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Integration, expression and inheritance of two linked T-DNA marker genes in transgenic lettuce

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Abstract

T-DNA integration and stability were assessed in *Agrobacterium*-derived transgenic lettuce lines carrying a chimaeric CaMV 35S promoter-driven *gus*-intron gene and a chimaeric *nos.nptII.nos* gene. T-DNA integration was predominantly complex in transgenic plants derived from an *A. tumefaciens* strain carrying the supervirulent plasmid ToK47. Truncation of the right side of the T-DNA was observed in first seed generation R_1 plants from one line. Complex T-DNA integration patterns did not always correlate with low transgene expression. Despite a high T-DNA copy number, ca. 30% of the lines analysed showed high transgene expression in the R_1 generation. High transgene expression was stable at least to the R_4 seed generation in selected high-expressing lines. Transgene expression was lost in the R_2 generation in a low expressing line, while complete, heritable transgene silencing from the R_0 to R_2 generations was also observed in another line. A 50-fold variation in β-glucuronidase (GUS) activity and a 16-fold variation in NPTII protein content were observed between R_1 plants derived from different R_0 parents. Reactivation of transgene expression with 5-azacytidine in partially silenced lines indicated that low expression was associated with DNA methylation.

Introduction

More than a decade has elapsed since the first report of *Agrobacterium*-mediated transformation of lettuce [48]. Since then, there have been several reports on the production of transgenic lettuce [8, 9, 10, 11, 12, 16, 19, 20, 24, 55, 67, 76]. However, despite being an important commercial crop which would benefit from genetic engineering, transgenic lettuce has yet to be released onto the market. This is probably due to the high degree of transgene instability in this crop. For example, it has been reported that 80% of transgenic first seed generation (R_1) lettuce plants resistant to lettuce mosaic virus (LMV) lost their resistance in the R_2 generation [24]. Falk et al. [20] produced transgenic lettuce and tobacco plants carrying the same three genes for resistance to LIYV (lettuce infectious yellows virus). LIYV resistance was observed after analysing a limited number of transgenic tobacco plants, but extensive studies did not reveal LIYV resistance in the transgenic lettuce plants. Dinant et al. [15, 16] produced transgenic lettuce and tobacco carrying the same LMV coat protein (LMV-CP) gene. A high degree of heterologous resistance to potato virus Y (PVY) was observed in transgenic tobacco, but in transgenic lettuce, the LMV-CP gene gave poor resistance to LMV, which was of no agronomic value.

In order to formulate a strategy to increase the frequency of high, stable expression of transgenes in lettuce, it is necessary to establish the principal causes of low transgene expression in this leafy vegetable. The principal causes of variability of transgene expression in plants have been reported to be the position of transgene integration into the plant genome (position effects), the integrity, copy number and methylation of transgenes and the environmental conditions under which the transgenic plants are grown [22, 47, 68].

Independent transformants, which carry single intact copies of the same transgene and which differ only in the site of genome integration, often show different levels of transgene expression. This situation is attributed to position effects, i.e. the effect of the chromosomal locus on the integrated transgene. Position effects are, in turn, partly explained by the methylation status of the integration site [56].

Current literature is conflicting on the relationship between transgene copy number and expression in plants. A positive correlation between high transgene copy number and expression has been reported in potato, tobacco and rice [23, 37, 65, 71]. In contrast, no strict correlation between gene copy number and expression was found in tobacco [28, 61], whereas a negative correlation was reported in petunia, maize and tobacco [21, 27, 31, 53, 72]. Hobbs *et al.* [28] found that inverted T-DNA repeats in tobacco gave low expression, while single T-DNA inserts gave high expression, regardless of copy number. These authors suggested that the type of insert influenced transgene expression rather than copy number. Other workers [69] observed that repeated T-DNA structures led to loss of transgene activity in the R_1 progeny of transgenic petunia and suggested that this occurred by repeat-induced-point mutation (RIPPING). In *Arabidopsis*, repeated sequences for antibiotic resistance on the same chromosome led to gene silencing, which was termed repeat-induced gene silencing (RIGS) [3].

Studies have also shown that silenced or low expressing transgenes are often methylated, although it is not known whether this is a cause or an effect of transgene silencing [69]. Transgene methylation is believed to depend on the integration site as certain randomly integrated transgene copies become hypermethylated and inactive, whereas others remain hypomethylated and active [47]. Demethylation of transgenes may occur spontaneously, or following treatment with the demethylating agent 5-azacytidine [73]. It has been proposed that in animal systems there is a DNA-methylation mechanism which specifically recognizes foreign DNA by its composition [5, 17]. A similar situation may occur in plants [22, 39, 47, 58].

Differences in transgene expression have been observed in plants derived from the same transformation event but grown under different environmental conditions. Growth in culture [57], heat stress [75] and seasonal variation under field conditions [44], have been shown to induce transgene instability. Meyer et al. [44] analysed ca. 30 000 isogenic F_1 petunia plants derived from a line which had shown high expression of the maize A1 gene. Ninety five percent of the plants showed high stable expression in the glasshouse, but only 37% exhibited high stable gene expression under field conditions. In the same report, transgenes which did not express in transgenic plants were found to have become highly methylated, whereas the surrounding chromatin remained hypomethylated, indicating that the transgenes had been specifically methylated [46].

Attempts have been made to limit the variability of transgene expression. These include the use of matrix-associated regions (MARs; synonymous with scaffold attachment regions, SARs) [1, 2, 7, 50, 64] to protect T-DNA inserts from the influence of the surrounding chromatin, reduction of T-DNA copy number by transforming root explants instead of leaf explants [25], and positioning the selectable marker gene adjacent to the left T-DNA border to reduce selection of plants containing truncated T-DNA inserts [4]. The major phenomena associated with variation in transgene expression have been reviewed [22, 34, 42, 47, 63].

In the present work, integration, expression, heritability and methylation status of the *npt*II and *gus*-intron genes were analysed in a large population of transgenic lettuce plants in order to determine the causes of the high degree of transgene instability in this vegetable.

Materials and methods

Plant material

Lettuce seeds (*Lactuca sativa* L. cv. Raisa) were supplied by Leen de Mos (P.O. Box 54, 2690 AB's-Gravenzande, Netherlands). Seeds were surface-sterilized by immersion in 10% (v/v) 'Domestos' bleach (Lever Industrial, Runcorn, UK) for 30 min, followed by 3 washes in sterile distilled water. The seeds were placed on half-strength agar-solidified (0.8% w/v) [51] medium with 1.0% (w/v) sucrose, pH 5.8 (20 ml/9 cm Petri dish; 30–40 seeds/dish). Seeds were germinated at 23±2 °C (16 h photoperiod, 18 μmol m^{-2} s^{-1}, daylight fluorescent tubes). Cotyledons were excised after 7 days for bacterial inoculation.

Bacterial strains and plasmids

A binary vector, pVDH65, based on the pBIN19 derivative pMOG18 [62] carried a T-DNA with the *nos.npt*II.*nos* gene next to the left border and a

35S.*gus*-intron.35S gene [70] adjacent to the right border (Figure 1). Plasmid VDH65, together with the supervirulent pToK47 [30], were introduced into *A. tumefaciens* LBA4404 [54] to give strain 1065 [9].

Bacteria were grown from −70 °C glycerol stocks at 28 °C on Luria broth (LB) [59] semi-solidified with 1.5% (w/v) agar and supplemented with the appropriate antibiotics 3–5 days prior to explant inoculation. Antibiotic selection of strain 1065 on agar medium employed kanamycin sulfate (100 mg/l), rifampicin (50 mg/l) and tetracycline (5 mg/l). Overnight liquid cultures were grown at 28 °C on a horizontal rotary shaker (180 rpm) and were initiated by inoculating 20 ml of liquid LB medium, containing kanamycin sulphate (50 mg/l), rifampicin (40 mg/l) and tetracycline (2 mg/l) in a 100 cm^3 conical flask. Bacterial cultures were grown to an OD_{600} of 1.0–1.5 prior to inoculation of explants.

Plant transformation

Cotyledons excised from 7-day old seedlings were inoculated with *A. tumefaciens* [9]. Shoots, which regenerated from explants on medium containing kanamycin (50–100 mg/l), were rooted *in vitro* in the presence of kanamycin (100 mg/l) and transferred to the glasshouse, where they were allowed to self-pollinate and set seed. Seeds were collected and stored at 4 °C. For controls, plants were regenerated from uninoculated cotyledons on antibiotic-free medium.

Double-antibody sandwich ELISA assay for quantification of NPTII protein in plant tissue

Leaf discs (4 per plant) were punched from randomly selected leaves of 28–42-day old plants in the glasshouse using the lids of 1.5 ml microfuge tubes. Discs within the tubes were frozen immediately in liquid nitrogen. Frozen samples were ground to a fine powder with a plastic microhomogenizer previously cooled in liquid nitrogen. A 500 μl aliquot of protein extraction buffer (0.25 M Tris-HCl pH 7.8, 1 mM phenylmethylsulfonylfluoride) was added to each sample and the latter vortexed (15 s) and placed on ice. Samples were centrifuged for 10 min at 10 000 × g in a microcentrifuge at 4 °C. The supernatants (200 μl) were removed to fresh tubes and stored on ice. Protein extracts were quantified [6], before storage overnight at −70 °C. The amount of NPTII protein in 80 μg of each plant protein extract was quantified using the NPTII ELISA kit (5 Prime → 3 Prime Inc., Boulder, USA) according to the manufacturer's instructions. Colour development of the final reaction in plant protein extracts and NPTII standards was quantified using a microtitre plate reader (A_{405nm}) (Microplate Reader, Dynatech, Billingshurst, UK).

Analysis of segregation of kanamycin resistance in seedlings

Seeds were surface-sterilized and germinated on 20 ml aliquots of half-strength MS-based medium with 1.0% (w/v) sucrose and kanamycin (200 mg/l) in 9 cm Petri dishes (maximum 20 seeds/dish) under the conditions described earlier. The kanamycin resistance of seedlings was scored one month after germination according to the categories resistant (green leaves) and sensitive (completely bleached and dead).

GUS assays

Fluorometric and histochemical GUS assays were performed essentially as described [29]. For GUS histochemical assays, discs were punched from leaves using the lid of a 0.5 ml microfuge tube. The discs were placed into individual wells of a 96-well microtitre plate, each well containing 200 μl of substrate solution, and incubated for 16 h at 37 °C. Protein extracts for the GUS fluorometric assay were prepared in the same way as for NPTII ELISA assays, except that the extraction buffer consisted of 50 mM sodium phosphate (pH 7.0), 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 0.1% (w/v) sodium lauryl sarcosine and 0.1% (v/v) Triton X-100. Protein samples (20 μl) were incubated in 200 μl of 1 mM MUG (4-methylumbelliferyl-β-D-glucuronide) substrate for 90 min at 37 °C. Reactions were terminated by addition of 90 μl of 0.2 mM sodium carbonate. Fluorescence readings were taken using a plate reader (Cytofluor 2300, Millipore, Watford, UK) with excitation and emission wavelengths of 365 nm and 455 nm, respectively. Readings were calibrated against 0.0–5.0 nmol MU (4-methylumbelliferone sodium salt) standards at 0.5 nmol intervals in triplicate.

T-DNA copy number analysis

Total genomic DNA was isolated from ca. 1 g of leaf material [14]. Ten μg of plant DNA were digested with 50 units of *Eco*RI restriction endonuclease for 16 h at 37 °C. A single *Eco*RI site was positioned between the *nos.npt*II.*nos* and 35S.*gus*-intron.35S on the T-DNA of pVDH65 (Figure 1). Cleavage of the *Eco*RI

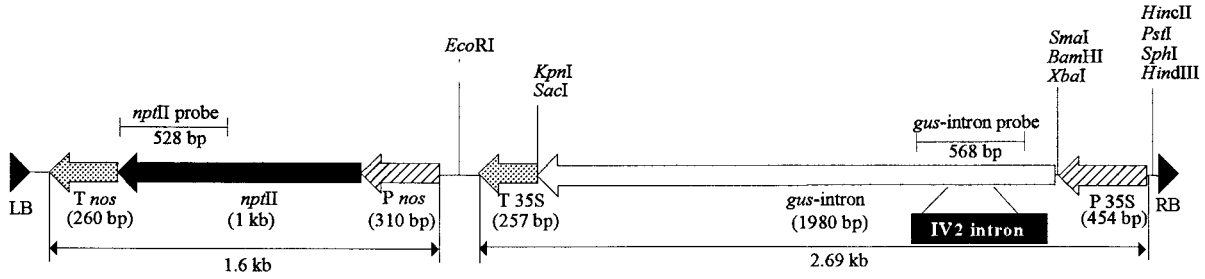

Figure 1. pVDH65 T-DNA.

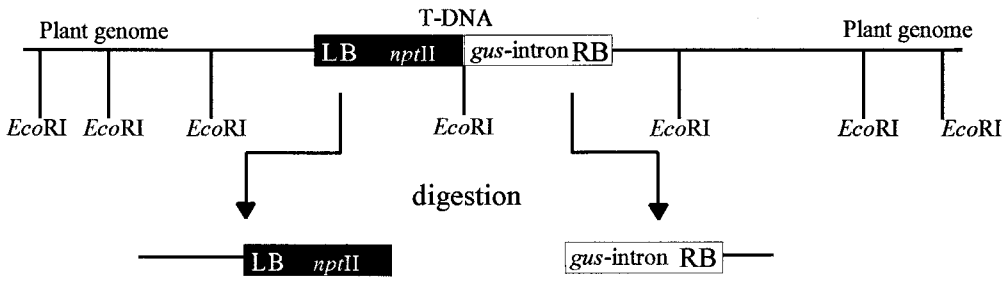

Figure 2. Digestion of pVDH65 T-DNA (integrated into the plant genome) with *Eco*RI to produce border fragments for determination of T-DNA copy number.

site of pVDH65 T-DNA integrated into plant genomic DNA gave border fragments of random size depending on the integration site (Figure 2). Non-radioactive Southern analysis was performed as descrbed previously [43].

Treatment with 5-azacytidine

Seeds were germinated on filter paper in 9 cm diameter Petri dishes (10 seeds per dish) each containing 10 ml of an aqueous solution of 5-azacytidine, at concentrations of either 0, 1, 10, 50 or 100 μM. Seedlings were removed for GUS histochemical analysis 14 days after germination.

Results

Copy numbers of nptII *and* gus *genes*

In *Eco*RI-digested genomic DNA from R_1 plants, the number of bands which hybridized clearly to *gus*-intron and *npt*II probes varied from 1 to 6. Additional faint bands, probably resulting from partial digestion of DNA, were also visible in some samples (Figure 3A). In many samples, the number of bands which hybridised to the *gus*-intron probe was different from the number which hybridized to the *npt*II probe, indicating rearrangement of the T-DNA. As there were no R_1 plants from the same line which all showed single bands following hybridisation with *gus*-intron and *npt*II probes, it was deduced that their R_0 parents did not have single intact T-DNA insertions. In R_1 plants from two R_0 plants (H and N), bands hybridised to the *npt*II probe but not to the *gus*-intron probe, indicating the absence of the right side of the T-DNA.

Unexpectedly, most of the R1 plants tested for lines T and P showed the same banding pattern for *npt*II and *gus*-intron probes (Figure 3A). Adjacent samples from different lines on the same Southern blot filter showed different banding patterns for the *npt*II and *gus*-intron probes. This confirmed that the bands for R_1 plants from lines T and P were a true representation of the T-DNA integration patterns. A possible explanation for this hybridisation pattern was that several T-DNA copies were integrated into the plant genome at the same locus without intervening *Eco*RI sites (Figure 3B).

Comparison of GUS activity in R_0, R_1 and R_2 generation transgenic plants

GUS histochemical analysis was performed on R_0 plants which had been transferred from culture to the glasshouse and on their seed-derived R_1 and R_2 progeny grown in compost in the glasshouse. There was wide variation in GUS activity between high and

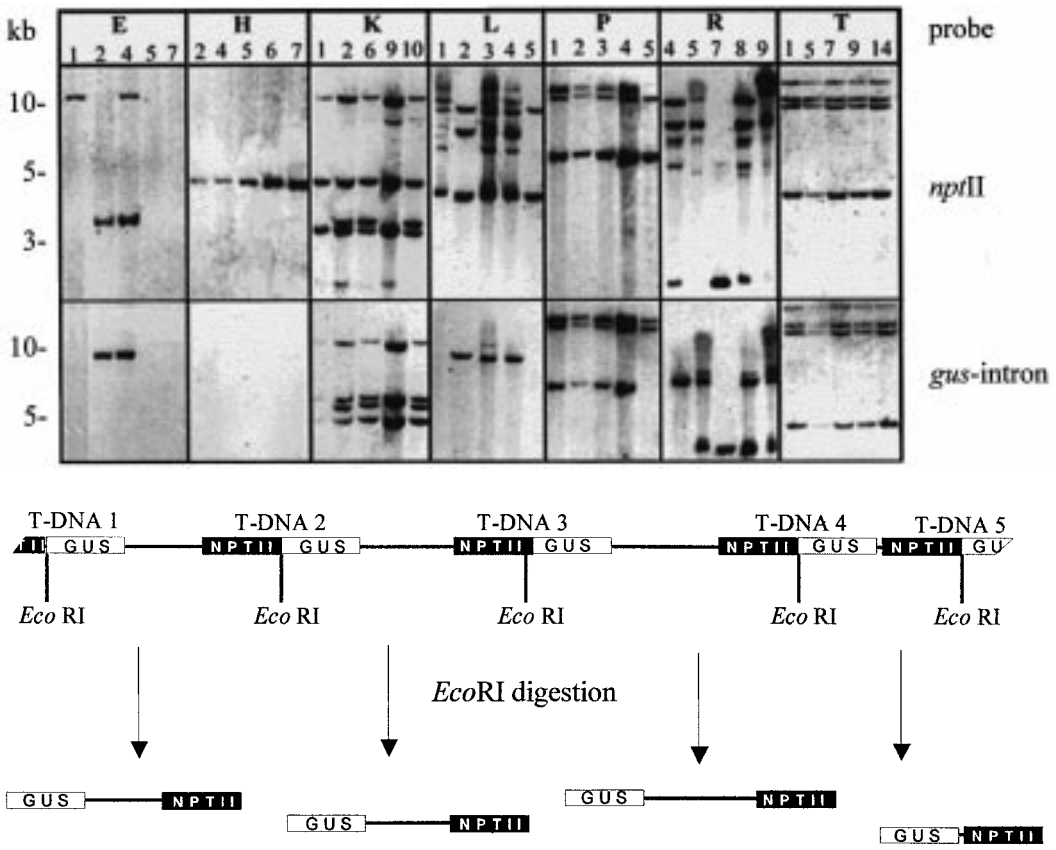

Figure 3. A (top). Representative Southern blot border fragment analysis of genomic DNA from lettuce 1065-transformed R_1 plants hybridized with *gus*-intron and *npt*II gene probes. B (bottom). Proposed T-DNA integration pattern (deduced from Southern blot shown in A, above) for plants of R_1 line T. *Eco*RI digestion of 5 T-DNA copies integrated at the same plant gene locus, with no *Eco*RI sites on the intervening plant DNA, resulting in 4 fragments each containing the *gus* and *npt*II genes. T-DNAs 1 and 5 are truncated. Southern hybridization to this digest would give the same banding pattern for both *npt*II and *gus* probes.

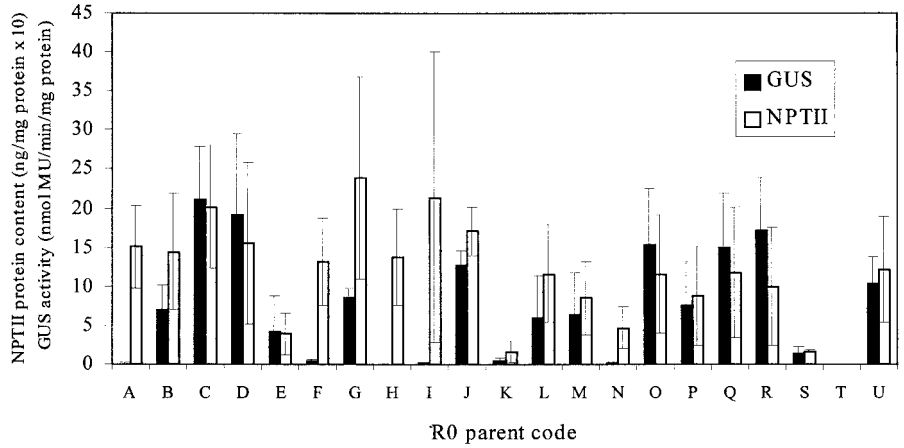

Figure 4. Mean NPTII protein content and GUS activity of seed-derived R_1 plants from 21 independent R_0 lettuce lines transformed by *A. tumefaciens* strain 1065 (10 R_1 plants per R_0 parent, error bars represent standard deviation). All samples were collected on the same day (65 days after sowing seeds in compost in the glasshouse) and assays were performed simultaneously. Values for 10 non-transgenic control lettuce plants were zero in all cases (data not shown).

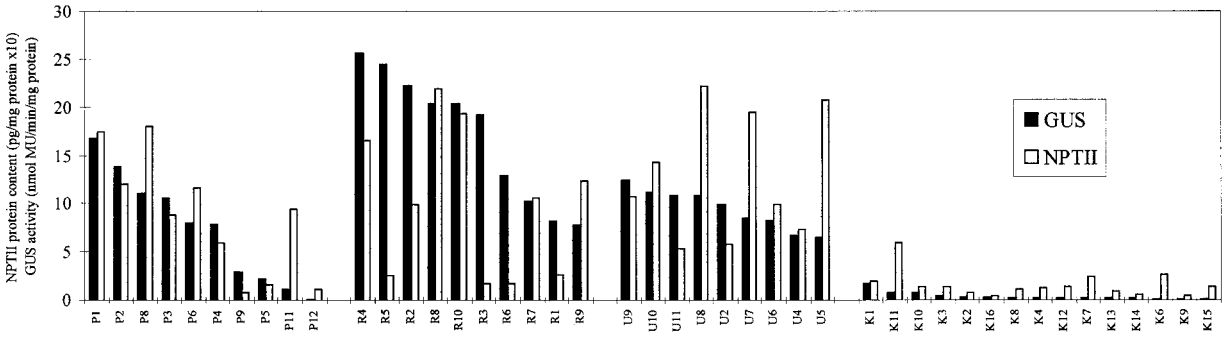

Figure 5. GUS fluorometric activity and NPTII protein content of individual R_1 transgenic lettuce plants from high- and low-expressing lines. All samples were collected on the same day (65 days after sowing seed in compost in the glasshouse) and assays were performed simultaneously.

low gene expressing plants. In many cases, GUS activity was higher in R_1 plants than in their R_0 parents (Table 1A). For example, in line S, GUS activity was not detected in the R_0 plant, but activity was detected in 8 of 10 R_1 plants. Lines C, G and R, which had high GUS activity in the R_1 generation, also had high GUS activity in the R2 generation (Table 1B). In line K, low GUS activity was detected in R_0 and R_1 plants, but was not observed in R_2 plants (Table 1A, B). This indicated loss of transgene expression during successive generations in this line.

GUS fluorometric analysis of R_1 transgenic lines

A fluorometric plate reader was not available at the time of R_0 plant analysis. Consequently, the first quantative GUS analyses were performed on R_1 plants. Fluorometric quantification of GUS activity in R_1 plants (10 plants per line) revealed extreme variation between lines (Figure 4), with about a 50-fold difference between the GUS-positive line with the highest activity (line C) and line K with the lowest activity. R_1 plants from lines A, H, I, N and T did not exhibit detectable GUS activity.

Considerable variation in GUS activity was also observed between individual GUS-positive R_1 plants from the same R_0 parent (Figure 5). For example, in plants derived from the R_0 parent P, GUS activity was intermediate to low in plants P5 and P9, but high in plants P1, P2, P3, P6, P8 and P11. GUS acitivity in plant P1 was almost 8-fold higher than in plant P5 (Figure 5).

NPTII ELISA analysis of R_1 transgenic plants

Comparison of the mean NPTII protein content of R_1 plants (10 plants from each line) showed that plants from lines E, K, N, S and T had low levels of NPTII,

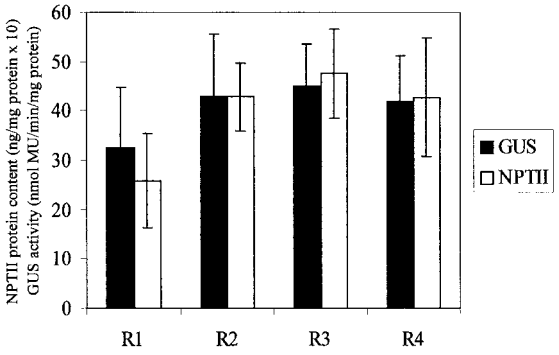

Figure 6. GUS activity and NPTII protein content in seed-derived R_1-R_4 generation transgenic lettuce plants (10 plants per generation) derived from R_0 parental line R (error bars represent standard deviation). All samples were collected on the same day (60 days after sowing seed in compost in the glasshouse) and assays were performed simultaneously.

while plants from the remaining lines had intermediate or high levels of NPTII protein (Figure 4). There was about a 16-fold difference in mean NPTII protein content of R_1 plants from line G with the highest value, and R_1 plants from line K with the lowest value. In general, R_1 plants with high GUS activity had high or intermediate values for NPTII protein content; R_1 plants with low NPTII protein content also had low GUS activity. However, R_1 plants from lines A, F, H, I and N had undetectable or low GUS activity, but intermediate or high levels of NPTII protein (Figure 4).

As with GUS activity, variation in NPTII protein content was also observed between R_1 plants derived from the same R_0 parent. Individual plants with high GUS activity did not always have high levels of NPTII protein. For example, plant P11 had high GUS activity, but low NPTII protein. Conversely, plant R5 had low GUS activity, but high NPTII protein (Figure 5).

Table 1A. GUS histochemical analysis of cv. Raisa 1065 R_0 plants and their R_1 progeny 60 days after sowing.

R_0 parent					R_1 progeny				R_0 parent					R_1 progeny			
plant code	GUS activity[1]				plant code	GUS activity[1]			plant code	GUS activity[1]				plant code	GUS activity[1]		
	a	b	c	d		a	b	c		a	b	c	d		a	b	c
B	++	++	++	++	B1	++	++	++	O	+	++	++	++	O1	+++	++	+++
					B2	++	++	++						O2	++	++	+++
					B3	++	++	++						O3	+++	+++	+++
					B4	++	++	++						O4	+++	+++	+++
					B5	++	++	++						O5	+++	+++	+++
C	++	+	+	+	C1	+++	+++	+++						O6	++	++	++
					C2	+++	+++	+++						O7	+++	+++	+++
D	++	++	++	++	D1	+++	+++	+++						O8	+++	+++	+++
					D3	+++	+++	+++						O9	−	−	−
					D4	+++	+++	+++						O10	+++	++	+++
					D5	++	++	++	P	+	+	+	+	P1	+++	++	++
E	+	+	+	+	E1	−	−	−						P2	+++	++	++
					E2	++	++	++						P3	++	++	++
					E4	++	++	++						P4	+++	++	++
					E5	−	−	−						P5	+	+	+
					E6	++	++	++						P6	++	+++	+++
					E7	−	−	−						P8	++	++	+++
F	+−	+	+	+	F1	−	−	−						P9	+	+	+
					F2	+−	+−	+−						P11	+++	+++	+++
					F3	−	−	−						P12	−	−	−
					F4	−	−	−	Q	++	++	++	++	Q1	+++	+++	+++
					F5	+−	+−	+−						Q2	−	−	−
G	+	+	+	+	G1	+++	+++	+++						Q3	++	++	+++
					G2	+++	+++	+++						Q4	+++	+++	+++
					G3	+++	+++	+++						Q5	++	+++	+++
					G4	++	++	++						Q6	++	++	+++
					G5	+++	+++	+++						Q7	++	+++	+++
J	++	++	++	++	J1	++	++	+++						Q8	+++	+++	+++
					J2	+++	++	+++						Q10	+++	+++	+++
					J3	+++	++	+++	R	++	++	++	++	R1	+++	+++	++
					J4	++	++	++						R2	+++	+++	+++
					J5	++	+++	+++						R3	+++	+++	+++
K	+	+	+	+	K1	−	+	++						R4	+++	+++	+++
					K2	+	++	+						R5	+++	+++	+++
					K3	−	−	+−						R6	+++	++	++
					K4	−	++	−						R7	++	++	+++
					K6	+	+	+						R8	+++	+++	+++
					K7	+	+	+						R9	++	+++	+++
					K8	+	+	+−						R10	+++	++	+++
					K9	−	−	+	S	−	−	−	−	S1	−	−	−
					K10	+	++	++						S2	+−	++	++
					K11	+	++	+−						S3	−	−	−
					K12	+−	+	−						S4	++	++	++
					K13	−	−	+−						S5	+	++	++
					K14	+−	+−	+−						S6	++	++	++
					K15	−	−	−						S7	++	++	++
					K16	+	+−	+						S8	++	++	++

Table 1A. Continued.

R_0 parent					R_1 progeny				R_0 parent					R_1 progeny			
plant code	GUS activity[1]				plant code	GUS activity[1]			plant code	GUS activity[1]				plant code	GUS activity[1]		
	a	b	c	d		a	b	c		a	b	c	d		a	b	c
L	++	+	+	+	L1	−	−	−						S9	+−	+	++
					L2	+++	++	++						S11	++	++	++
					L3	++	++	++	U	+	+	+	+	U2	+++	+++	+++
					L4	++	++	++						U3	+++	+++	+++
					L5	−	−	−						U4	+++	+++	+++
M	+	+	+	+	M1	−	−	−						U5	+++	+++	+++
					M2	+++	++	++						U6	+++	+++	+++
					M3	+++	+++	+++						U7	+++	+++	+++
					M4	−	−	−						U8	+++	+++	+++
					M5	+++	+++	+++						U9	+++	+++	+++
					M6	++	++	++						U10	+++	+++	+++
					M7	++	++	+++									

[1]Scoring of intensity of blue staining on wounded surfaces of individual leaf discs (a, b, c and d) after GUS histochemical staining: + + + = completely stained, dark blue, ++ = intermediate, + = weak, +− = questionable, − = no visible staining, nd = not determined. Plants were grown in compost in 9 cm pots under glasshouse conditions. R_1 progeny plants have the same first character as their R_0 parent *i.e.* B1, B2 and B3 are the R_1 progeny of R_0 plant B. Plants from the same generation were analysed simultaneously.

Analysis of kanamycin resistance in R_1 and R_2 seedlings

Kanamycin-resistant R_1 seedlings were produced by all R_0 plants, except plant T. Segregation of kanamycin-resistant and kanamycin-sensitive seedlings was observed in R_1 progeny derived from 10 of 21 R_0 parents. R_1 plants from only 3 lines exhibited a segregation ratio of ca. 3:1 (kanamycin-resistant/sensitive), indicating that an active *npt*II gene(s) was integrated at a single locus or linked loci in the R_0 parents. Segregation ratios in R_1 seedlings derived from 15 R_0 plants indicated that 2 or more active *npt*II genes had been integrated at unlinked loci in the R_0 parents. Unexpected segregation ratios were observed in R_1 progeny derived from R_0 plants K and S, in which only 2 or 3 of 20 seedlings were kanamycin-resistant (Table 2). Variation in kanamycin resistance was apparent between individual R_1 seedlings from R_0 parents A, E, G, H, K, O and R (Table 2).

R_2 seedlings, from 17 glasshouse-grown R_1 plants, which were shown by ELISA to contain NPTII protein, were also analysed for segregation of kanamycin resistance (Table 2). Kanamycin resistance was observed in R_2 seedlings from 16 of the 17 selected R_1 parents. Segregation of kanamycin resistance was observed amongst R_2 seedlings derived from 10 of the 17 selected R_1 parents. R_2 seedlings from 6 of the R_1 parents were all resistant to kanamycin, while those from the R_1 plant K6 were all sensitive to kanamycin.

Simultaneous quantification of GUS and NPTII expression in R_1, R_2, R_3 and R_4 transgenic plants

Fluorometric GUS activity and NPTII protein content were quantified in a heterogeneous population of R_1 plants derived from R_0 line R, and homozygous R_2, R_3 and R_4 plants from the same parental line (10 plants per generation) (Figure 6). Seeds from all generations were germinated and grown simultaneously under the same conditions. High GUS activity and NPTII protein content were recorded in R_1, R_2, R_3 and R_4 plants from line R, demonstrating stable heritable transgene expression in lettuce throughout four seed generations.

Relationship between transgene copy number and expression

A wide range of transgene expression was observed amongst those R_1 plants which showed 1, 2 or 3 bands after Southern hybridization with the *gus*-intron or the *npt*II probes (Figure 7). Most plants exhibiting 4 hybridization bands with either probe had low transgene expression. However, at least 3 plants which showed 4 bands after hybridization with the *npt*II probe, contained relatively high levels of NPTII protein. This in-

Table 1B. GUS histochemical analysis of cv. Raisa 1065 R_1 plants and their R_2 progeny 60 days after sowing.

R_1 parent				R_2 progeny				R_1 parent				R_2 progeny			
plant code	GUS activity[1]			Plant code	GUS activity[1]			plant code	GUS activity[1]			plant code	GUS activity[1]		
	a	b	c		a	b	c		a	b	c		a	b	c
B1	++	++	++	B1.1	++	++	++	G5	+++	+++	+++	G5.1	+++	+++	+++
				B1.2	+++	+++	+++					G5.2	+++	+++	+++
				B1.3	+++	+++	+++					G5.3	+++	+++	+++
				B1.4	+++	+++	+++					G5.4	+++	+++	+++
				B1.5	+++	+++	+++					G5.5	+++	+++	+++
B4	++	++	++	B4.1	+++	+++	+++					G5.6	+++	+++	+++
				B4.2	+++	+++	+++					G5.7	+++	+++	+++
				B4.3	+++	+++	+++					G5.8	+++	+++	+++
				B4.4	+++	+++	+++					G5.9	+++	+++	+++
				B4.5	+++	+++	+++					G5.10	+++	+++	+++
				B4.6	+++	+++	+++	L1	−	−	−	L1.1	−	−	−
				B4.7	+++	+++	+++					L1.2	−	−	−
				B4.8	+++	+++	+++					L1.3	−	−	−
				B4.9	+++	+++	+++					L1.4	−	−	−
				B4.10	+++	+++	+++					L1.5	−	−	−
B5	++	++	++	B5.1	+++	+++	+++					L1.6	−	−	−
				B5.2	+++	+++	+++					L1.7	−	−	−
				B5.3	+	+	+					L1.8	−	−	−
				B5.4	+++	+++	+++					L1.9	−	−	−
				B5.5	+++	+++	+++					L1.10	−	−	−
				B5.6	+++	+++	+++	L2	+++	++	++	L2.1	+++	+++	+++
				B5.7	+++	+++	+++					L2.2	+++	+++	+++
				B5.8	+++	+++	+++					L2.3	+++	+++	+++
				B5.9	+++	+++	+++					L2.4	+++	+++	+++
				B5.10	+++	+++	+++					L2.5	+++	+++	+++
C1	+++	+++	+++	C1.1	+++	+++	+++					L2.6	+++	+++	+++
				C1.2	+++	+++	+++					L2.7	+++	+++	+++
				C1.3	+++	+++	+++					L2.8	+++	+++	+++
				C1.4	+++	+++	+++					L2.9	+++	+++	+++
				C1.5	+++	+++	+++					L2.10	+++	+++	+++
				C1.6	+++	+++	+++	K6	+	+	+	K6.1	−	−	−
				C1.7	+++	+++	+++					K6.2	−	−	−
				C1.8	+++	+++	+++					K6.3	−	−	−
				C1.9	+++	+++	+++					K6.4	−	−	−
				C1.10	+++	+++	+++					K6.5	−	−	−
E4	+	+	+	E4.1	++	++	++					K6.6	−	−	−
				E4.2	++	++	++					K6.7	−	−	−
				E4.3	++	++	++					K6.8	−	−	−
				E4.4	++	++	++					K6.9	−	−	−
				E4.5	++	++	++					K6.10	−	−	−
				E4.6	++	++	++	K12	+−	+	−	K12.1	−	−	−
				E4.7	++	++	++					K12.2	−	−	−
				E4.8	++	++	++					K12.3	−	−	−
				E4.9	++	++	++					K12.4	−	−	−
				E4.10	++	++	++					K12.5	−	−	−
F4	−	−	−	F4.1	−	−	−					K12.6	−	−	−
				F4.2	−	−	−					K12.7	−	+	−

Table 1B. Continued.

R_1 parent				R_2 progeny				R_1 parent				R_2 progeny			
plant code	GUS activity[1]			Plant code	GUS activity[1]			plant code	GUS activity[1]			plant code	GUS activity[1]		
	a	b	c		a	b	c		a	b	c		a	b	c
				F4.3	–	–	–					K12.8	–	–	–
				F4.5	–	–	–					K12.9	–	–	–
				F4.6	–	–	–					K12.10	–	–	–
				F4.7	–	–	–	R4	+++	+++	+++	R4.1	+++	+++	+++
				F4.8	–	–	–					R4.2	+++	+++	+++
				F4.9	–	–	–					R4.3	+++	+++	+++
				4.10	–	–	–					R4.4	+++	+++	+++
F5	+–	+	+	F5.1	+–	+–	+–					4.5	+++	+++	+++
				F5.2	+–	+–	+–					R4.6	+++	+++	+++
				F5.3	+–	+–	+–					R4.7	+++	+++	+++
				F5.4	+–	+–	+–					R4.8	+++	+++	+++
				F5.5	+–	+–	+–					R4.9	+++	+++	+++
				F5.6	+–	+–	+–					R4.10	+++	+++	+++
				F5.7	+–	+–	+–	Control				Cont 1	–	–	–
				F5.8	+–	+–	+–					Cont 2	–	–	–
				F5.9	+–	+–	+–					Cont 3	–	–	–
				F5.10	+–	+–	+–					Cont 4	–	–	–

[1]See Table 1A. Plants were grown in compost in 9 cm pots under glasshouse conditions. R_2 progeny have the same first two characters as their R_1 parents, i.e. B1.1, B1.2 and B1.3 are the R_2 progeny of R_1 plant B1. Plants from the same generation were analysed simultaneously.

dicated that there was no distinct correlation between high copy number and low transgene expression.

Treatment with 5-azacytidine

Of the 4 R_1 lines B, K, U and T which were germinated on medium containing 5-azacytidine, seedlings of lines B and K from the 10 μM 5-azacytidine treatments showed higher GUS activity (as determined by histochemical staining) than those treated with 0 or 1 μM 5-azacytidine (Figure 8). This suggested that low GUS expression in these lines was associated with DNA methylation. Seedlings from line T did not exhibit expression in any treatment, indicating that methylation was not associated (or reversible) with transgene silencing in this line. As expected, high GUS activity in seedlings from line U was observed in all treatments. Azacytidine at 10 μM and above reduced seedling growth; seed germination on 50 and 100 μM 5-azacytidine was severely reduced. Consequently, the latter concentrations were not suitable for this analysis.

Discussion

The variation in transgene expression observed in the lettuce cv. Raisa was similar to that seen in other plants, such as tobacco. The level of protein coded for by a heterologous transgene commonly varies by 10- to 50-fold amongst individual transformants within the same experiment [27], while up to 16-fold differences in gene expression have been reported amongst genotypically identical clones [31]. In the present work, there was a 50-fold difference in mean GUS activity between line K and line C (lowest and highest *gus* expressers), but only a 16-fold difference in mean NPTII protein content between line K (lowest value) and line G (highest value). Furthermore, despite the fact that the *gus*-intron and *npt*II genes were introduced on the same T-DNA, 5 lines (A, H, I, N and T) lacked GUS activity, but all lines, except line T, contained significant concentrations of NPTII protein. Therefore, variation of GUS activity between R1 lines was greater than variation in NPTII protein content. This can be explained partly by Southern analysis, which revealed that the right side of the T-DNA, containing the *gus*-intron gene, was absent in many of the T-DNA copies in R_1 lines. Such a result is unexpected, as it is re-

Table 2. Growth of R_1 and R_2 seedlings, derived from cv. Raisa 1065 transformed R_0 lines, one month after germination on medium supplemented with kanamycin (200 mg/l).

R_0 parent plant code	Growth of R_1 seedlings on kanamycin-resistant/sensitive[1]	R_1 parent plant code	Growth of R_2 seedlings on kanamycin-resistant/sensitive[1]
A	20:0	A1	17:3
		A5	16:4
B	20:0	B4	15:5
		B5	12:8
C	10:0	nd	–
D	19:1	D3	14:6
E	19:1	E4	17:3
F	15:5	F4	14:6
		F5	15:5
G	20:0	G5	20.0
H	20:0	H2	18:2
		H4	20:0
		H5	20:0
		H6	20:0
		H7	20:0
I	19:1	nd	–
J	15:5	nd	–
K	3:17	K6	0:20
L	18:2	L1	16:4
		L2	20:0
M	18:2	nd	–
N	20:0	nd	–
O	20:0	nd	–
P	16:4	nd	–
Q	10:0	nd	–
R	20:0	nd	–
S	2:18	nd	–
T	0:20	nd	–
U	20:0	nd	–

[1]Number of resistant seedlings (green true leaves): number of sensitive seedlings (bleached and dead).

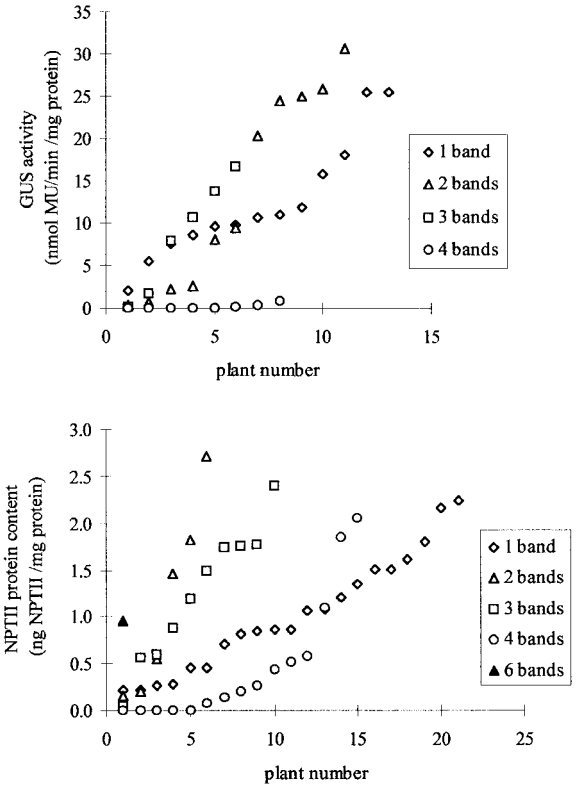

Figure 7. Relationship between number of bands from *Eco*RI-digested genomic DNA from transgenic lettuce plants and the level of transgene expression.

ported that the left end of the T-DNA integrated into plant genomes, is usually poorly conserved (i.e. is usually truncated by 3 to 100 nucleotides), whereas the right end is often conserved up to the nucleotide that is attached to the Vir D2 protein [66]. Moreover, T-DNA integration into plant genomes requires the presence of the right border [66]. Therefore, it would be expected that, as the selectable marker was positioned at the left end of the T-DNA in pVDH65, kanamycin-resistant plants resulting from transformation with this construct would contain full-length T-DNAs [4]. However, it is possible that T-DNA was deleted in R_0 plants after integration. In this respect, Ulian et al. [69] reported that T-DNA insertions in R_0 plant genomes were not completely stable, after observing that R_1 transformants of petunia had *npt*II-hybridizing *Hin*dIII fragments that differed in size from those of the parent. Post-integrational T-DNA deletion is debatable, as most reports claim that T-DNA instability is restricted to the integration process and, once integrated, T-DNA is stably inherited by seed-derived progeny [66].

The differences in transgene expression between the R_0 and R_1 generations of transgenic lettuce may also be explained by loss of unstable T-DNA inserts in the R_0 parent. However, Southern analysis of R_1 plants from line T showed that the *npt*II and *gus*-intron genes were both stably integrated, but inactive. As primary transformants were selected on kanamycin-containing medium, the *npt*II gene must have been active in the R_0 parental plant of line T during culture. Similarly, R_1 plants from lines E, K, N and S, which showed low expression of the *npt*II gene, were likely to have had high NPTII activity in the R_0 parent in order to have survived selection. Therefore, the *npt*II gene in these plants must have been inactivated or suppressed either in the R_0 generation after transfer from culture to the glasshouse, or some time after meiosis. Loss of transgene activity in petunia has also been observed after transfer of primary transformants from culture to the glasshouse, or after germination of R_0-derived seed [69].

GUS activity was lower in some R_0 plants than in their R_1 progeny. Suppressed *gus*-intron gene expression in R_0 plants may have been due to epigenetic factors, such as culture-induced stress and, therefore, was not meiotically heritable. Several reports have described non-heritable transgene silencing in plants [12, 13, 18, 26]. In line T, complete transgene silencing was inherited by R_1 and R_2 progeny, as was low transgene expression in line K. Stable inheritance of silenced transgenes through several generations was also reported previously [41, 45]. Other workers have observed at least partial meiotic transmission of silenced transgenes in plants [38, 40, 45, 49, 52, 74]. Current evidence suggests that the more stable forms of silencing are usually meiotically heritable, result from transcriptional inactivation and are often associated with transgene methylation [35]. However, in line T, it was not possible to demonstrate that transgene silencing was associated with methylation, as 5-azacytidine did not reactivate transgene expression.

The majority of transgenic lettuce lines showed multiple and/or truncated T-DNA inserts. Interestingly, only limited analysis of transgene insertion in lettuce has been performed previously. For example, only 2 of 16 *Agrobacterium*-derived lettuce transformants, produced with binary and co-integrate vectors carrying the chimaeric *nos.npt*II.*nos* construct, contained T-DNA insertions which were not rearranged [48]. All 16 of these plants showed monogenic segregation of kanamycin resistance (75% resistant seedlings) in the R_1 generation, indicating

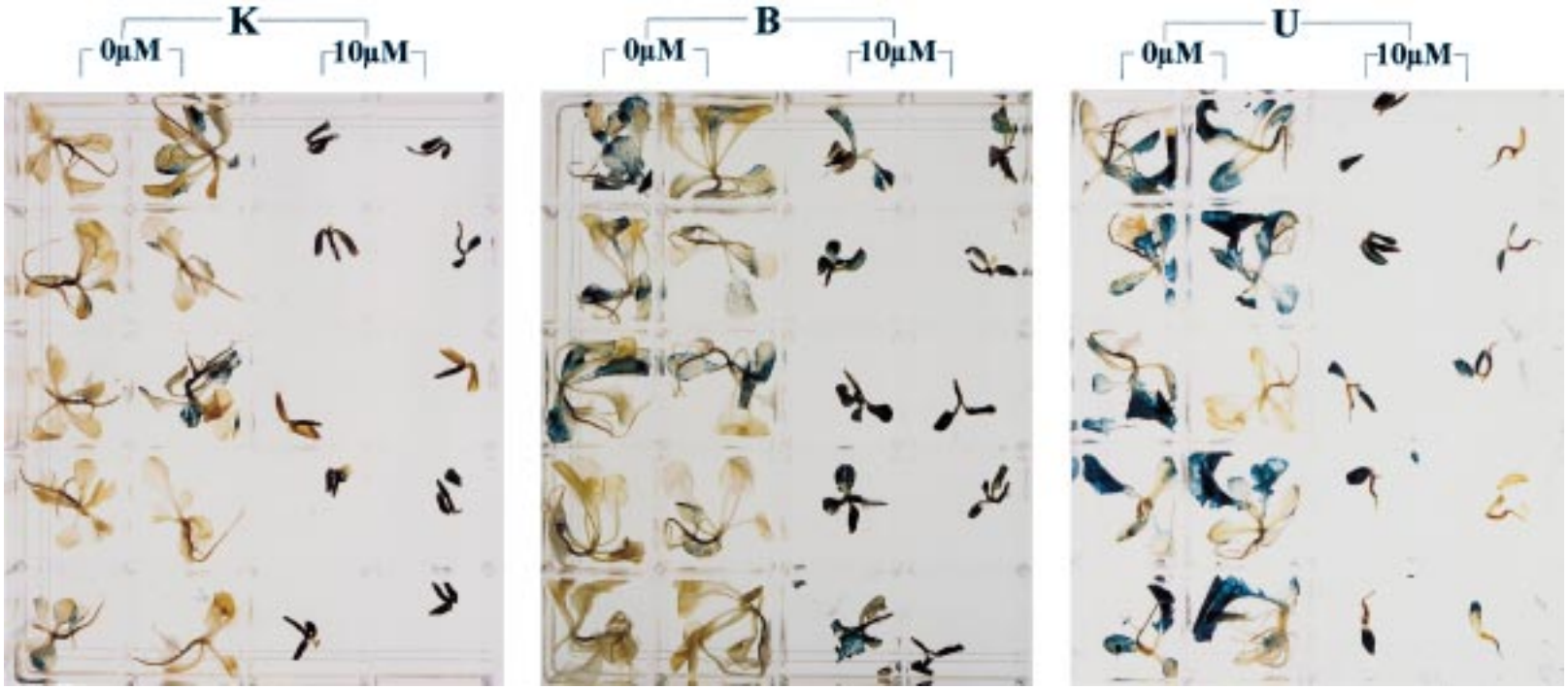

Figure 8. GUS histochemical analysis of R1 seedlings from transgenic lettuce lines K, B (low transgene expressers) and U (high transgene expresser) 14 days after germination on 0 μM and 10 μM 5-azacytidine. Increased intensity of blue colouration in seedlings from the 10 μM 5-azacytidine treatment, compared with those on the 0 μM treatment indicates demethylation of the *gus*-intron gene in lines K and B. Seedlings from different lines were assayed simultaneously with the same substrate solution.

single or linked integration sites for active *npt*II genes. Others workers [8] analysed 4 transformants, from electroporation-mediated DNA transformation of lettuce protoplasts, which contained 1, 2, 3 and 5 copies of the *npt*II gene respectively. In this case, 2 of 3 transformants showed monogenic segregation of kanamycin resistance in the R_1 generation; the transformant with 5 copies of the *npt*II gene exhibited unusual segregation (55% resistant seedlings), which indicated gene silencing. T-DNA rearrangement was found in only 1 of 13 *Agrobacterium*-derived lettuce transformants carrying the 35S.*gus.nos*-35S.*npt*II.*nos* genes [19]. Monogenic segregation of kanamycin resistance in the R_1 generation was observed for all 13 lines.

It is clear from published and present work, regardless of whether transgene copy number influences transgene expression, that multiple inserts cause complications and are an undesirable variable in plant transformation. Increasing the frequency of single inserts in a transformation system is difficult, as the cause of multiple T-DNA insertions is unclear. Jorgensen et al. [32] found that T-DNA was organised mostly as inverted repeat structures in plants transformed with agrobacteria derived from *A. tumefaciens* strain C58. Thus, the bacterial strain may determine T-DNA integration patterns. Unpublished data of the present authors has shown that the presence of a supervirulent plasmid in *Agrobacterium* strains may increase the frequency of T-DNA insertions in transgenic lettuce.

An apparent increase of GUS activity in lettuce lines K and B, after treatment with 5-azacytidine, indicated that methylation was associated with transgene silencing or low expression. In transgenic pearl millet callus, inactivation of the *gus* gene was associated with methylation and could be reactivated with 10 μM 5-azacytidine [36]. However, in the present work, lettuce line T, which was completely silenced, did not respond to even the highest concentration of 5-azacytidine, implying that methylation was not associated with gene silencing in this line. Similarly, other workers [27, 40] were unable to increase gene expression by treating seedlings from low expressing transgenic tobacco lines with 5-azacytidine, even though analysis with methylation-sensitive restriction enzymes confirmed T-DNA in these lines to be methylated. Shao et al. [60] reported that, while NPTII activity in tobacco was increased by 5-azacytidine, *npt*II gene expression remained unaffected and suggested a post-translational effect of 5-azacytidine.

The choice of promoters has also been found to be important for stable expression of transgenes in lettuce. For example, the present authors have shown recently that a truncated plastocyanin promoter from pea gives increased frequency of stable inheritance of the *bar* gene in lettuce. As the pea plastocyanin promoter contains fewer methylation sites than the CaMV 35S promoter, this provides additional evidence for the role of methylation in transgene silencing in lettuce. The data obtained in the present investigation improve our understanding of T-DNA integration and expression in lettuce, which should facilitate the formulation of strategies to alleviate transgene instability in this vegetable crop.

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