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Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters

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Abstract

Forty-five individually transformed clonal tobacco callus lines were simultaneously assayed for both chloramphenicol acetyltransferase (CAT) and β-glucuronidase (GUS) activity resulting from expression of introduced reporter genes driven by the adjacent and divergent mannopine (*mas*) promoters. Excluding lines in which one or both of the enzyme activities was essentially zero, the activities of the reporter genes varied by as much as a factor of 136 (CAT) and 175 (GUS) between individual transformants. Superimposed upon the high degree of inter-clonal expression variability was an intra-clonal variability of 3–4-fold. The observed degree of intra-clonal reporter gene activity may be more extreme because of the regulatory characteristics of the mannopine promoters, but must still be addressed when considering the limitations of reporter gene-based analysis of transgene function and structure. There was no consistent correlation between the expression levels of the introduced CAT and GUS genes since the ratio of GUS to CAT activities (nmol min^{-1} mg^{-1}) within individual lines varied from 0.05 to 49. Even divergent transcription from two directly adjacent promoter regions (both contained within a 479 bp TR-DNA fragment) is insufficient to guarantee concurrent expression of two linked transgenes. Our quantitative data were compared to published data of transgene expression variability to examine the overall distribution of expression levels in individual transformants. The resulting frequency distribution indicates that most transformants express introduced transgenes at relatively low levels, suggesting that a potentially large number of *Agrobacterium*-mediated transformation events may result in silent transgenes.

Introduction

The development of efficient procedures for introducing *in vitro* manipulated DNA into higher eukaryotic cells has contributed greatly to recent progress in understanding many important developmental, cellular and molecular processes of eukaryotes. The same techniques have also allowed researchers to introduce, into both plants and animals, engineered genes designed to augment the normal genetic content of the target organisms by providing desirable traits difficult or impossible to obtain using more traditional procedures. However, very little is currently known about the actual molecular processes acting upon the foreign DNA during uptake, intracellular

transport and stable integration into the genomes of eukaryotic cells. It is clear that genetic material newly introduced into eukaryotic cells must be subject to considerable modification and processing between its entry into the target cell (usually as DNA of prokaryotic or synthetic origin) and its subsequent expression as part of the structurally organized chromatin of the resulting transgenic cell or organism. Of specific importance to the genetic engineer is the question of how the process of genetic transformation affects both the character and stability of expression of chromosomally integrated foreign genes. One such widely reported effect is a seemingly random clonal variability in the level of expression of newly introduced transgenes, each containing initially identical regulatory and structural DNA sequences.

Expression level variability between different transgenic cell lines or organisms has been observed after introduction of many unrelated genes, both natural and chimeric, into numerous plants species [4, 12, 13, 14, 15, 18, 19, 20, 22, 23, 29, 41, 45, 52, 57, 62, 65]. The observed variability has often been referred to as 'position effect', based on the as yet unproved assumption that expression levels of the introduced genes are directly influenced by host DNA sequence or chromosomal structure/composition at or near to the site of integration.

Despite the nearly ubiquitous occurrence of 'position effect', the nature of the molecular factors contributing to transgene expression variability remains elusive. In general, transgene variability has failed to correlate with the copy number of stably integrated transgenes [30, 41, 57, and this paper], although a significant correlation between gene copy number and transgene expression has been described [22]. Co-transformation of up to 23 kb of plant DNA flanking a petunia ribulose bisphosphate carboxylase (*rbcS*) gene does not appear to influence the level of transgene variability upon reintroduction into tobacco plants [15].

Some indication of the molecular resolution of the processes producing transgene variability is given by investigation of expression variability of two linked genes co-transferred on the same T-DNA. Expression levels of linked nopaline synthase (*nos*) and octopine synthase (*ocs*) genes [30], as well as closely adjacent neomycin phosphotransferase II (NPTII) and CAT reporter genes [4] were found to vary independently between individual transformants. However, significant co-variation was reported between independent transgenotes containing linked CAT and GUS genes driven by the the 35S promoter of the cauliflower mosaic virus [20]. Interestingly, covariance of linked genes driven by two *rbcS* promoters and divergently expressed chlorophyll *a/b*-binding protein (*Cab*) genes was found to be greatly influenced by either the particular combination of promoters used [14] or the location of the transgenes within the T-DNA of the plant transformation vector [18, 23].

In this paper we report the quantitative analysis of simultaneous independent transgene expression level variability using two reporter genes (CAT and GUS) fused to an extremely closely linked (479 bp, ATG-ATG) divergent promoter pair, the mannopine promoters (*mas*) from *Agrobacterium tumefaciens*. To date, function of the mannopine promoters in plants has only been examined either separately [16, 53, 60] or under conditions in which simultaneous activity of both promoters is required for reporter gene activity (the *luxA* and *luxB* genes [33]).

Materials and methods

DNA manipulation and cloning

Figure 1 shows pGC4-OO and pGC4-NP. The pGC40-OO plasmid, a binary vector, contains a pair of divergently oriented reporter genes, chloramphenicol acetyltransferase (CAT) from Tn9 [65] and β-glucuronidase (GUS) encoded by the *uidA* locus of *Escherichia coli* [27] driven by the two divergent mannopine promoters, 1′ (Pmas1′) and 2′ (Pmas2′), isolated from the TR-DNA of *A. tumefaciens* [66]. This construction includes the 1.0 kb *Cla* I-*Eco* RI fragment from pCAP212 which contains the CAT coding

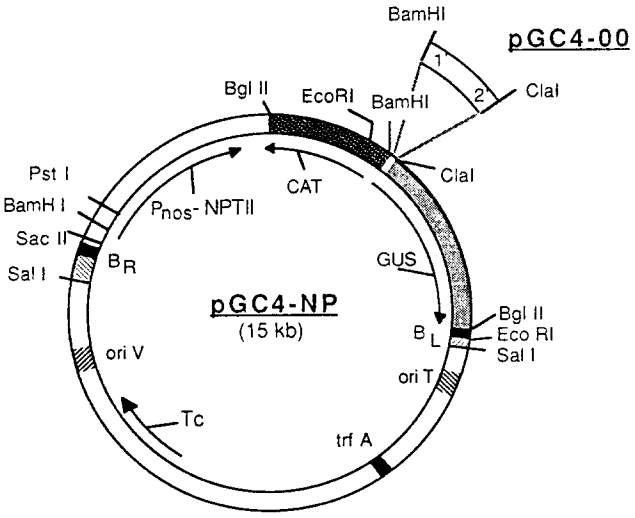

Fig. 1. The circular map shown, pGC4-NP, is based upon the binary vector, pGG102 (pGA470 [3] in which a *Bgl* II linker has been inserted into the unique *Hind* III site (W.M. Ainsley, personal communication)). Inclusion of the *mas* dual promoter fragment [66] at the indicated *Bam* HI-*Cla* I sites creates pGC4–00. Construction of the CAT ← Pmas1′-Pmas2′ → GUS cassette is described in [47]. Symbols: Tc, tetracycline resistance gene (from pTJS75); CAT, chloramphenicol acetyltransferase (*Tn9*) coding region fused to the g7 polyadenylation signal [65]; GUS, β-glucuronidase coding region fused to the nos 3′ polyadenylation signal [27]; Pnos-NPTII, nopaline synthase promoter fused to the NPTII (kanamycin resistance) gene of Tn5 and the nopaline synthase 3′ polyadenylation/termination signal; BR, the right border of T-DNA (from pTiT37); BL, the left border of T-DNA (from pTiT37); oriV, origin of vegetative growth (pRK4); oriT, origin of transfer (pRK4).

region and polyadenylation signal from TL-DNA gene 7 [65]. The GUS gene was obtained from pRAJ275 [27]. The 2.1 kb *Bam* HI-*Eco* RI GUS cassette includes Kozak's transcriptional initiator [32] 5′ to the GUS coding sequence and 3′ nopaline synthase termination signal. These fragments were directionally ligated into pGG102 (pGA470 [3] modified to contain a *Bgl* II linker at the unique *Hind* III site (W.M. Ainley, personal communication)), a binary vector containing right and left borders of T-DNA, suitable for *Agrobacterium*-mediated transformations. pGC4-OO contains the Pmas1′ and Pmas2′ dual promoter fragment from pOP4434 [65] inserted between the CAT and GUS reporter genes. The negative control plasmid, pGC4-NP (no promoter), is identical to pGC4-OO (Fig. 1) except that it lacks the Pmas1′-Pmas2′ promoter fragment.

Plant transformation and maintenance

The plasmids, pGC4-OO and pGC4-NP, were moved into *A. tumefaciens* strain C58C1(*rif*) containing the pGV3850 Ti plasmid [69] by the freeze/thaw method of An *et al.* [2] and the structure of the T-DNA confirmed by Southern hybridization of restriction-digested total *A. tumefaciens* DNA. Mesophyll protoplasts were isolated as described previously [55] from *Nicotiana tabacum* cv. Petit Havana SR1 [34] plants sterilely maintained on 1/2 MS hormone-free agar media [40]. Regenerating protoplasts were transformed by co-cultivation with *Agrobacterium* harboring pGC4-OO or pGC4-NP [37, 66, 68]. Micro-calli embedded in agarose were cultured on liquid K3 media [41] plus sucrose (0.4 M → 0.05 M) supplemented with 1 mg/l naphthaleneacetic acid (NAA), 0.2 mg/l kinetin, 100 μg/ml kanamycin and 500 μg/ml cefotaxim (Claforan, Hoechst Chemicals). Individual transformed micro-calli appeared in 6–8 weeks. Only well separated micro-calli were further propagated for analysis.

CAT, GUS and protein assays

Chloramphenicol acetyltransferase activity was assayed by a modification of that described by Neumann *et al.* [44]. A more detailed description of the CAT kinetic assay employed is given by Peach and Velten [46]. A previously reported spectrophotometric assay [28] was used to measure GUS activity using the the substrate p-nitrophenyl β-D-glucuronide. Both CAT and GUS activity values for individual extractions were normalized to total protein content as determined by the method of Bradford [7].

DNA isolation and analysis

Total DNA was prepared from individual callus tissues by the method of Doyle and Doyle [17] and further purified by cesium chloride-ethidium bromide density gradient centrifugation [35]. This procedure yielded 1–2 μg DNA per g fresh tissue weight. Total callus DNA was digested with restriction enzymes, electrophoretically separated on 1% agarose gels and transferred to Zeta-Probe blotting membrane (Bio-Rad) for Southern hybridization analysis [58].

Results

Activities of the Pmas1'-CAT and Pmas2'-GUS reporter genes vary extensively among clonal callus lines

Transcriptional activities of the mannopine promoters in regenerated transformed plants are known to display tissue specificity, hormone sensitivity and wound inducibility [33, 47, 53, 60]. Additionally, the expression levels of transgenes within regenerated plants have been reported to show considerable, and difficult to control, environmental and developmental dependence [15]. Based upon the presumption that reporter gene activity within relatively homogeneous, undifferentiated callus tissue (grown under controlled tissue culture conditions) is less subject to environmentally and developmentally related gene regulation, we chose to use independently transformed, clonal tobacco callus lines for our analysis. Differences in reporter gene activity among individual callus clones was expected to predominantly reflect 'position effect' or general inter-clonal variability in transgene expression levels.

Clonal callus lines were produced by co-cultivation of protoplasts with *Agrobacterium* harboring a binary T-DNA vector containing the dual reporter gene construct and a *nos* promoter-NPTII kanamycin (Km) resistance marker gene (see Figure 1). Transformed protoplasts were embedded in agarose and incubated in liquid media under continuous Km selection until small, well separated micro-calli developed. Due to continuous uniform exposure of the co-cultivated protoplasts to kanamycin, each separated micro-calli has a high probability of being clonally derived. The resulting micro-calli were individually propagated and assumed to be the result of an independent transformation event.

To reduce the number of variables and to minimize assay inaccuracy, the activities of both reporter genes were measured from the same extract and were determined by enzyme kinetic analysis instead of single-point assays. Enzyme activities were normalized to total soluble protein in each common extract. Both the CAT and GUS assays are linear with respect to added extract and are highly reproducible, displaying standard deviations of 1.5% of mean and 2% of mean, respectively (a more detailed description of accuracy of the CAT kinetic assay is published elsewhere [46]).

The measured activities of both reporter genes within 45 clonal lines are presented in Table 1 (the values given are the mean of 2 or more independent assays). The largest observed inter-clonal differences in activities were 136-fold for CAT and 175-fold for GUS (Table 1). When different portions of the same clonal callus line were independently assayed, it was noted that essentially all the lines show a 3–4-fold variability in CAT and GUS activities (e.g. Table 2). Intra-clonal variability of phenotypes within the same cell line has been reported for different traits (e.g. [5, 50]) and may result from micro-heterogeneity in general cell physiology or ploidy levels within each callus. In our case the intra-clonal transgene expression variability is much smaller in magnitude than the inter-clonal variability and, considering the hormone dependence of the mannopine promoters [33], may result from differential exposure of callus to hormones within the the media.

Consistent with the findings of others [30, 57], we found no correlation between observed transgene activity and DNA content within the clonal callus lines. Transgene DNA dosage within twelve individual callus lines was estimated by densiometric scannings of autoradiograms from

Table 1. Pmas1′ → CAT and Pmas2′ → GUS activities within clonal transgenic tobacco callus lines.

Callus line identifier	CAT activity[1] (nmol min^{-1} mg^{-1})	GUS activity[1] (nmol min^{-1} mg^{-1})	Ratio of activities[2] GUS/CAT
GC4-NP	78.99	0	NC
GC4-00.1	29.52	15	0.19
GC4-00.2	41.80	285	9.65
GC4-00.3	34.86	1434	34.30
GC4-00.4	25.84	980	28.11
GC4-00.5	2.43	609	23.55
GC4-00.6	60.13	16	6.53
GC4-00.7	1.86	1298	21.58
GC4-00.8	71.51	15	8.02
GC4-00.9	98.59	272	3.81
GC4-00.10	0	485	4.92
GC4-00.11	40.91	0	NC
GC4-00.12	56.94	1109	27.11
GC4-00.13	60.87	597	10.49
GC4-00.14	53.40	192	3.16
GC4-00.15	22.30	18	0.34
GC4-00.16	14.45	0	NC
GC4-00.17	0	419	29.03
GC4-00.18	73.29	0	NC
GC4-00.19	30.89	578	7.88
GC4-00.20	247.39	1513	48.99
GC4-00.21	97.60	14	0.66
GC4-00.22	0	625	6.40
GC4-00.23	33.89	0	NC
GC4-00.24	65.39	731	21.58
GC4-00.25	0	631	9.65
GC4-00.26	65.39	0	NC
GC4-00.27	23.00	471	20.49
GC4-00.28	26.55	328	12.36
GC4-00.29	62.29	1337	21.46
GC4-00.30	56.13	1423	25.35
GC4-00.31	38.16	1214	31.80
GC4-00.32	3.49	0	NC
GC4-00.33	135.11	33	0.25
GC4-00.34	67.39	805	11.95
GC4-00.35	36.21	1290	35.63
GC4-00.36	49.48	849	17.16
GC4-00.37	43.26	847	19.58
GC4-00.38	54.48	2447	44.92
GC4-00.39	253.33	14	0.05
GC4-00.40	62.16	1492	24.01
GC4-00.41	0	0	NC
GC4-00.42	4.67	0	NC
GC4-00.43	17.36	810	46.66
GC4-00.44	36.18	881	24.34
GC4-00.45	57.80	0	NC

[1] Values indicated by '0' were less then two times the background values for the no promoter (GC4-NP) callus (CAT < 0.12 nmol min^{-1} mg^{-1} and GUS < 4.5 nmol min^{-1} mg^{-1}).

[2] GUS/CAT ratios were not calculated (NC) when one or both activities were zero.

Table 2. Repeated GUS and CAT assays from separate extracts of callus line GC4-00.2.

Callus line identifier	CAT activity (nmol min^{-1} mg^{-1})	GUS activity (nmol min^{-1} mg^{-1})	Ratio of activities GUS/CAT
GC4-00.2A1	35.93	313	8.70
GC4-00.2A2	40.16	192	4.77
GC4-00.2A3	22.74	227	9.97
GC4-00.2A4	24.15	264	10.93
GC4-00.2A5	26.74	280	10.48
GC4-00.2B1	13.52	283	20.96
GC4-00.2B2	27.59	240	8.70
GC4-00.2B3	22.54	352	15.63
GC4-00.2B4	29.40	309	10.51
GC4-00.2B5	19.74	360	18.24

Southern blots in which total callus DNA was digested with *Eco* RI and hybridized to the 479 bp dual promoter fragment (which detects a 2.9 kb DNA band containing all of the GUS gene, both promoters and part of the CAT gene; see Figure 1). Based upon the lack of any secondary bands on the Southern blot, none of the lines analyzed showed any evidence of gross rearrangement within the *Eco* RI fragment probed (data not shown).

When the transgene dosage of the twelve clonal lines (transgene DNA content varied 20–30-fold between lines) was compared with both GUS and CAT activities, correlation coefficient of 0.172 (CAT activity vs. transgene content) and 0.116 (GUS activity vs. transgene DNA content) were obtained. For example, callus lines GC4-00.4 and GC4-00.11 both contained approximately 10–12 integrated copies of the dual promoter, yet displayed vastly different GUS (GC4-00.4 = 980 versus GC4-00.11 = 0 nmol min^{-1} mg^{-1}) and CAT (GC4-00.4 = 34.86 versus GC4-00.11 = 0 nmol min^{-1} mg^{-1}) activities (see Table 1).

Activities of the Pmas1'-CAT and Pmas2'-GUS reporter genes vary independently between clonal callus lines

Somewhat surprisingly, examination of the data presented in Table 1 clearly indicates that, even discounting lines in which one or both of the activities are essentially zero, the activities of the two reporter genes show a large degree of independent variability, with the ratio of GUS to CAT in separate clonal lines ranging from 0.05 to 49 (Table 1). Not all of the inter-clonal variation between the linked reporter gene activities (correlation coefficient of 0.165) can be directly attributed to 'position effect' since repeated assays of *different* samples collected from the same callus tissue also show a lower level of independent variation of GUS and CAT activities (Table 2). Ratios of GUS to CAT activities ranging from 4.8 to 21 (Table 2) were observed within different samples from a single callus line (GC4-00.2), giving a correlation coefficient between GUS and CAT activities of only 0.35. Smaller numbers of repetitive assays from other clonal lines often, but not always, displayed a similar range of GUS-CAT ratios (data not shown). It would, thus, appear that enzymatic activities from the two reporter genes can respond differentially to whatever factors contribute to the observed micro-heterogeneity of transgene activities within the same clonal transgenic callus line.

The frequency distribution of transgene expression level variability

Published quantitative examinations of inter-clonal transgene expression level variability have measured reporter gene activities at the level of

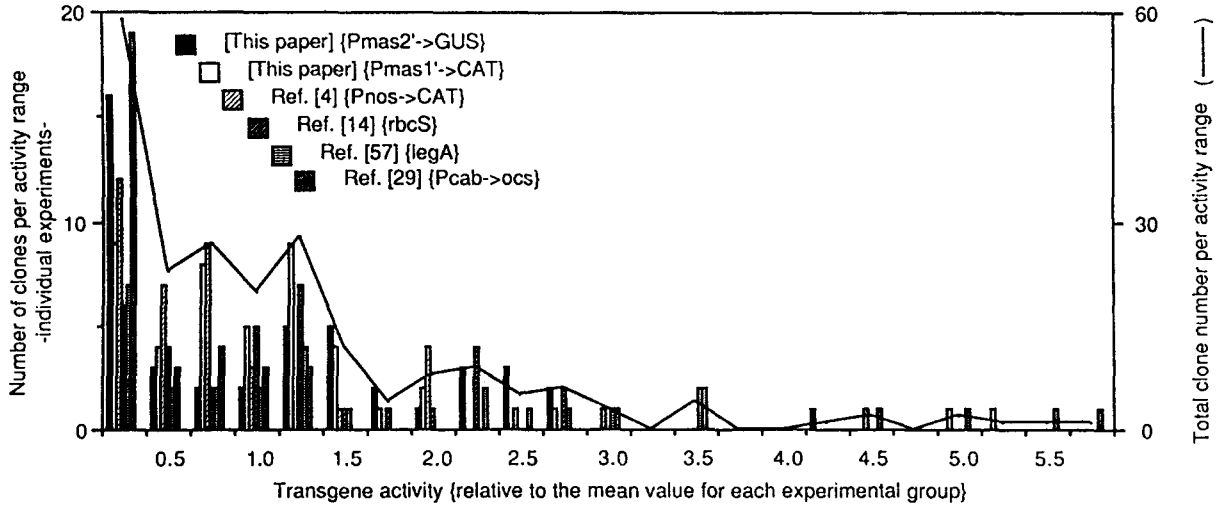

Fig. 2. Frequency distribution of transgene expression variability. The number of clones displaying relative activities within the specified range have been plotted against the corresponding activity ranges. Activity values are relative to the average activity for each experimental group and are clustered into ranges of 0 to 0.249, 0.250 to 0.499, 0.500 to 0.999, etc. The line plot represents a summation of all experimental data groups.

mRNA [14, 18, 19, 23, 29, 30], enzyme activities [4, 13, 22, 52, 53] and immunologically detectable protein [57]. We felt it would be of interest to compare the overall frequency distribution of these independent measurements of transgene expression variability to our own data. In order to compare the different data sets, it was necessary to present transgene activity values as the ratio of each measured value to the mean for that data group. The results of the comparison are presented in Fig. 2. The distributions for all the data groups (measured activities for the directly selected marker gene, NPTII [4], were not included due to probable selection bias) are qualitatively the same, with the number of clones displaying each activity range decreasing steadily as relative transgene activity levels increase. Summation of the results from all data groups follows essentially the same distribution as the individual data groups (Fig. 2).

Discussion

*Transgene expression variability of reporter genes driven by the mannopine synthase (*mas*) promoters: inter-clonal ('position effect') verses intra-clonal variability*

Our results clearly indicate that, similar to results reported for many other genes introduced into plants, expression of both the co-transferred *mas* promoter-driven reporter genes are subject to a high degree (136–175-fold) of variability between independently transformed transgenic callus lines. Our original intent was to confirm the applicability of the CAT and GUS reporter genes to an analysis of the *mas* dual-promoter system. Clonal callus lines were chosen for this work in order to better focus on the inter-clonal 'position effect' and to avoid difficult to control tissue, developmental and environmental regulatory effects on gene expression levels known to occur in regenerated plants. It is clear, however, that at least with respect to CAT and GUS activities driven by the *mas* promoters, even clonally derived tobacco callus is not a simple homogeneous collection of uniform cell types. Nearly all of the 45 indepen-

dently transformed callus lines were found to display a 3–4-fold intra-clonal variation in reporter gene expression levels superimposed upon the much larger inter-clonal variability.

Our data are consistent with the overall premise of reporter gene usage, i.e. that the large variability of inter-clonal reporter gene enzyme activity is representative of differences in transgene transcription levels. The observed intra-clonal variability is more likely to reflect physiological or biochemical micro-heterogeneity within the callus lines, especially considering the hormone-responsive nature of at least one of the *mas* promoters [33]. There are reported precedents for micro-heterogeneity in callus gene expression characteristics (e.g. [5, 49]). In our case, because the co-transformed genes in each clonal callus line were assayed at the level of CAT and GUS enzyme activities, intra-clonal variability could result from localized (within the callus) differences in transcription, mRNA stability, translation, protein stability or overall cellular protein concentration (since the enzyme activities were normalized to total soluble protein within a common extract). The fact that the CAT and GUS activities from different samples of the same callus line failed to co-vary, essentially eliminates normalization to total soluble protein as the basis for intra-clonal variability, and indicates that localized differences in the other potential mechanisms are not general effects, but instead differentially influence the two reporter genes, or their gene products. Whatever the basis of the observed intra-clonal variability, it must be taken into consideration when using reporter gene enzyme activities to compare transgene expression levels.

Resolution of the molecular factors producing transgene expression level variability: transgene expression from divergent promoters within 479 bp of DNA vary independently

In this and previous reports, independent expression level variability has been documented using several different co-transformed transgenes (Table 3). It is apparent that the molecular factors contributing to transgene expression variability can differentially affect even 5′ adjacent genes such as the divergent petunia *Cab* genes [14, 23] and *mas* promoter driven CAT and GUS chimeric genes (Table 1). The ability of co-transformed genes to exhibit coordinated transgene expression levels in plants seems to be sensitive to as yet poorly defined variables related in some way to the nature of the two promoters being compared and the location of the transgenes within an artificial T-DNA [18, 23]. To the best of our know-

Table 3. Co-transferred transgenes reported to display independent transgene expression level variability.

Transgene arrangement	Promoters	Reporter genes	Inter-gene distance (inclusive)[1]	Inter-promoter distance (inclusive)[2]	Reference
Tandem (→ →)	*nos* *nos*	CAT NPTII	ca. 6 kb	ca. 4.5 kb	[4]
Tandem (→ →)	*rbcS* 301 *rbcS* 301	*ocs* CAT	ca. 10 kb	ca. 9 kb	[14]
Divergent (← →)	*cab* 21 *cab* 22	*Cab* 21 *cab* 22	ca. 4 kb	ca. 1.2 kb	[18]
Divergent (← →)	*mas* 1′ *mas* 2′	CAT NPTII	ca. 3.5 kb	ca. 0.5 kb	[This paper]

[1] The inter-gene distance is the length of DNA between and including both transgenes (Tandem = 5′-gene 1 to 3′-gene 2, Divergent = 3′-gene 1 to 3′-gene 2).

[2] The inter-promoter distance is the minimum DNA length between and including both promoters.

ledge, no 'locus control regions' able to eliminate 'position effect' with animal β-globin genes [21, 24, 51, 61] have been yet identified in plants.

Based upon reported data [30, 57] and limited Southern analysis of the transferred genes in our callus lines, differences in copy number or rearrangement of the transgenes cannot account for the complete lack of coordination in the expression levels of the linked CAT and GUS reporter genes. Both of *mas* promoters are contained within a 479 bp (start codon to start codon) DNA fragment (the actual regulatory sequences are more likely to be confined to the central 360 bp of DNA, Pmas1′-transcription start to Pmas2′-transcription start (unpublished data from this lab and Winter *et al.* [67]). It would, thus, appear that the molecular factors contributing to transgene variability are either able to efficiently discriminate between two adjacent promoters, or, alternatively, produce the observed expression level variability through changes to, or interactions with, other portions of the affected transgenes. If one assumes that modification of transgene promoter function is the primary basis of expression level variability, then the observed independent variability of the *mas* promoter driven reporter genes provides useful insight into potential molecular mechanisms of 'position effect'.

Inherent in the term 'position effect' is the concept that some characteristic (or characteristics) of the genetic material in the vicinity of the transgene insertion site produce the observed expression level variability. The high resolution of transgene variability suggests the phenomenon does not result from some generalized effect such as chance insertion of the foreign DNA in the neighborhood of general transcriptional enhancers. This conclusion is supported by the results of Dean *et al.* [15] in which inclusion of 23 kb of flanking plant DNA with a petunia *rbcS* gene failed to influence the degree of transgene expression level variability observed in transformed tobacco plants.

In the context of transgene variability, it is important to consider that virtually all the sources of DNA used for plant genetic engineering share a common lack of any predetermined chromatin structure and/or pattern of DNA modification normally provided by the parental gametes (e.g. genomic imprinting [39, 50, 54]). During the process of genetic transformation, foreign DNA enters the cell normally as DNA of prokaryotic origin and therefore lacks any of its eventual chromatin structure of eukaryotic-specific modification (e.g. CG or CXG cytosine methylation [25]). Foreign DNA stably integrated in the nuclei of plants cells has been found to display normal chromatin structure (e.g. [11, 59]), and to respond to changes in DNA methylation [1, 26, 48, and unpublished data from our lab]. Thus, the eventual state, both biochemical and functional, of the inserted transgene must result from interactions between the initially 'naked' foreign DNA and host (and donor?) cell proteins and enzymes present during the process of transformation, integration and eventual gene expression. It is certainly conceivable that flanking host DNA, and its current chromatin content or pattern of modification, could influence the final state of the introduced transgenic DNA. However, essentially random patterns of modification and/or association of incoming DNA with chromatin proteins during transformation, but prior to integration, are also possible. Inter-clonal expression level variation could result from such random factors and be essentially unrelated to the eventual site of transgene integration.

Differences in either local chromatin fine-structure or DNA methylation patterns have sufficient molecular resolution to functionally discriminate between the two divergent *mas* promoters. DNA methylation has been clearly demonstrated to be capable of affecting local patterns of gene expression in native plant genes (e.g. transposon activity [6, 8, 9, 10, 36, 56] and integrated foreign DNA [38]). Apparent regulatory interaction between independently introduced foreign genes (co-suppression [43, 63]) has been correlated with DNA methylation changes within promoters [38], and must be considered in the context of transgene expression level variability.

Based on currently available data, it is premature to attempt to assign any specific molecular

mechanism or mechanisms as the primary basis of transgene variability. It seems likely that the phenomenon of transgene expression level variability or 'position effect' will be found to have multiple underlying molecular origins, at least some of which reflect higher-order gene regulatory mechanisms normally active in plants.

The frequency distribution of inter-clonal transgene expression levels indicates high activity levels are considerably less common than low or no transgene activity

The distribution presented in Fig. 1 clearly indicates that a majority of transformants express introduced transgenes at levels well below the potential maximum expression levels. This observation is clearly pertinent in considering how many different transformants need to be examined in order to obtain one or more clones expressing the introduced gene(s) at desired (usually high) levels. The observed distribution is consistent with the possibility that many plant cells receiving new DNA may fail to express introduced genes or express them at very low levels. In functional terms, it is possible that introduced foreign DNA either only rarely escapes inactivating modification or, conversely, is only rarely activated to maximal levels of expression. A more trivial explanation in which a majority of the population of low to zero expressing clones have simply failed to receive, or have reorganized, the transgene or transgenes displaying reduced activity, cannot be completely ruled out without extensive analysis of the T-DNA structure within each clonal line. However, when such an analysis was performed on T-DNA containing a chimeric *ocs* gene, a group of nineteen low expressing transgenic plants (expression levels from undetectable (13 of 19) to 0.25 times the group mean activity), contained only two lacking the predicted *ocs* gene structure [30].

It will be interesting in terms of a basic understanding of overall plant gene regulation, and important to the long-term successful application of plant genetic engineering, to better define the molecular nature of transgene expression level variability.

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