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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

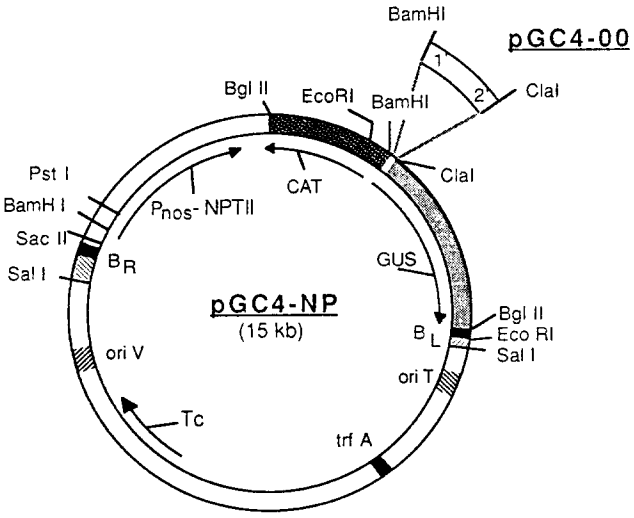

Fig. 1. The circular map shown, pGC4-NP, is based upon the binary vector, pGG102 (pGA470 [3] in which a *Bgl* II linker has been inserted into the unique *Hind* III site (W.M. Ainsley, personal communication)). Inclusion of the *mas* dual promoter fragment [66] at the indicated *Bam* HI-*Cla* I sites 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.

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