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Transgene inheritance and silencing in hexaploid spring wheat

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Abstract Inheritance and expression of the *Act1D-uidA*::*nptII* transgene cassette inserted into the genome of a spring wheat cultivar, 'Fielder', was studied in T_4 and T_5 transgenic wheat lines. Southern blot and PCR analyses demonstrated that the transgene was inherited for five generations of selfed plants. The multiple integration pattern displayed in the T_1 generation was maintained up to the T_5 generation with no evidence of transgene rearrangement. There was no cytoplasmic effect on the inheritance of the transgene as observed by GUS histochemical assays in F_1 seeds of reciprocal crosses (T_4 crossed with untransformed 'Fielder'). Based on the histochemical GUS activity a clear Mendelian segregation ratio was not obtained in the F_2 seeds of the crosses, although there was a tendency towards a two-locus insertion ratio. For one cross (A1//FD/A1), some of the transgenic plants produced low GUS and NPTII enzyme activities in seeds, even though Southern blot and PCR analyses indicated the presence of an intact transgene expression cassette. The transgene of these plants was methylated based on Southern blot analysis of genomic DNA restricted with methylation-sensitive enzymes. Northern blot analysis revealed that the plants with the methylated transgene did not accumulate the *uidA*::*nptII* fusion gene transcript.

Key words DNA methylation · GUS activity · Spring wheat · Transgene expression · Transgene inheritance

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Introduction

Successful production of transgenic wheat (*Triticum aestivum*) by microprojectile bombardment or *Agrobacterium*-mediated transformation techniques has been reported by several research groups (Becker et al. 1994; Cheng et al. 1997; Nehra et al. 1994; Vasil et al. 1992; Weeks et al. 1993). Wheat plants have also been transformed with useful genes such as the high-molecular-weight glutenin subunit genes that enhance the bread-baking qualities of flour (Altpeter et al. 1996; Barro et al. 1997; Blechl and Anderson 1996). However, the success of developing improved wheat cultivars through genetic engineering depends on stable and predictable expression of the inserted gene. Gene silencing and the instability of transgenes are some of the problems encountered in transgenic plants (Finnegan et al. 1998; Senior 1998). The mechanism of gene silencing is not fully understood, but the phenomenon can occur at both the transcriptional and post-transcriptional levels (Stam et al. 1997). Transgene expression level can be affected by the surrounding DNA into which a transgene integrates (Pröls and Meyer 1992) as well as by environmental factors and endogenous parameters (Meyer et al. 1992; Walter et al. 1992). Unstable gene expression is often observed when several copies of a transgene are integrated into the plant genome or when inserted genes contain sequence homology to an endogenous gene (Müller et al. 1996). However, gene silencing has also been reported for transgenic plants with single-copy insertions containing no apparent sequence homology to the host genome (Elmayan and Vaucheret 1996).

For production of commercial cultivars, transgenes must be inherited in a predictable manner in successive generations, and the expression must be stable. Among cereal crops, transgene expression and inheritance in advanced generations has so far only been reported for maize (Armstrong et al. 1995; Register et al. 1994). We have previously reported on the production of self-fertile transgenic wheat (cv 'Fielder') carrying the *Act1D-uidA*::*nptII* expression cassette, (pRC62; Nehra et al.

1994). The regenerated T_1 wheat lines exhibited high levels of β-glucuronidase (GUS) and neomycin phosphotransferase II (NPTII) enzyme activities. In this investigation, we analyzed the inheritance and expression of the *Act1D-uidA::nptII* expression cassette in the T_4 and T_5 generations. The T_4 transgenic lines were reciprocally crossed with the parental cultivar ('Fielder') to study the inheritance of the transgene. The segregation analyses of F_1, F_2 and BC_1F_1 plants together with the molecular analyses carried out to investigate the transgene silencing phenomenon observed are presented in this report.

Materials and methods

Plant materials

The transgenic wheat lines were produced by microprojectile bombardment of isolated scutella with gold particles containing the pRC62 construct (Nehra et al. 1994). Seeds were collected from two independent T_4 transgenic wheat lines, designated as A1 and A6, which were grown in the field. The presence and expression of the *uidA::nptII* fusion gene was detected by histochemical GUS assays (Jefferson et al. 1987). GUS-positive seeds from a single spike were used for the study. Only the non-embryo portion of the seed was histochemically assayed for GUS activity, whereas the embryo portion was germinated to produce a plant for making crosses.

Plants used for crossing were grown in a growth chamber maintained at 22°±2° C day and 20°±2°C night, 16 h light (photon flux density of 100±10 μ Einsteins $m^{-2}s^{-1}$) and 8 h dark. Plants were watered every second day and fertilized once a week with 0.4 g/l 20N:20P:20K greenhouse fertilizer.

Isolation and hybridization analysis of nucleic acids

The procedures used for DNA isolation, Southern blot analysis, determination of NPTII activity and histochemical and fluorometric GUS activities have been previously described (Nehra et al. 1994). DNA methylation analysis was performed on 15 μm g of genomic DNA digested with the methylation-sensitive restriction enzymes, *Hpa*II and *Msp*I, in combination with *Hind*III. The number of transgene integration sites was assessed by Southern blot analysis of 18–20 μg genomic DNA digested with *Hind*III. The Southern blots were probed with a radiolabeled 1.8-kb *uidA* fragment isolated from pRC62.

Polymerase chain reaction (PCR)

For PCR, DNA was extracted from 30–40 mg fresh leaf tissue using the CTAB (cetyltrimethyl-ammonium bromide) protocol (Doyle and Doyle 1987). PCR was performed in 0.5-ml microfuge tubes containing 25 μl of a reaction mixture consisting of 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 m*M* each dNTP, 0.4 μ*M* of each oligonucleotide primer, 1.0 unit of *Taq* DNA polymerase (Boehringer, Mannheim, Germany) and 20–25 ng genomic DNA. The *nptII* PCR primers pair used was PSGA (GAGGCTATTCGGCTATGACTG) and PSGB (CAAGCTCTTCAGCAATATCACG). The PCR primers pair designed to recognize sequences within the *Act1* promoter and *uidA* region, respectively, were: ACT1 (GCTGCTCGTCAGGCTTAGAT) and GUS9 (GTCCGCATCTTCATGACGAC). DNA amplification was performed in a thermal cycler using 35 cycles of 1.0 min at 94°C, 1.0 min at 65°C, and 2.0 min at 72°C. One additional cycle was performed at 72°C for 10 min for complete extension of the PCR products. Amplification products were separated by electrophoresis in 1.4% (w/v) agarose gels.

Northern blot analysis

Total RNA was isolated from 0.1 g of 7-day-old kernels using a modified hot-phenol extraction method (Maes and Messens 1992). Five micrograms of total RNA was fractionated on a 1.5% agarose-formaldehyde gel according to Sambrook et al. (1989). The RNA was blotted onto a Hybond™-N+ nylon membrane (Amersham), and hybridized to a DIG-labeled 640-bp *nptII* DNA fragment according to Engler-Blum et al. (1993). The probe was labeled with DIG-dUTP according to the manufacturer's instructions (Boehringer Mannheim).

Statistical analyses

Segregation ratios of GUS positive and GUS-negative seeds were analyzed for χ^2 goodness of fit by MINITAB for Windows release 10.

Results

Southern blot analysis of transgene inheritance Genomic DNA was restricted with *Hind*III and analyzed by Southern blot hybridization using 1.8 kb *uidA* fragment as a probe. The *Act1D-uidA*::*nptII* gene cassette contains only one *Hind*III site positioned in the 5' region of the *Act1D* promoter. The analysis of T_1 plants revealed seven to eight hybridization signals, which indicated that the transgenic plants contained multiple copies of the transgene (Nehra et al. 1994). The hybridization patterns of T_5 and F_2 plants of A1//FD/A1 (Table 1) were very similar to that of the T_1 plant (Fig. 1). This suggests that there was no or undetectable rearrangement of the transgene during the successive generations.

Determination of GUS activity in F_1, F_2 and BC_1F_1 seeds

Seeds were collected from a single spike of 2 independent T_4 transgenic wheat plants (designated as A1 and A6) and tested for GUS activity by a histochemical GUS assay. The non-embryo portions of all the T_4 transgenic seeds, as well as the T_4-derived T_5 seeds, were GUS-positive, which indicated homozygosity for *uidA::nptII*. All

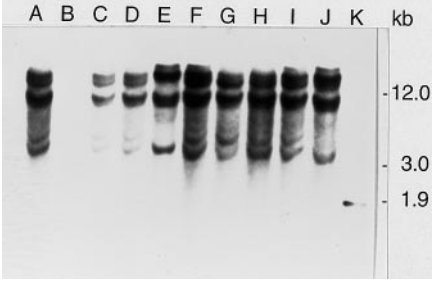

Fig. 1 Analysis of transgene integration by Southern blot analysis. Genomic DNA was digested with radiolabeled *Hind*III and probed with the *uidA* fragment. *A*=T_5 (A1), *B*='Fielder' (control), *C*, *D*, *G*, and *H* are DNA samples of A1//FD/A1 (F_1), *E*, *F*, *I* and *J* are DNA samples of A1//FD/A1 (F_2), *K* 7 pg of the *uidA* DNA fragment

Table 1 Histochemical GUS activity in F_1, F_2 and BC_1F_1 seeds

Cross[a]	GUS+	GUS–	Observed Total	Observed Ratio	Expected Ratio	χ^2	P
1) FD/A1-F_1	66	0	66				
2) A1/FD-F_1	90	0	90				
3) FD/A6-F_1	96	0	96				
4)A6/FD-F_1	110	0	110				
5) FD/A1-F_2	727	33	760	22:1	15:1	4.72	0.03
6) A1/FD-F_2	719	44	763	16.3:1	15:1	0.31	0.58
7) FD//FD/A1-F_1	257	111	368	2.3:1	1:1	57.9	0.0
					3:1	5.2	0.02
8) A1//FD/A1-F_1	241	33	274				
9) FD/A6-F_2	788	76	864	10.4:1	15:1	9.56	0.002
10) A6/FD-F_2	783	81	864	9.6:1	15:1	14.4	0.0001
11) FD//FD/A6-F_1	92	84	176	1.2:1	1:1	0.36	0.55
					3:1	48.5	0.0
12) A6//FD/A6-F_1	220	0	220				
13) A1-T_4	12	0	12				
14) A1-T_5	360	0	360				
15) A6-T_4	16	0	16				
16) A6-T_5	192	0	192				
17) FD	0	192	192				

[a] Transgenic lines used for reciprocal crosses (nos 1–4) were T_4 seeds of A1 (no.13) and A6 (no.15). In BC_1F_1 (crosses 7, 8, 11 and 12), 'Fielder' (FD) and T_5 transgenic lines (A1 or A6) were used as the seed source, whereas the F_1s (FD/A1 or FD/A6) were used as the pollen source

the F_1 seeds of the reciprocal crosses were GUS-positive, as expected, and showed no difference in GUS activity levels, suggesting a lack of cytoplasmic effect on inheritance of the transgene (Table 1, nos. 1–4). The F_1 seeds from reciprocal crosses were planted to generate F_2 seeds for further analysis. The F_2 seeds of A1/FD (T_4 crossed with untransformed 'Fielder') showed a 16.3:1 segregation ratio, which fits a two-gene insertion model (15:1) for unlinked loci (Table 1). For the reciprocal cross FD/A1, the expected 15:1 ratio was not obtained, but the observed ratio of 22:1 was closer to that of a two-locus inheritance than to either a single (3:1) or a three-locus (63:1) inheritance. The segregation data obtained for FD//FD/A1 (2.3:1) did not fit either a single-locus or a two-locus inheritance.

The segregation analysis performed with the A6 transgenic line revealed a complex inheritance pattern. The segregation data for F_2 seeds of the FD/A6 reciprocal cross did not fit a two-locus inheritance model due to an excess of GUS-negative segregants (Table 1). For the F_1 seeds of A6//FD/A6 (T_5 crossed with F_1) all 220 seeds were GUS-positive. The F_1 seeds of FD//FD/A6 (untransformed 'Fielder' crossed with F_1) exhibited a 1:1 segregation ratio, fitting a one-locus inheritance. This was in contrast to the F_2 ratios of FD/A6, which did not fit a one-locus model. Our data showed that inheritance of the transgene in some of the lines was unpredictable based on the assay of GUS activity.

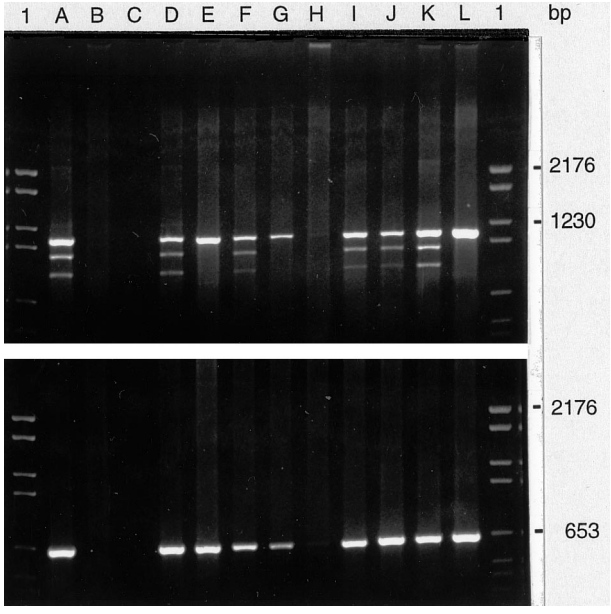

Fig. 2 PCR amplification of representative samples: *upper* ACT1 and GUS9 primers (a 1.1-kb DNA fragment is amplified), *lower* PSGA and PSGB primers, (a 0.64-kb DNA fragment is amplified. *1* DNA marker, *A* T_1, *B* 'Fielder' (negative control), *C* no DNA added to the PCR reaction, *D* R5 (A1), *E* T_5 (A6), *F* F_1 (A1/FD), *G* F_1 (A6/FD), *H* F_2 (A1/FD), *I* F_2 (A1/FD), *J*, *K* F_2 (A1//FD/A1), *L* F_2 (A6/FD). Note that *J* and *K* produced the expected DNA fragments but that the DNA is methylated as shown in **Fig. 3**, *lanes D* and *E*

Low GUS activity was associated with DNA methylation status

All seeds derived from the A1//FD/A1 cross were expected to be GUS-positive, however 33 of the 274 seeds did not exhibit GUS activity when tested by histochemical GUS assays (Table 1, no. 8). To investigate if the plants carried the transgene expression cassette, we analyzed DNA from 27 of the 33 GUS-negative seeds by PCR, using the primer pair ACT1 and GUS9 that recognizes sequences within the *Act1* promoter and *uidA*, respectively. The PCR reactions produced a 1.1 kb DNA fragment for 26 of the 27 DNA samples (Fig. 2, upper

part), which strongly suggested that the *Act1-uidA* portion of the expression cassette was intact. With the *nptII* primers, PSGA and PSGB, a 0.64-kb DNA fragment was obtained in all 27 DNA samples, confirming the presence of *nptII* (Fig. 2, lower part). Thus, the PCR analysis data suggested that the GUS-negative plants carried an intact *Act1D-uidA::nptII* expression cassette. Furthermore, leaf samples from some of these GUS-positive (A1//FD/A1) plants exhibited very low NPTII and GUS enzyme activities. F_2 seeds were generated from these F_1 seeds with low GUS activity. There was very little GUS activity in F_2 seeds derived from the weakly GUS-positive F_1 seeds (Table 2). For some of the plants, all 80 seeds tested from weakly GUS-positive half-seeds showed no histochemical GUS-activity.

Detailed molecular analysis was carried out on 11 transgenic wheat plants including the control (Table 3). The T_1 plant had the highest GUS and NPTII enzyme activities followed by T_5 plants. In contrast, the progeny of A6 plants maintained high NPTII and GUS enzyme activities (Table 3, H, I, J and K). The F_2 plants of A1//FD/A1 (Table 3, F and G) had low GUS and NPTII enzyme activities, even though they still carried the *nptII* and *uidA* genes as confirmed by Southern blot hybridization and PCR analyses (Figs. 1 and 2).

To study whether enzymatic activity level encoded by the transgene was associated with *uidA::nptII* methylation, we analyzed plants with high and low levels of GUS and NPTII enzyme activities by Southern blot hybridization. For this analysis, genomic DNA was digested with *HindIII* in combination with one of the methylation-sensitive enzymes, *HpaII* or *MspI*. *HpaII* and *MspI* are isoschizomers that recognize and cleave DNA at the unmethylated CCGG sites but differ in their recognition of the methylated CCGG sites: *HpaII* activity is inhibited by the methylation of either of the two C, whereas MspI activity is inhibited only when the outer C is methylated. The digested DNA was analyzed by Southern blot hybridization using a *uidA* fragment as the probe. Analysis of the transgenic lines (A1//FD/A1, GUS-negative plants) with low GUS and NPTII enzyme activities showed hybridization to DNA fragments of higher molecular weights as compared to DNA from plants with high GUS and NPTII enzyme activities (Fig. 3, lanes D and E). The same hybridization pattern was observed when the methylation-sensitive enzyme, *MspI* was used to digest the DNA (data not shown). The F_1 and F_2 DNA samples from line A6 (producing relatively high levels of GUS activity) did not reveal any methylation of the transgene (Fig. 3, lanes G, H, I and J). Our data showed that the plants with reduced GUS activity contained

Table 2 Examples of variation in histochemical GUS activity results from F_2 seeds of A1//FD/A1

Plant no.	F_1 seeds[a]	F_1-derived F_2 seeds[b] Number of seeds	
	$GUS^{+/-}$	GUS^+	GUS^-
3A	+	12	68
	+	56	24
	–	0	80
9A	+	0	80
	+	0	80
	–	0	80

[a] Histochemical GUS activity in nonembryo parts of half-seeds derived from A1//FD/A1.
[b] GUS activity in F_2 seeds derived from embryo portions of F_1 seeds of A1//FD/A1

Table3 DNA and enzyme analyses of representative transgenic wheat lines (*ND* not determined)

Transgenic lines	Histochemical GUS staining[a] (seeds)	PCR[b] (leaves)	Southern[c] (leaves)	NPTII activity[d] (leaves)	GUS activity[e] (leaves)	GUS activity[f] (pollen)
A) T_1	+	+	+	+++	++++	++
B) Fielder	–	–	–	–	–	–
C). T_5 (A1)	+	+	+	+	++	+
D) F_1 (A1/FD)	+	+	+	++	++	++
E) F_2 (A1/FD)	–	-	–	–	–	–
F) F_2 (A1//FD/A1)	+	+	+	–	+	–
G) F_2 (A1//FD/A1)	+	+	+	–	–	–
H) T_5 (A6)	+	+	+	++	+++	++
I) F_1 (A6/FD)	+	+	+	++	++	++
J) F_2 (A6/FD)	–	+	+	–	++	+
K) F_2 (A6/FD)	+	+	+	ND	ND	++

[a] Half-seed without the embryo portion was incubated with X-Gluc, and the embryo portion of the half-seed was planted to generate leaves and seeds for PCR, enzyme activity, pollen staining and Southern analyses. Transgenic line E does not carry the *uidA*::*nptII* fusion transgene, whereas transgenic lines F and G have methylated DNA
[b] The two primer pairs (PSGA and PSGB and ACT1 and GUS9) gave identical DNA amplification
[c] Genomic DNA was digested with *Hin*dIII and *Hpa*II and probed with the *uidA* fragment
[d] NPTII activity was assayed by dot blot procedure: +++=high, ++=medium, +=low, –=background NPTII activity, respectively
[e] Activity, is expressed in picomole MU h^{-1} mg^{-1} of GUS activity: ++++=>8000, +++=>4000, ++=500–1000, +=5–100, –=0. Leaf tissues were analyzed 5 weeks after planting, and analysis of GUS activity was carried out on duplicate samples
[f] Pollen staining with X-Gluc: ++=dark blue, +=light and dark blue, –=no staining

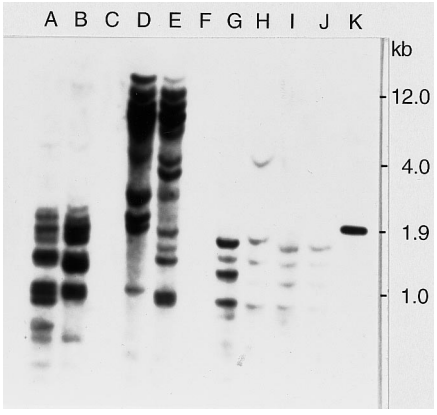

Fig. 3 Analysis of DNA methylation pattern in transgenic wheat lines. Genomic DNA (15 µg) was double-digested with *Hin*dIII and *Hpa*II and probed with a radiolabeled *uidA* fragment. *A* T_1 (A1), *B* F_1 (A1), *C* 'Fielder' (negative control), *D*, *E* F_2 (A1//FD/A1), **F** empty lane, *G* T_5 (A6/FD), *H* F_1 (A6/FD), *I* F_2 (A6/FD), *J* $_2$ (A6/FD), *K* 10 pg *uidA* DNA fragment

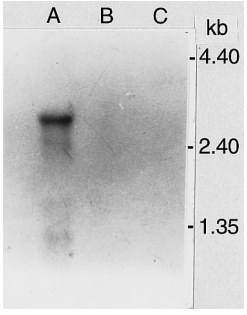

Fig. 4 Effect of transgene methylation on gene expression. Five micrograms RNA fractionated on 1.5% agarose gel and transferred to nylon membrane was hybridized to a 640-bp *nptII* DNA fragment using a non-radioactive method. *A* RNA from nonmethylated T_1 plant, *B* RNA from control plant ('Fielder'), *C* RNA from methylated F_2 plant (A1//FD/A1)

higher level of methylated CCGG sites than plants with high GUS activity.

Plants with the methylated transgene lacked *uidA::nptII* transcripts

Northern blot analysis was performed to study if transgene methylation could be correlated with the expression level of the transgene. The RNA analysis of a T_1 plant, which was GUS-positive and indicated no transgene methylation, revealed hybridization to a 3.0-kb transcript. The length of the hybridized RNA corresponded to the size of the expected transcript from the *uidA::nptII* fusion transgene. On the other hand, analysis of RNA from transgenic plants with the methylated transgene did not show any hybridization signal (Fig. 4). These data suggested that DNA methylation of the inserted *uidA::nptII* results in reduced accumulation of the *uidA::nptII* transcripts.

Discussion

The success of plant transformation for production of new cultivars depends on stable and predictable inheritance of the inserted gene. In this study, the inheritance and stability of an *Act1D-uidA::nptII* expression cassette was studied in T_4 and T_5 transgenic plants of the spring wheat cultivar, 'Fielder'. There was stable inheritance of the transgene without any cytoplasmic effect based on histochemical detection of GUS activity in F_1 seeds. However, based on GUS activity assays, a clear Mendelian genetic ratio was not obtained for all crosses, even though there was a tendency towards a two-locus inheritance pattern. Studies on wheat (Kluth et al. 1998; Müller et al. 1996; Srivastava et al. 1996), oat (Pawlowski et al. 1998) and petunia (Ulian et al. 1996) have also shown a non-Mendelian inheritance of transgenes for some of the transgenic lines. The causes for the unpredicted inheritance of transgenes are not well understood (Finnegan et al. 1998; Senior 1998), but gene silencing or gene instability may play a significant role. In oat, it was suggested that the large genome combined with a small percentage of highly expressed sequences favors insertion of transgenes into silent or low-expressed genomic regions which are susceptible to chromatin condensation (Pawlowski et al. 1998). *Arabidopsis* triploid plants obtained from a diploid by tetraploid cross showed reduced transgene expression as compared with diploid plants, implying that an increase in ploidy number can result in epigenetic gene silencing (Mittelsten-Scheid et al. 1996). Thus, the complex wheat genome (2n=6x=42) may be relatively susceptible to epigenetic gene silencing.

Gene silencing is a common phenomenon in transgenic plants (Stam et al. 1997). The two kinds of gene silencing include (1) transcriptional gene inactivation, as a result of promoter in-operation, and (2) post-transcriptional gene inactivation that occurs when produced mRNA fails to accumulate or encode a product. It was recently shown in tobacco co-suppression studies that a sequence-specific gene inactivation signal can be transmitted by grafting non-silenced scions onto silenced rootstocks (Palauqui and Vaucheret 1998). Crossing of low-*uidA*-expressing tobacco plants with homozygous high-*uidA*-expressing plants resulted in low GUS activity in F_1 and F_2 plants with both types of inserts (Hobbs et al. 1993), indicating suppression of the *uidA* expression by the low-expressing insert. These data indicate that once silencing is triggered it can be transmitted to other parts of the same plant or to the next generation.

A very low enzyme activity (histochemical GUS, GUS-fluorometric and NPTII assays) encoded by the transgene was detected in one of our crosses (A1//FD/A1). This loss of transgene activity did not seem to be due to the loss of the inserted expression cassette as judged by our Southern and PCR analyses. Southern blot analysis performed with the methylation-sensitive enzyme, *Hpa*II, showed that the transgene in GUS-negative plants was highly methylated relative to the transgene in GUS-positive plants. Several previous

reports including data on clonal cell lines and plants have demonstrated a relationship between the methylation status of a transgene and gene silencing (Müller et al. 1996; Srivastava et al. 1996). Some of these studies have suggested that a multiple integration pattern and copy number is also associated with DNA methylation (Hobbs et al. 1993; Müller et al. 1996). However, gene silencing has also been observed in the case of single-copy insertion (Elmayan and Vaucheret 1996). In our study, multiple integrations of the transgene were observed, which could have triggered transgene methylation causing gene silencing and distortion of segregation ratios. However, progeny derived from 1 of the transgenic plants (A6) did not show DNA methylation. A selection from field-grown A6 plants had consistently shown high GUS-activity (Hucl et al. 1998).

DNA methylation plays a pivotal role in establishing and maintaining an inactive state of the gene by rendering the chromatin structure inaccessible to the transcription machinery (Razin 1998). Therefore, methylation of DNA is expected to result in reduced gene expression, as observed in our study. DNA methylation may interfere with transcription by preventing transcription factors from binding to regulatory regions (Kass et al. 1997). The other possibility is that specific repressors are able to recognize and bind to methylated residues and thereby turn off transcription either independently or together with other components (Kass et al. 1997).

The problem of gene silencing could be minimized by optimizing methods for simple integration patterns (Meyer 1995). Directing the integration of the transgenes towards transcribed regions may help in producing plants with simple integration patterns (Birch 1997). Efficient promoter and reporter gene constructs could also help in reducing transgene silencing, even though strong promoters in some cases may lead to silencing of the transgene they drive (Elmayan and Vaucheret 1996). Promoters and gene sequences isolated from cereals may also help in reducing the gene-silencing problem (Båga et al. 1999). The use of matrix-associated regions (MARs) or scaffold attachment regions (SARs) to insulate transgenes from the surrounding chromatin is reported to reduce variability in transgene expression (Mlynárová et al. 1996), but the impact of MARs on gene expression in cereals has not been studied so far. *Agrobacterium tumefaciens* has been reported to have been successfully used to genetically transform some cereals (Aldemita et al. 1996; Cheng et al. 1997; Ishida et al. 1996; Tingay et al. 1997). This technique may facilitate the incorporation of simple integration patterns as compared with those obtained using the particle bombardment method, thereby producing predictable segregation patterns and reducing gene silencing.

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