration (position effects). Since T-DNA insertion occurs preferentially in transcribed regions (Koncz et al. 1989; Herman et al. 1990), it is not unlikely that plant promoter activity or the action of regulatory plant sequences might interfere with the tran-

scription and regulation of the transgene. We previously demonstrated that transcriptional interference can diminish the activity of a gene located downstream in opposite orientation (Ingelbrecht et al. 1991). Accordingly, it has been shown in animal cells that transcriptional read-through from one gene into a second present in the same orientation can reduce the expression level of the latter (Proudfoot 1986). In both cases the effect could be alleviated by inserting a terminator sequence in between the two transcription units.

In our study, an additional 3′*nos* region was inserted between the transgene and the RB in such an orientation that it would stop possible transcripts coming from the flanking plant DNA. The presence of 3′*nos* resulted in transgenic populations with 1.5- to 2-fold higher mean *gusA* expression and a coefficient of variation 20–60% lower compared to populations carrying the constructs without 3′*nos*. Also the number of transformants with extremely low expression (<25 U/mg) was reduced from 35 or 50% to 15 or 20% respectively. This indicates that the inserted 3′*nos* can at least partially dampen influences diminishing the expression, which are exerted from the flanking plant DNA. The findings also suggest that plant transcriptional activities do interfere with the transcription of the reporter gene in some of the transformants, leading to low activity levels.

The finding that the effect of inserting the 3′*nos* region is related to the specific molecular function of the fragment rather than to the fact that the reporter gene is separated from the plant sequences by a random piece of DNA was indicated after substituting 3′*nos* by the 35S promoter. In cases where the reporter gene is oriented towards the plant DNA (pNG3) there is no obvious effect, either on the expression levels and their variability or on the frequency distribution, indicating that only particular DNA fragments have the potential to change the expression pattern. However, inverting the reporter gene so that the promoter is close to the RB with the P35S located between them, resulted in a mean expression level that was increased by a factor of 2 and an inter-transformant variability that was 50% less compared to the control without P35S. The most likely explanation is that the close proximity of the 35S enhancer sequences to P*nos* results in an increase of *gusA* expression, as was also observed in transient expression analysis.

In summary, the configuration of a reporter gene within the T-DNA was demonstrated to have a significant effect on its expression pattern. This can be explained partially by transcriptional activity at the integration site, which differentially interferes with reporter gene expression. Interestingly, cloning the reporter gene with its promoter close to the right border of the T-DNA with a 3′ end in between, can result in 3- to 4-fold higher mean expression without increasing the inter-transformant variability compared to constructions carrying the gene in the middle of the T-DNA. Transcriptional interference is, however, probably not a major cause of position effects since there always remained a high degree of inter-transformant variability independently of the T-DNA construct used. Other molecular causes such as the local DNA structure and/or the higher-order chromatin arrangement (Breyne et al. 1992) possibly also have an important role in the overall level of gene expression.

Acknowledgements. We wish to thank Ivan Ingelbrecht for critical reading of the manuscript and Olivier Haemerlinck for initial help with the statistical analysis. Thanks also to Vera Vermaercke and Karel Spruyt for preparing the figures and photographs and to Martine De Cock for the layout. This work was supported by grants from the Services of the Prime Minister (IUAP 120CU192) and the "Vlaams Actieprogramma Biotechnologie" (174KP490).

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Communicated by J. Schell