

Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integrations

Kirk E. Francis[†] and Steven Spiker^{*}

Department of Genetics, North Carolina State University, Raleigh, NC 27695-7614, USA

Received 16 April 2004; revised 2 November 2004; accepted 8 November 2004.

^{*}For correspondence (fax +919 515 3355; e-mail steven_spiker@ncsu.edu).

[†]Present address: Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

Summary

Several recent investigations of T-DNA integration sites in *Arabidopsis thaliana* have reported 'cold spots' of integration, especially near centromeric regions. These observations have contributed to the ongoing debate over whether T-DNA integration is random or occurs preferentially in transcriptionally active regions. When transgenic plants are identified by selecting or screening for transgenic activity, transformants with integrations into genomic regions that suppress transcription, such as heterochromatin, may not be identified. This phenomenon, which we call selection bias, may explain the perceived non-random distribution of T-DNA integration in previous studies. In order to investigate this possibility, we have characterized the sites of T-DNA integration in the genomes of transgenic plants identified by pooled polymerase chain reaction (PCR), a procedure that does not require expression of the transgene, and is therefore free of selection bias. Over 100 transgenic *Arabidopsis* plants were identified by PCR and compared with kanamycin-selected transformants from the same T₁ seed pool. A higher perceived transformation efficiency and a higher frequency of transgene silencing were observed in the PCR-identified lines. Together, the data suggest approximately 30% of transformation events may result in non-expressing transgenes that would preclude identification by selection. Genomic integration sites in PCR-identified lines were compared with those in existing T-DNA integration databases. In PCR-identified lines with silenced transgenes, the integration sites mapped to regions significantly underrepresented by T-DNA integrations in studies where transformants were identified by selection. The data presented here suggest that selection bias can account for at least some of the observed non-random integration of T-DNA into the *Arabidopsis* genome.

Keywords: transcriptional gene silencing, selection bias, plant transformation, T-DNA integration, matrix attachment region, selectable marker removal.

Introduction

Agrobacterium tumefaciens-mediated integration of T-DNA sequences into plant genomes has recently been exploited as an insertional mutagen in several plant species (Chen *et al.*, 2003; Krysan *et al.*, 1999). Insertional mutagenesis is very effective in *Arabidopsis thaliana*, and is responsible (in one case) for insertions in or near approximately 74% of the predicted *Arabidopsis* genes (Alonso *et al.*, 2003). These projects have provided an invaluable tool that has led to a better understanding of many plant genes. Projects to map the locations of T-DNA integrations by sequencing flanking genomic regions have provided a genome-wide view of

T-DNA integration (Alonso *et al.*, 2003; Ichikawa *et al.*, 2003; Pan *et al.*, 2003; Rosso *et al.*, 2003; Sessions *et al.*, 2002; Szabados *et al.*, 2002). These and other efforts have repeatedly demonstrated that identified sites of transgene integration via *Agrobacterium*-mediated transformation do not appear to be randomly distributed across the *Arabidopsis* genome. Specifically, fewer T-DNA integrations have been observed in centromeric, non-genic, and other regions than would be expected if integration were random (Alonso *et al.*, 2003; Brunaud *et al.*, 2002; Krysan *et al.*, 2002; Szabados *et al.*, 2002). This non-random distribution is frequently

suggested to be a consequence of a preference for T-DNA integration into genic regions or open chromatin (Barakat *et al.*, 2000). These suggestions are consistent with previous reports that T-DNA preferentially targets actively transcribed genes (Koncz *et al.*, 1989; Lindsey *et al.*, 1993; Topping *et al.*, 1991) and that non-genic or heterochromatic regions may physically prevent T-DNA integration (Herman *et al.*, 1990; Topping and Lindsey, 1995). These views are in contrast to reports that T-DNA integration is random (Azpiroz-Leehan and Feldmann, 1997; Fobert *et al.*, 1991; Forsbach *et al.*, 2003; Thomas *et al.*, 1994).

There are, in fact, two possible explanations for the observed non-randomness of mapped T-DNA integration sites. It is possible that genic regions are preferred sites for T-DNA integration (either through a preference for genic regions or an avoidance of non-genic or heterochromatic regions). However, it is also possible that T-DNA integration occurs randomly throughout the entire genome, but integrations into some regions are not recognized due to the repressive nature of the surrounding chromatin or nearby transcriptional regulators. If the selectable or screenable markers contained in the T-DNA are not expressed, those transgenic plants will never be identified. The result would be a pattern of identified integration sites with a bias for active chromatin regions. We refer to this phenomenon as selection bias and believe that selection bias is at least partially responsible for the perceived non-randomness of T-DNA integration.

In addition to the genome-wide non-randomness discussed above, non-randomness can be manifested within individual genes. For example, Alonso *et al.* (2003) reported that T-DNA is preferentially targeted to 5' and 3' UTRs. Non-randomness at this scale is unlikely to be due to antibiotic selection bias (although other forms of selection may be involved), and we do not address this form of non-randomness in this paper.

In order to investigate genome-wide apparent non-randomness of T-DNA integrations, we have taken advantage of several recently developed transformation systems that do not depend on activity of selectable markers (Dominguez *et al.*, 2002; Permingeat *et al.*, 2003; de Vetten *et al.*, 2003). These systems use polymerase chain reaction (PCR) to identify transformants regenerating in the absence of selection and are therefore free from selection bias. It is interesting that some of these selection-free systems have reported a high occurrence of transcriptional gene silencing (TGS) (Dominguez *et al.*, 2002).

We have conducted a PCR-based screen to identify transgenic *Arabidopsis* seedlings based on the presence of integrated transgenes, not their activities. Our screen revealed that more transgenic seedlings are recovered when selectable marker expression is not a prerequisite for identification. We also demonstrate that PCR-identified lines are more likely to contain inactive or silenced transgenes

than kanamycin-selected lines. Many identified transgenic plants contain inactive transgenes that appear to have integrated into regions characterized by a low density of previously mapped T-DNA integrations. The data presented here suggest that many successful transformation events are not identified by conventional methods and that T-DNA integration may be more nearly random than previously thought.

Results

Significantly more transformants are identified by PCR screening than by kanamycin selection

Agrobacterium carrying the binary vector pBI121 (Jefferson *et al.*, 1987) was used to transform 20 T₀ plants by the floral dip procedure (Clough and Bent, 1998). Samples from a central pool of homogeneously mixed T₁ seed were plated on medium without kanamycin, and seedlings were grown for 10–14 days. PCR was used to screen DNA isolated from pooled samples for T-DNA sequences using primers A and B located in the interior of the T-DNA region (Figure 1). Preliminary screening indicated that a single transgenic leaf from a 10-day-old seedling could be easily detected in a pool of 10 untransformed leaves. Five separate screens were conducted on samples taken from the pooled T₁ seed. Each screen resulted in the identification of multiple transgenic plants (Table 1). False-positive results were uncommon and quickly identified in subsequent analysis. Efforts were taken to reduce the occurrence of false-negative results; however, it is possible that some transgenic seedlings were not identified, and therefore our PCR-based estimations of transformation are conservative.

In addition to PCR-screening, kanamycin selection was used to identify transformants from the same pool of T₁ seed. Estimated transformation efficiencies for PCR-identified and kanamycin-selected approaches are presented in Table 1. In total, 2959 seedlings were screened by PCR for the presence of T-DNA sequences, and 104 transgenic seedlings were identified (3.5% transformation efficiency). Three screens were conducted on seedlings that were plated on medium containing kanamycin at 50 mg l⁻¹. In total, 1895 seedlings were plated on kanamycin, and 47 kanamycin resistant seedlings were identified (2.5% transformation

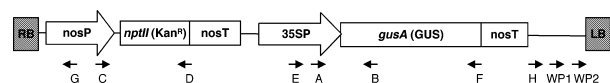


Figure 1. Map of pBI-121 T-DNA.

nptII is driven by the *nosP* promoter (*nosP*), which is located at the right border (RB). β -glucuronidase is driven by the 35S promoter (35SP) and is located internally. PCR primer sites and orientations are indicated by solid black arrows. Walking primers (WP1 and WP2) are located just inside the left border (LB). Regions are not drawn to scale.

Table 1 Transformation efficiencies based on method of identification

	PCR-identified			Kanamycin-selected			
	Screened ^a	PCR-positive ^b	Efficiency (%)		Screened ^a	Kanamycin-resistant ^c	Efficiency (%)
Screen 1	411	17	4.1	Screen A	843	19	2.3
Screen 2	783	27	3.4	Screen B	284	9	3.2
Screen 3	412	15	3.6	Screen C	768	19	2.5
Screen 4	612	12	2.0				
Screen 5	741	33	4.5				
Total	2959	104	3.5		1895	47	2.5

^aSeeds that failed to germinate and aborted seedlings were not considered.

^bBased on A/B amplification from individual seedlings.

^cBased on kanamycin tolerance at 2 weeks on kanamycin at 50 mg l⁻¹.

efficiency). As all screens were conducted on samples from the same homogeneously mixed pool of seed, any differences in the estimates of transformation efficiency should be the result of differences in the methods of detection. A statistical comparison of the methods used to identify transgenic seedlings using Fisher's exact test indicated that PCR screening identified significantly more transgenic seedlings than kanamycin selection ($P = 0.0250$).

Verification and analysis of T-DNA integration

In both PCR-identified and kanamycin-selected lines, full-length open reading frames for *nptII* and *gusA* were verified by PCR. Additionally, PCR was used to verify the presence of non-T-DNA sequences and to determine integration characteristics of some lines. Non-T-DNA pBI121 vector sequences were present in 69 of 102 PCR-identified lines (68%) and in 33 of 42 kanamycin-selected lines (79%). These frequencies are similar to those reported by others (Wenck *et al.*, 1997). Tandem integrations were present in 50 of 71 PCR-identified lines (70%) and in 43 of 63 kanamycin-selected lines (68%). Attempts to determine the presence of inverted T-DNA sequences around the left or right border by PCR were unsuccessful, probably due to self-competition (De Buck *et al.*, 1999). A chi-square test comparing the presence of tandem repeats and binary vector sequences in PCR-identified and kanamycin-selected lines was conducted. No significant difference was observed, suggesting that differences between the two identification methods are reflected by neither the presence of non-T-DNA vector sequences nor tandem T-DNA repeats. An analysis of the combined PCR-identified and kanamycin-selected data (not shown) revealed that the presence of tandem T-DNA repeats and vector sequences was not independent of one another ($P < 0.0001$). Lines containing tandem repeats were more likely to also contain non-T-DNA vector sequences.

It is possible that *Agrobacterium* from the floral dip of the T₀ plants could remain as a systemic contaminant in the T₁ plants. This could potentially result in false-positive

amplification by PCR for sequences present in pBI121. To minimize this possibility, all T₁ seedlings were grown in the presence of timentin at 200 mg ml⁻¹ to eradicate contaminants. It is still possible, however, that low levels of bacteria could remain in the plant tissues and result in false PCR results (Cubero *et al.*, 1999). To test for the presence of contaminating *Agrobacterium*, all DNA samples from T₁ plants were amplified by PCR with primers for endogenous *Agrobacterium VirG*. No plant genomic DNA samples ever amplified the *VirG* fragment, while the positive control preparation of plant tissue dipped in an overnight culture of *Agrobacterium* strain GV3101:pMP90 always amplified the *VirG* fragment.

A higher frequency of *nptII* silencing occurs in PCR-identified lines

Because standard kanamycin selection is carried out on germinating seedlings, it is impossible to directly determine if PCR-selected transformants (identified after 2 weeks) would have survived kanamycin selection. Thus, in order to gain an estimate of kanamycin resistance of the T₁ plants, T₂ seedlings from PCR-identified lines were assayed for kanamycin tolerance and compared with those of kanamycin-selected lines. Between 18 and 72 sterilized T₂ seed from all T₁ plants that produced seed were plated on medium containing kanamycin at 50 mg l⁻¹. At 2 weeks, seedlings were scored by comparing the number of kanamycin-tolerant seedlings to the total number of germinated seedlings for that line. In general, germination frequencies were highly consistent (average = 94.2%); however, three lines (two PCR-identified lines and one kanamycin-selected line) had germination frequencies lower than 80%. Low T₂ germination frequencies could be an indicator of integration into essential genes. Because such lines would not be expected to conform to a 3:1 segregation ratio for kanamycin resistance, they were not included in subsequent analysis. The segregation ratios for kanamycin tolerance of the remaining 89 PCR-identified lines and 42 kanamycin-selected lines

were tested against the expected 3:1 ratio by chi-square goodness of fit tests. Lines with significantly fewer kanamycin-tolerant seedlings than expected were considered to be experiencing some degree of silencing of the *nptII* transgene and are referred to as '*nptII*-silencing' lines throughout this study. All other lines are considered to be '*nptII*-expressing.' One-third of the PCR-identified lines (30 lines, 34%) displayed *nptII* silencing, while only around one-tenth of the kanamycin-selected lines (five lines, 12%) displayed *nptII* silencing. The proportions of the PCR-identified and kanamycin-selected lines that failed the chi-square test were compared with each other using Fisher's exact test. A significantly higher proportion of the PCR-identified lines were found to deviate from the expected ratio than the kanamycin-selected lines ($P = 0.0046$). As the *nptII* genes are unaltered, we attribute the absence of kanamycin tolerance to *nptII* silencing. An alternative explanation is that the T_1 plants are chimeric, which would result in non-Mendelian segregation of the *nptII* transgene. However, PCR screening of individual T_2 seedlings, demonstrated normal Mendelian segregation of the T-DNA (Table S2, Supplementary Material).

In addition to the difference in the frequency of silencing between PCR-identified and kanamycin-selected lines, the distribution of kanamycin tolerance frequencies within each group is strikingly different. Figure 2 shows the histograms of the frequencies of kanamycin tolerance in PCR-identified and kanamycin-selected lines. As expected, the distribution of kanamycin tolerance frequencies within the kanamycin-selected lines indicates that the vast majority of the lines have frequencies of 0.75 or higher, a few lines are slightly lower than 0.75, and a single line has a very low kanamycin tolerance frequency. Two or more unlinked copies of *nptII* could account for frequencies of higher than 0.75. The distribution of kanamycin tolerance frequencies in the PCR-identified lines is very different. The distribution is clearly bimodal. The upper mode is largely composed of lines with frequencies over 0.75, while the lower mode is heavily skewed towards zero (Figure 2b). If the lines in the lower mode (frequencies below 0.40) of the PCR-identified distribution are excluded from analysis, only 10 of the remaining 69 lines (14%) have significantly fewer kanamycin-tolerant seedlings than expected. This is not significantly different from the number of kanamycin-selected lines failing the chi-square test (12%). Collectively, these data suggest that the PCR-identified lines contain a subset of kanamycin-sensitive lines, represented by the lower mode of Figure 2(b), which are absent from the kanamycin-selected population. Quantitative measurements of *nptII* expression were conducted on a sample of lines using ELISA. The results from ELISA on pools of 2-week-old T_2 seedlings (data not shown) paralleled the results obtained by assessing kanamycin tolerance on selective medium.

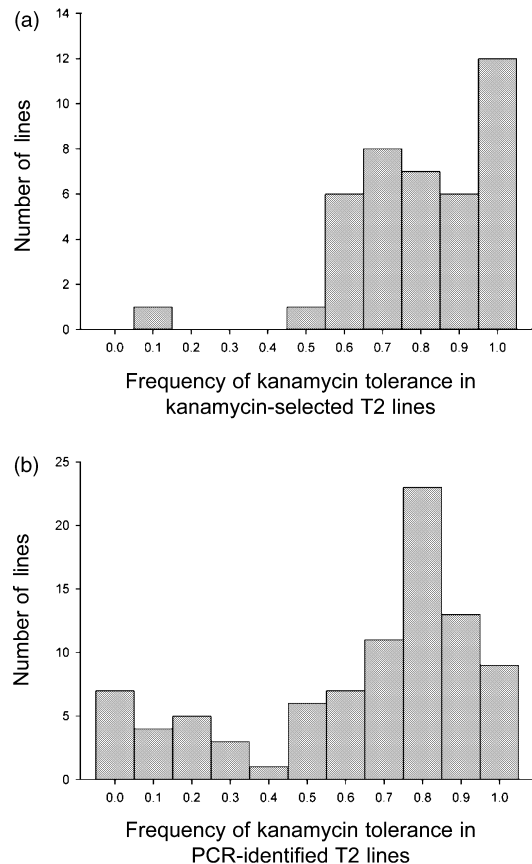


Figure 2. Kanamycin tolerance in kanamycin-selected and PCR-identified lines.

Histograms for kanamycin tolerance in 2-week-old T_2 seedlings are presented for 42 kanamycin-selected (a) and 89 PCR-identified (b) lines. In the absence of silencing, the expected frequency for a single-locus line is 0.75. Low frequencies are indicative of silencing and high frequencies are indicative of multiple loci. PCR-identified lines are bimodally distributed with frequencies lower than 0.40 belonging to the lower mode.

Correlation of *nptII* and *gusA* expression

Although we are testing for selection bias and are therefore primarily interested in the expression of *nptII*, we have also assayed the expression of GUS in PCR-identified and kanamycin-selected lines. Evidence in the literature indicates that expression of linked transgenes may be disparate (Mlynarova *et al.*, 2002; Peach and Velten, 1991). Nevertheless, GUS expression in T_1 plants (as assayed by histochemical staining, Jefferson *et al.*, 1987), was in general agreement with *nptII* expression in the T_2 generation. At approximately 4 weeks post-germination, leaf tips from seedlings were sampled for GUS by histochemical X-gluc staining. A qualitative assessment of strong, weak, or no staining determined the staining intensity of each seedling. All kanamycin-selected transformants demonstrated strong histochemical staining, while only 73% of PCR-identified transformants stained at this intensity (7% had weak staining and 20% had

no staining). The absence of kanamycin-selected samples with weak or no staining is significant ($P < 0.0001$). To quantitatively measure GUS activity, MUG assays (Jefferson *et al.*, 1987) adapted to a 96-well plate reader (see Supplementary Material) were conducted on PCR-identified and kanamycin-selected T_2 seedlings. Pools of 10 T_2 seedlings germinated on SG medium without kanamycin were harvested at 14 days post-germination and used for quantitative measurements of GUS activity (Figure 3). There was no significant difference in levels of GUS activity between PCR-identified and kanamycin-selected T_2 lines. However, *nptII*-silencing PCR-identified lines had significantly lower levels of GUS activity (average = 70.0 pmol MU min⁻¹ mg⁻¹) than *nptII*-expressing PCR-identified lines (average = 134.6 pmol MU min⁻¹ mg⁻¹) ($P = 0.0073$) (see Table S3, Supplementary Material).

Analysis of genomic integration

Identification of sites of integration. Using a ligation-mediated PCR approach initially reported by Siebert *et al.* (1995) and modified by Alonso *et al.* (2003), the genomic sites of T-DNA integration in several of the transgenic lines were identified. Candidate lines for sequencing were determined based on *nptII* activity in T_2 seedlings. Approximately equal numbers of PCR-identified *nptII*-expressing and PCR-identified *nptII*-silencing lines were chosen for analysis. Some kanamycin-selected lines were also analyzed, but because very large numbers would be needed to detect differences between all kanamycin-selected lines and all PCR-identified lines, we focused on detecting differences between PCR-identified lines with differences in kanamycin tolerance. Due to the nature of the ligation-mediated PCR approach used (Siebert *et al.*, 1995), attempts to identify flanking genomic sequences from some lines failed. All

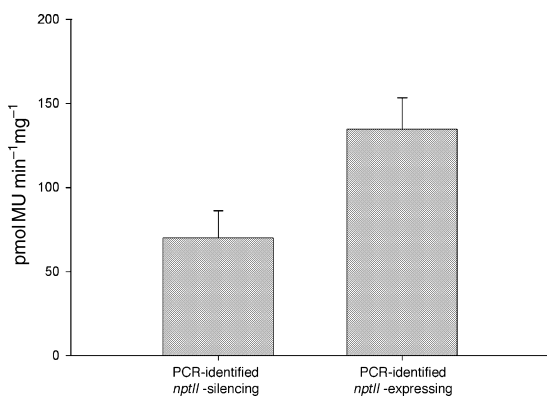


Figure 3. GUS activities in T_2 seedlings.

GUS activities based on MUG assays of pools of 10 T_2 seedlings at 14 days post-germination from 31 PCR-identified *nptII*-silencing lines and 53 PCR-identified *nptII*-expressing lines are presented. Lines with *nptII*-silencing have significantly less GUS activity than *nptII*-expressing lines ($P = 0.0073$). Error bars indicate standard errors.

procedures commonly used to identify genomic sequences flanking T-DNA integration sites have inherent biases, which may exclude the identification of integrations in some lines due to the nature of the integration locus (Cottage *et al.*, 2001; Hui *et al.*, 1998). Possible causes of failure include the absence of appropriate restriction enzyme sites in close proximity to the integration site and the presence of flanking sequences not amenable to PCR amplification. Because our intentions were to compare our integrations with those of the SIGnAL project (Alonso *et al.*, 2003), it was critical that we used an identical approach to mapping our integrations. All flanking sequences were analyzed for homology by BLAST comparison against Arabidopsis sequences in the SIGnAL database (<http://signal.salk.edu>) and all existing sequences in Genbank (<http://www.ncbi.nlm.nih.gov/blast/>) (see Table S1 Supplementary Material).

The distribution of T-DNA inserts encompassed all five chromosomes of Arabidopsis for both *nptII*-silencing and *nptII*-expressing PCR-identified lines. Two *nptII*-silencing lines contain T-DNA integrations in known heterochromatic regions. Line S3-GC3 contains an integration site in the Arabidopsis 25S rDNA repeat region. The 25S rDNA regions in Arabidopsis are found exclusively in large repeats contained in the nuclear organizing regions (NOR2 and NOR4) adjacent to the telomeres of chromosomes II and IV (Copenhaver and Pikaard, 1996). It can then be concluded that this line contains a T-DNA integration in a NOR, although further mapping analyses would be necessary to determine whether the integration occurred into NOR2 or NOR4. Line S7-BB1 contains a T-DNA integration in the gene At4g05150 located near the centromere of chromosome IV. In total, two of 14 (14%) mapped *nptII*-silencing lines have inserts in known heterochromatic regions. No mapped integrations in *nptII*-expressing lines identified in this study appear to have occurred in similar regions.

All mapped integrations were analyzed for T-DNA left border (LB) truncation. Large LB truncations can occur frequently during T-DNA integrations (Tinland, 1996), although truncation may be less severe than previously thought (Brunaud *et al.*, 2002; Forsbach *et al.*, 2003). In general, we found truncation was common, but limited to fewer than 25 bp in most lines. No difference was observed in the frequency or degree of truncation between *nptII*-expressing and *nptII*-silencing lines.

Comparison to SIGnAL integration profiles. Using the T-DNA integration data reported in the supplemental material of Alonso *et al.* (2003), a sliding window analysis was conducted across the entire Arabidopsis genome for the number of SIGnAL inserts within 5 kb windows at 1 kb steps. It was then possible to graphically represent the SIGnAL integration densities around the sites of our PCR-identified T-DNA integrations. Integration profiles of two

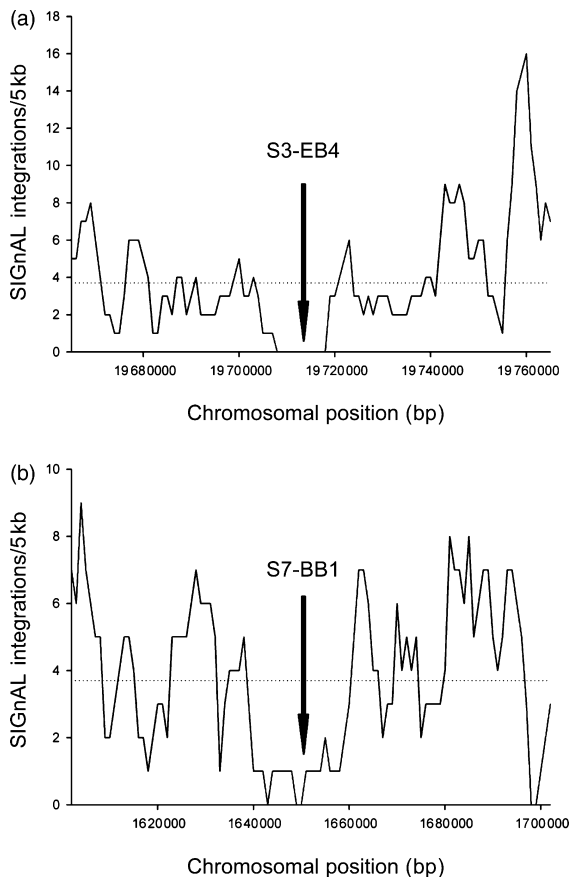


Figure 4. Profiles of SIGnAL project integrations surrounding two PCR-identified integrations. Bold arrows indicate the position of integration of PCR-identified lines S3-EB4 in chromosome 5 (panel a) and S7-BB1 in chromosome 4 (panel b). Surrounding these sites, the profiles of integrations are shown as the number of SIGnAL project (Alonso *et al.*, 2003) integrations within a 5-kb window with incremental steps of 1 kb. The dotted line shows the average number of SIGnAL integrations per 5 kb across the 100 kb regions.

PCR-identified lines are represented in Figure 4. We find it interesting that the majority of low or non-expressing PCR-identified lines (such as S3-BB1 and S3-EB4) integrated into regions underrepresented by T-DNA integrations in the SIGnAL project. In contrast, the majority of expressing PCR-identified lines and kanamycin-selected lines integrated into regions with either moderate or high numbers of SIGnAL integrations.

T-DNA integration profiles similar to those shown for two transgenes in Figure 4 have been constructed for all the mapped PCR-identified lines. For each line, the number of SIGnAL project T-DNA integrations in 1 kb windows was determined for a 100 kb region centered on the point of integration. The number of integrations in each window is averaged for 13 *nptII*-silencing lines in the lower mode in Figure 2(b) (lines that appear to represent a population of lines with severe silencing), and plotted in Figure 5(a). In Figure 5(b), the averages for 12 *nptII*-expressing lines are plotted. A comparison of these two profiles reveals the presence of a clear decrease in the average number of SIGnAL integrations within a few kb of the integration sites of *nptII*-silencing lines. No such decrease is observed for the *nptII*-expressing lines. Furthermore, the average number of SIGnAL integrations is lower for the *nptII*-silencing lines than for the *nptII*-expressing lines (dashed lines in Figure 5a,b).

Fewer SIGnAL integrations are observed in regions where *nptII*-silenced PCR-identified lines have integrated. The number of SIGnAL integrations within 5, 10, 100, and 200 kb centered on the integration sites of each mapped PCR-identified line is presented in Table 2 along with the T_2 segregation frequencies for kanamycin resistance. The average numbers of integrations for all of the *nptII*-silencing PCR-identified lines, and for the subset of those lines that compose the lower mode in Figure 2(b) are presented in

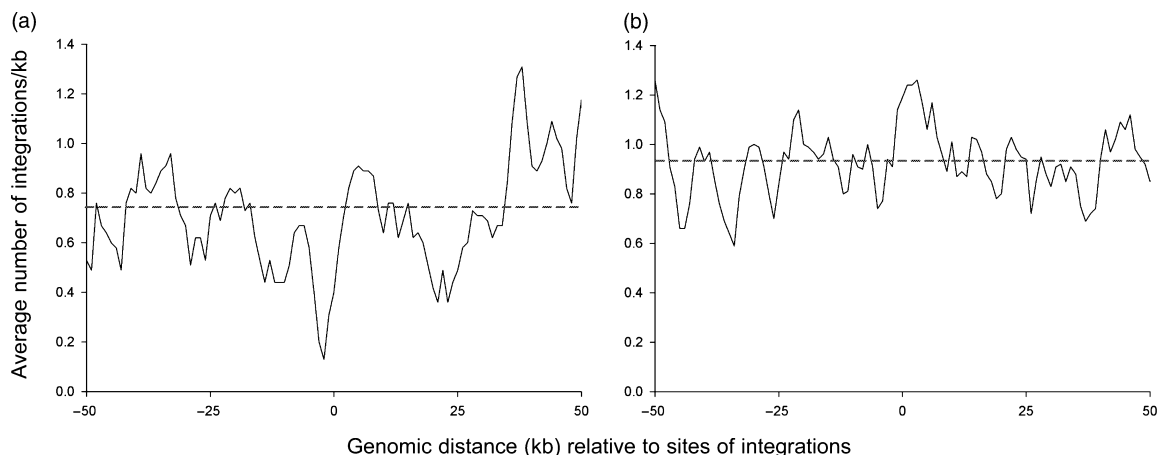


Figure 5. Average profiles of SIGnAL project integrations surrounding integrations of *nptII*-silencing and expressing lines identified by PCR. Profiles of SIGnAL project integrations (Alonso *et al.*, 2003) surrounding 13 *nptII*-silencing PCR-identified lines (panel a) and 12 *nptII*-expressing PCR-identified lines (panel b) are presented as the average number of integrations in 1 kb windows. These profiles are plotted at positions relative to the sites of integration (0 on x-axis) in PCR-identified transformants. Dashed lines indicate the average number of integrations across the entire 100 kb window.

Line	T ₂ Kan. Res.	SIGnAL integrations within window ^h				Nearest MAR ^d (kb)
		5 kb	10 kb	100 kb	200 kb	
S3-EB4	0.00 ^{a,b}	0	0	79	163	None
S7-OE1	0.00 ^{a,b}	1	7	107	205	None
S7-BB1	0.00 ^{a,b}	1	3	68	142	1.8
S7-LA6	0.00 ^{a,b}	2	4	43	94	1.0
S4-WE2	0.05 ^{a,b}	1	1	77	153	None
S7-LD1	0.08 ^{a,b}	2	4	84	172	1.1
S7-JD6	0.13 ^{a,b}	3	9	57	96	3.4
S7-HB5	0.14 ^{a,b}	6	9	110	216	1.1
S7-KB2	0.25 ^{a,b}	1	1	63	139	None
S7-BD5	0.30 ^{a,b}	1	2	55	79	None
Average (integrations/ kb) ^e		1.8 ^c (0.36)	4.0 ^c (0.40)	74 ^c (0.74)	146 ^c (0.73)	
S4-QB5	0.44 ^a	7	16	92	166	4.4
S4-SA3	0.48 ^a	5	10	99	211	None
S1-CG6	0.49 ^a	7	10	86	185	1.7
S3-GC3	0.53 ^a	–	–	–	–	na
Average (integrations/ kb) ^f		2.9 ^c (0.57)	5.8 ^c (0.58)	78 ^c (0.78)	155 ^c (0.78)	
S3-CC5	0.64	4	5	93	168	3.9
S4-YC1	0.69	6	6	108	200	3.2
S7-FB5	0.69	13	16	115	192	None
S7-TE3	0.75	7	10	87	197	1.7
S3-AD1	0.75	2	3	105	218	None
S4-CE2	0.76	4	10	82	171	4.4
S7-IC1	0.76	7	20	na	na	None
S7-NA5	0.79	9	12	82	175	0.6
S4-GB6	0.94	5	16	87	159	None
S7-GA3	0.94	3	7	106	199	None
S7-AC5	0.95	5	5	58	128	2.2
S7-AC6	0.95	4	12	99	193	None
Average (integrations/ kb) ^g		5.8 (1.15)	10.2 (1.02)	93 (0.93)	181 (0.91)	

^aT₂ kanamycin resistance frequencies are significantly lower than the expected 3:1 ratio ($\alpha = 0.01$).

^b*nptII*-silencing lines represented in the lower mode of Figure 2b.

^cNumber of integrations is significantly lower than *nptII*-expressing lines ($\alpha = 0.05$).

^dPredicted MARs based on MAR-Wiz (<http://www.futuresoft.com>).

^eAverage integrations/kb of *nptII*-silencing lines in lower mode of Figure 2b.

^fAverage integrations/kb of all *nptII*-silencing lines.

^gAverage integrations/kb of *nptII*-expressing lines.

^hNumber of SIGnAL integrations reported within windows of varying sizes (kb) centered on T-DNA integration sites of PCR-identified lines.

na, not applicable.

Figure 6. For comparison, the average numbers of integrations for *nptII*-expressing PCR-identified lines are also presented. The average number of integrations in 5, 10, 100, and 200 kb windows centered on *nptII*-silencing lines is significantly lower than the number of integrations in *nptII*-expressing lines ($P = 0.0073$, 0.0206, 0.0365, and 0.0485, respectively, for each of the windows) (Table 2). No difference is observed when the window is enlarged to 500 kb (Figure 6). If only the lines with the most severe silencing (lower mode of Figure 2b) are compared with the *nptII*-expressing lines, the difference is even more pronounced

($P = 0.0014$, 0.0041, 0.0346, and 0.0378, respectively) (Table 2).

Analysis of predicted matrix attachment regions. It has been reported that transgenes frequently integrate in close proximity to matrix attachment regions (MARs) (Dietz *et al.*, 1994; Makarevitch *et al.*, 2003; Sawasaki *et al.*, 1998; Shimizu *et al.*, 2001). In some reports MARs near transgenes were identified by *in vitro* binding assays (Dietz *et al.*, 1994; Sawasaki *et al.*, 1998; Shimizu *et al.*, 2001), while in other reports 'candidate' MARs were identified by using MAR-Wiz

Table 2 Summary of SIGnAL integrations and MAR potentials centered on T-DNA integration sites for *nptII*-silencing and *nptII*-expressing lines

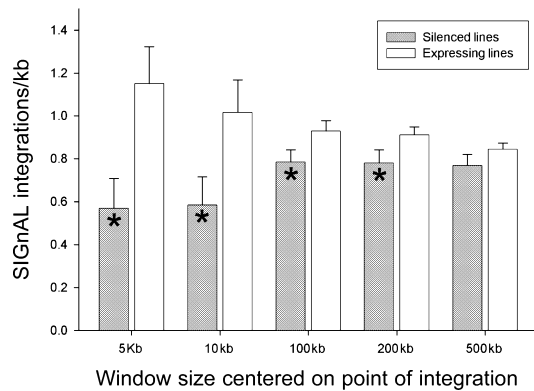


Figure 6. Comparison of average numbers of SIGnAL project integrations in regions surrounding *nptII*-silencing and *nptII*-expressing PCR-identified lines. Bars represent the average number of SIGnAL project (Alonso *et al.*, 2003) integrations in 5, 10, 100, 200, and 500 kb windows centered on integration sites of PCR-identified lines. Lines with signs of *nptII* silencing are represented with gray bars, and lines with expected patterns and levels of *nptII* expression are represented with white bars. Error bars represent the standard error of the mean. Asterisks indicate significant differences at the $\alpha = 0.05$ level.

(<http://www.futuresoft.org/MAR-Wiz/>) (Makarevitch *et al.*, 2003; Qin *et al.*, 2003) or other approaches based on sequence motifs and patterns commonly found in known MARs (Takano *et al.*, 1997). If MARs increase the level of expression of nearby transgenes as has often been suggested (Allen *et al.*, 2000; Bode *et al.*, 2000), it is possible that selection bias may result in the preferential identification of transformants that have integrated in close proximity to MARs. The presence of MAR candidates within a 10 kb region centered on the point of integration was estimated using MAR-Wiz (Table 2). Nearby MAR candidates were predicted for only about half of the sites analyzed, and the presence of MAR candidates was not correlated with the kanamycin tolerance of the lines examined. MARs may also facilitate transgene integration because of their A+T-richness, DNA-bending propensity, and presence of topoisomerase sites (Sawasaki *et al.*, 1998). For this reason, the presence of MAR candidates was also examined in regions of high SIGnAL T-DNA integration density. No relationship was observed (data not shown). Collectively, these data suggest that proximity to predicted endogenous MAR candidate sequences does not correlate with the integration sites of T-DNAs in transgenic plants identified by PCR-screening or by kanamycin selection.

Discussion

Differences in perceived transformation efficiencies

Significantly more transgenic *Arabidopsis* seedlings from the same pool of T₁ seeds were identified by PCR screening than by selecting for kanamycin resistance. If PCR identification represents a conservative estimate of the true

transformation efficiency, we can conclude that identification of transgenic seedlings by kanamycin selection fails to identify about 30% of all integration events in *Arabidopsis*. It is likely that kanamycin selection failed to identify some transformants because the selectable marker (*nptII*) was not expressed or expressed at a low level at the time of selection. Many possibilities exist as to why a transgene may not express. These can be summarized in three general categories: (i) mutations in the transgene, (ii) post-transcriptional gene silencing (PTGS), and (iii) chromatin-related TGS. Although we cannot rule out involvement of phenomena relating to any of these general categories, several lines of evidence indicate that TGS may be the cause of the lack of expression of the *nptII* transgene.

Mutations in the transgene. Of the various possible mutations that could result in transgene inactivity, truncation associated with T-DNA integration is the most likely. Many reports of T-DNA truncation exist in the literature (reviewed in Tinland, 1996). Truncations at the LB are more commonly reported, although right border truncations also occur (Gheysen *et al.*, 1990). Recently, several high-throughput approaches to examining T-DNA integration have reported that left and right border truncations are perhaps less common than previously thought (Brunaud *et al.*, 2002; Forsbach *et al.*, 2003). No truncation that would be expected to preclude transgene expression was observed in any of the lines described here. Point mutations and internal deletions are also possible, although rarely reported for *Agrobacterium*-mediated integration and more frequently associated with particle bombardment-mediated integration (Sawasaki *et al.*, 1998; Ülker *et al.*, 2002). Because at least some T₂ plants from nearly all of the PCR-identified lines showed kanamycin tolerance, it is unlikely that our data can be explained by mutations in the transgenes.

Post-transcriptional gene silencing. Complex transgene arrangements and high expression levels have been reported to result in PTGS in some cases (De Buck *et al.*, 2001; Elmayan and Vaucheret, 1996). We used PCR to detect direct repeats and inclusion of binary vector sequences in the transgenic lines. Examples of such complex integrations were found in several lines, but there was no difference in frequency between kanamycin-selected and PCR-identified lines. The presence of inverted repeats, more commonly thought to be associated with PTGS (Ma and Mitra, 2002), was determined for a sample of lines by DNA blot hybridization and found to occur at similar frequencies for PCR-identified and kanamycin-selected lines (data not shown). PTGS is significantly less active in very young and actively dividing tissues (Mitsuhara *et al.*, 2002). Because kanamycin selection of transgenic seedlings was performed within the first 10–14 days after germination, the possibility that PTGS played a major role in silencing *nptII* in the very young T₁

seedlings is diminished. Szittyá *et al.* (2003) reported that growing seedlings at 15°C appears to block or reduce the effects of PTGS. We grew T₂ seedlings at 15°C, and although a few lines demonstrated slight changes in kanamycin tolerance, the overwhelming majority of lines were not significantly different at 15 and 22°C (data not shown). Additionally, the PCR-identified lines continued to follow a bimodal distribution. Taken together, these data suggest that PTGS may not have been a major contributor to the perceived differences in transformation efficiencies when transformants are identified by kanamycin selection versus PCR screening.

Transcriptional gene silencing. The assays we have made under conditions that minimize PTGS and our studies on the sites of T-DNA integrations suggest that TGS accounts for the higher perceived transformation efficiency when PCR is used to identify transformants. That is, some transgenic lines may not be identified by kanamycin selection because the T-DNAs in those lines have integrated into genomic regions that repress transgene expression. Such repression of transgene expression by chromatin or nearby genomic elements has been characterized in many systems (for general reviews see van Holde, 1989; Wolffe, 1998) and may account for the higher percentage of transformants identified by PCR.

Silencing in PCR-identified lines

Polymerase chain reaction-identified lines are more likely than kanamycin-selected lines to demonstrate *nptII* silencing in the T₂ generation. Lines with T₂ kanamycin tolerance ratios significantly lower than the expected 3:1 ratio were considered to be *nptII*-silenced. Silencing of *nptII* was observed in 34% of the PCR-identified lines, while only 12% of kanamycin-selected lines had *nptII* silencing. The majority of PCR-identified lines with *nptII* silencing demonstrated severe silencing (lower mode in Figure 2b), while only one kanamycin-selected line demonstrated silencing to this degree. Furthermore, in seven of 89 PCR-identified lines analyzed, none of the T₂ seedlings showed kanamycin tolerance. No kanamycin-selected line demonstrated this degree of *nptII*-silencing. Although *in planta* transformation can result in chimeric transformation events (Bechtold *et al.*, 2003; Ye *et al.*, 1999), the absence of kanamycin tolerance in T₂ seedlings does not appear to be a consequence of distorted segregation patterns resulting from chimeric T₁ plants (which could yield wt T₂ seeds from untransformed sectors). Amplification of T-DNA regions from individual T₂ seedlings from a sample of *nptII* silencing lines indicated normal Mendelian segregation of the T-DNA (see Table S2, Supplementary Material). It is interesting that the 34% of PCR-identified lines with *nptII* silencing corresponds closely to the approximately 30% increase in transformation efficiency

when transformants were identified by PCR. Similar levels of silencing have been reported in Mexican lime plants identified without selection (Dominguez *et al.*, 2002).

Again, it is possible that T₂ silencing could be a result of PTGS; however, kanamycin tolerance was assayed in very young seedlings that may not be capable of PTGS (Mitsuhashi *et al.*, 2002). Furthermore, kanamycin tolerance for PCR-identified and kanamycin-selected T₂ seedlings was not significantly different at 15°C, a temperature reported to block PTGS (Szittyá *et al.*, 2003). PTGS can cause meiotically stable changes in transgene methylation that can result in a heritable loss of transgene activity (Wassenegger *et al.*, 1994). This phenomenon, called RNA-directed DNA methylation (Aufsatz *et al.*, 2002), may explain why some kanamycin-selected lines showed a partial loss of kanamycin resistance in the T₂ generation but cannot explain why more silencing was observed in PCR-identified lines. Taken together, these data suggest that PTGS may not have been a major contributor to silencing of *nptII* in PCR-identified T₂ seedlings. It is therefore likely that the silencing observed in the T₂ generation was largely a result of TGS. Although it seems unlikely, we cannot rule out the possibility that the small number PCR-identified lines with no indication of kanamycin tolerance in the T₂ generation could be the result of point mutations, rearrangements, or truncations not detected by the approaches used.

Differences in active and silent T-DNA integration loci

Genomic integration sites were identified in a sample of PCR-identified lines. Two lines with significant *nptII* silencing appeared to have integrated in genomic regions usually considered to have chromatin structures that would preclude transcription. Line S7-BB1 integrated into the pericentromeric region of chromosome IV, and line S3-GC3 integrated into rDNA repeats in either NOR2 or NOR4. Integration sites in the remaining lines were compared with over 100 000 integration sites reported in the supplemental material of Alonso *et al.* (2003) to determine whether any integrations identified without selection bias occurred in regions with few or no SIGnAL T-DNA integrations (referred to by Alonso *et al.* as 'cold spots'). A clear difference in the density of SIGnAL integrations at the sites of integration between PCR-identified *nptII*-expressing and *nptII*-silencing lines was seen. PCR-identified lines with *nptII* silencing integrated into regions with roughly half the number of SIGnAL integrations when compared with *nptII*-expressing lines (Table 2). Furthermore, several of the lines with complete or nearly complete *nptII* silencing had T-DNA integrations in regions either completely devoid of SIGnAL integrations or containing only a single integration within 5 and 10 kb windows around the integration locus. None of the *nptII*-expressing lines (either PCR-identified or kanamycin-selected) appeared to have integrated into one of

these integration 'cold spots.' An examination through the SIGnAL website of the genomic regions surrounding T-DNA integration sites in *nptII*-silencing lines reveals occasional integrations from other collections (e.g. GABI and FLAG) into integration 'cold spots.' Even when these integrations are taken into account, these regions are still underrepresented in T-DNA integrations. We do not know if these integrations were found because of differences in mapping approaches or if they truly represent differences in integration and/or recovery of transformation events. Further analyses and comparisons of different T-DNA insert collections could help resolve this issue.

It is possible that these regions are identified as integration 'cold spots' because they are either incapable of supporting transgene expression or are capable of supporting expression only at a level too low to facilitate identification by kanamycin selection. This may explain why PCR-identified lines that have integrated into these regions have little or no detectable transgene expression. The expression patterns of our *nptII*-silencing lines may be reflected in the endogenous genes surrounding the integration sites; however, we have no evidence at this time to suggest this is true.

We have clearly demonstrated that T-DNA insertions into some integration 'cold spots' are possible. It is therefore likely that the reason some genomic regions exist that contain very few or no T-DNA integrations is not because the physical structure of the surrounding chromatin physically blocks T-DNA integration. A more plausible explanation is that integrations into these regions can occur, but are rarely reported because the selectable markers contained within those T-DNAs are not expressed. It is important to emphasize, though, that simply because integration is possible in some integration 'cold spots,' there still may be some genomic regions incapable of receiving T-DNA integrations.

The silent second T-DNA model

An examination of the distribution of SIGnAL T-DNA integrations reveals numerous 5–10 kb regions that completely lack integrations. There are also many considerably larger regions that contain only a very few integrations. A complete absence of integrations could be explained by either the complete inability of T-DNAs to integrate into those regions or by the complete inability of those regions to support transgene expression. Regions where identified T-DNA integrations exist, but with low frequency, could be explained by the occurrence of multiple T-DNA integrations. *Agrobacterium*-mediated transformation often results in the insertion of multiple T-DNAs during a single transformation event (De Buck *et al.*, 1998). These multiple integrations can occur at a single locus or at multiple loci. Indeed, Alonso *et al.* (2003) found the average number of unlinked loci per line to be approximately 1.5. This number is very consistent with other studies (Feldmann, 1991; Ichikawa *et al.*, 2003; Rios

et al., 2002). In the data presented here, over 25% of the kanamycin-selected lines demonstrated T₂ segregation ratios that suggest the presence of multiple active loci. The presence of multiple independent integrations can play a role in the effect of selection bias. Under selective conditions, only one T-DNA integration is required to have occurred in a transcriptionally competent region. As the expression of all other independently integrated T-DNAs in that line are not required, they may be perceived as being free of selection bias. In order to identify sites of integration in a high-throughput manner, the methods used by Alonso *et al.* (and used here as well), involved the identification of a single integration site per line, even if that line contained multiple integrations. The mapping methods used do not distinguish between active and inactive T-DNAs. It is therefore possible that some of the integration sites identified by the SIGnAL project are integration sites of inactive T-DNAs and are thus free of selection bias. We refer to these integrations as 'silent second T-DNAs' and suggest that identification of integrations into silent chromatin may be possible only through these means when transformants are identified under selective conditions. Furthermore, some SIGnAL integrations may be located in regions that have a transcriptionally repressive chromatin structure at the time of selection, but are activated through changes in chromatin structure at a later developmental time (e.g. flowering). If selection bias precludes identification of transformants with T-DNA insertions in or near such genes, it may be impossible to obtain such insertion mutants except by 'silent second T-DNAs.'

It is well known in the Arabidopsis community, that it is not uncommon for some SIGnAL lines obtained from the Arabidopsis Biological Resource Center (ABRC) to have 'lost' kanamycin resistance (for comments see <http://signal.salk.edu>). While silencing of some previously active T-DNAs may have occurred, it is possible that some T-DNAs were never active because they integrated into genomic regions that repress expression. If these regions segregate from actively expressed T-DNA insertions, it would appear as though the transgenic line had retained the T-DNA insert but that the transgene had been silenced subsequent to selection.

Consequences of selection bias

Only a minor proportion of the small and simple Arabidopsis genome is composed of heterochromatin (Barakat *et al.*, 2000; Fransz *et al.*, 2002). This may explain why T-DNA integrations can be identified throughout the majority of the genome with only a few regions lacking or low in observed integrations. Organisms with more complex genomes and greater proportions of heterochromatin, such as rice (*Oryza sativa*) (Barakat *et al.*, 1997), might be expected to display a different pattern of identified integrations. That is, indeed, what is observed. In rice, identified sites of T-DNA integration occur predominantly in genic regions, with fewer than

3% occurring in repetitive regions (Barakat *et al.*, 2000; Chen *et al.*, 2003). Such repetitive regions would likely be in a heterochromatic state and may be incapable of supporting transgene expression. It would be interesting to see if an approach similar to the one taken here with *Arabidopsis* would result in the identification of significantly more integrations into repetitive genomic regions in rice.

Under most circumstances, selection bias should not have a significant effect on experimental outcome. Indeed, a transformant that is incapable of expressing its transgenes is of limited experimental and practical use. There are, however, particular circumstances where selection bias must be considered. Selection bias could result in the inability to completely saturate any genome by insertional mutagenesis, although the integration of multiple independent insertions may help to decrease the impact of selection bias on insertional mutagenesis. It may be particularly difficult to achieve a large number of insertional mutants in regions inactive at the time of selection (i.e. developmental or stress-regulated regions), although these regions may be active later in the plant life cycle. Limited integrations in these regions may be achievable, however, through 'silent second T-DNA' integrations. Studies concerning the effects of particular elements such as MARs or insulators could be heavily impacted by selection bias. If such elements act by allowing transgene expression in genomic regions normally repressed by surrounding chromatin, they may functionally act to decrease the effects of TGS-related selection bias in transformants containing those elements. Organisms transformed with control constructs lacking these elements would be subject to selection bias, and transformants lacking gene expression would not be identified. The elimination of non-expressing transformants from the control populations would result in observing an artificially smaller effect on transgene expression levels than what may be truly occurring. One other possible consequence of selection bias relates to transformation-recalcitrant species. Many species considered to be difficult to transform contain very large and complex genomes (Emani *et al.*, 2002; Gelvin, 2003; Janakiraman *et al.*, 2002; Wang *et al.*, 2001). It is possible that these organisms are actually transformed at a relatively high rate, but the fraction of the genome capable of supporting transgene expression is so small that, under selective conditions, transformants are only rarely identified. It is also possible that some species are so effective at identifying and silencing foreign DNA insertions that the majority of integrations are immediately silenced and consequently unidentified. If either of these possibilities were the cause of transformation-recalcitrance, it would be prudent to take a different approach in improving transformation efficiencies, such as including elements that may increase the likelihood of transgene expression. The inclusion of MARs or insulators can improve transformation efficiencies in both plants (Han *et al.*, 1997) and *Drosophila*

(Roseman *et al.*, 1995). It is possible that these elements function by allowing transgene expression in normally repressed genomic regions, therefore reducing selection bias and allowing for the identification of transformants under selective conditions.

We have shown that using PCR to detect transformants results in the identification of T-DNA integration sites not likely to be found by selection. It is, however, not our intention to suggest that T-DNA integration is completely random. Rather, we simply wish to raise the issue that selection bias is likely responsible for one component of the observed 'non-randomness' of T-DNA integration and has possibly clouded previous assessments of T-DNA integration patterns. Previously reported observations of 'non-random' integration within genes (Alonso *et al.*, 2003) cannot easily be explained by selection bias and may result from an unrelated phenomenon. Our results suggest that true transformation efficiencies in *Arabidopsis* are significantly higher than the efficiencies observed when expression of selectable or screenable markers is a prerequisite for identification. It is likely that these observations will also apply to other species. We have also demonstrated that plants containing silenced transgenes may contain integrations in genomic regions that are largely underrepresented in T-DNA insertional mutagenesis collections. These findings may lead to advancements in the general understanding of T-DNA integration and in the goal of generating T-DNA insertions in every gene in *Arabidopsis* and in other important plant species.

Experimental procedures

Identification of transgenic T₁ seedlings by PCR

Twenty *A. thaliana* (var. Columbia) plants were transformed with *Agrobacterium tumefaciens* strain GV3101:pMP90 (Koncz and Schell, 1986) containing the binary vector pBI121 (Jefferson *et al.*, 1987) essentially as described in Clough and Bent (1998). Approximately 10 000 seeds were collected from the 20 T₀ plants. The pool of T₁ seed was then repeatedly and thoroughly mixed to ensure that subsequent sampling from the tube resulted in a random sample of seed from the original 20 T₀ plants. Repeated sampling consistently resulted in transformation frequencies via kanamycin selection of around 2.5%.

Samples of T₁ seed were sterilized in 20% commercial bleach with 0.01% Triton X-100 and plated on seed germination (SG) medium [MS salts (Gibco, Grand Island, NY, USA) at 0.5× concentration, 5 g l⁻¹ sucrose, pH 5.7, solidified with 6 g l⁻¹ phytagar (Gibco)] containing 200 mg l⁻¹ timentin. A low sucrose concentration was used to slow the growth rate of any contaminants that may result from repeated sampling and transfers expected in the PCR screening. Seeds were plated in 6 × 6 grids. Plates were then wrapped with micropore tape (3M Healthcare, St. Paul, MN, USA) and placed in a dark 4°C chamber for 4 days. After cold treatment, plates were transferred to a 22°C short-day (12 h light, 12 h dark) chamber. When seedlings had reached a stage where the first two true leaves were approximately 3 mm across and the third and fourth true leaves were beginning to emerge (10–14 days after germination), tissues were sampled. From each seedling, the two 3 mm leaves

were excised, pooled with the other leaves from the same row and column to form row pools and column pools, placed in a microcentrifuge tube containing approximately 0.2 ml of 1 mm glass beads, and frozen in liquid nitrogen. Pooled tissues were removed from liquid nitrogen and immediately homogenized for 5 sec using a dental amalgamator. The resulting fine powder was resuspended in DNA extraction buffer [1.4 M NaCl, 20 mM EDTA, 100 mM TrisHCl (pH 8.0), and 3% CTAB]. After a 30-min incubation at 68°C, an equal volume of chloroform was added, and the phases were separated by centrifugation. DNA was precipitated from the aqueous phase with an equal volume of isopropanol and resuspended in 100 µl sterile water.

Polymerase chain reaction was performed on each DNA sample of pooled tissues. For each reaction, 1 µl of isolated DNA template was used in a 25-µl reaction with 200 µM of each dNTP, 0.5 units of taq polymerase (Fisher, Pittsburgh, PA, USA), and reaction buffer A (Fisher) to a concentration of 1× resulting in a final Mg concentration of 1 mM. Primers were added to final concentration of 400 nM. Each reaction consisted of two primer sets, which resulted in two distinct product sizes. To verify the presence of T-DNA, primers A (5'-atgacgcacaatcccactat-3') and B (5'-gtgggttagagcattacgct-3') were used to generate a 648-bp product corresponding to a region in the center of the T-DNA (Figure 1). As a control to verify that Arabidopsis genomic DNA is present and in amplifiable quality, primers DDM1 (5'-cacctttcttttgcgtccac-3') and DDM2 (5'-tgggggttctgttaaagggtc-3') were used to generate a 490-bp product corresponding to the single-copy Arabidopsis gene *ddm1* (Jeddell-oh *et al.*, 1998). The amplifications were carried out as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 60 sec. This was followed by 5 min at 72°C and a hold at 4°C until the PCR products could be separated by gel electrophoresis. Positive amplification in rows and columns indicated putative transformants, and seedlings that individually tested positive for T-DNA amplification were transferred to seed germination enhanced (SGE) medium [MS salts (Gibco) at 1× concentration, 10 g l⁻¹ sucrose, pH 5.7, solidified with 6 g l⁻¹ phytagar (Gibco)] containing 200 mg l⁻¹ timentin. Amplification of *VirG* for the detection of contaminating *Agrobacterium* was achieved with primers *VirG*-up (5'-gcaatgattctctcaactgctcg-3') and *VirG*-dn (5'-gatttcagacgatagccctggtaac-3').

Identification of transgenic T₁ seedlings by kanamycin selection

Samples of sterilized T₁ seed from the same T₁ seed pool used for PCR-screening were plated on SG medium containing 200 mg l⁻¹ timentin and 50 mg l⁻¹ kanamycin. Between 100 and 200 seeds were plated on each plate. Plates were wrapped with micropore tape and placed in a dark 4°C chamber for 4 days. After cold treatment, plates were transferred to a 22°C short-day (12 h light, 12 h dark) chamber. Kanamycin tolerance was scored at 2 weeks. After 2 weeks on kanamycin, untransformed seedlings failed to develop true leaves, and cotyledons were completely chlorotic. Any seedling that showed any degree of kanamycin tolerance above that of untransformed seedlings (i.e. development of true leaves or retention of green color) was considered to be kanamycin resistant, and transferred to SGE medium containing 200 mg l⁻¹ timentin and no kanamycin.

Characterization of T-DNA by PCR

The presence of non-truncated reporter genes was verified by PCR with primers C (5'-aggctattcgctatgactggc-3') and D (5'-tttcata-gatggcggcgggtg-3') for *nptII* verification and primers E

(5'-gatagtggaaggagggtggctc-3') and F (5'-ttgttgattcattgttgcctcc-3') for *gusA* verification. The structure and complexity of T-DNA integration was also partially assessed by PCR. All transgenic plants were assayed for the presence of direct tandem repeats using primers G (5'-tgatagtacatttagggcagc-3') and H (5'-gaaaccctggcgt-tacc-3') (Figure 1). A product resulted only if T-DNAs integrated as direct tandem repeats. The presence of non-T-DNA binary vector sequence was identified by PCR amplification with primers I (5'-tgtttatcggcagctcgtagagc-3') and J (5'-tgtggcagcaggtgttgag-3'), which amplify a 672-bp fragment in the pBI121 vector approximately 2 kb outside of the LB.

Segregation of kanamycin tolerance in T₂ seedlings

Kanamycin tolerance in T₂ seedlings was measured by plating between 18 and 72 T₂ seeds from PCR-identified and kanamycin-selected lines on SG medium containing 50 mg l⁻¹ kanamycin. Kanamycin tolerance was scored 2 weeks after germination. Seedlings displaying tolerance at any level above that of untransformed Arabidopsis were considered to be demonstrating some degree of kanamycin tolerance. Kanamycin tolerance frequencies for each line were calculated based on the number of germinated seedlings showing kanamycin tolerance. For each line with a kanamycin tolerance frequency lower than 0.75, a chi-square goodness of fit test was conducted against the predicted 3:1 segregation at the $\alpha = 0.01$ level. Lines with significantly fewer kanamycin-tolerant seedlings (lines that failed the chi-square test) were considered to be silenced for *nptII*. Because a very liberal definition of kanamycin tolerance was applied, we consider this a very conservative estimate of silencing. All other lines were considered to be *nptII*-expressing.

Identification of flanking genomic sequences

Flanking genomic sequences adjacent to T-DNA LBs were identified by ligation-mediated PCR (Siebert *et al.*, 1995) as described in Alonso *et al.* (2003). Adapters for ligation to *EcoRI*-cut ends were generated by annealing ADAPS-E1 (5'-aattcacctgcccgg/3AmMc7/-3') with a 3' amino-terminal end and ADAPL-E1 (5'-ctaatacactactactagggctcgagcggcggcggcaggtg-3'). Primary products were generated by amplification with AP1 (5'-ggatcctaatacactactactagggc-3') and PBI121LB-WP1 (5'-ctgttgcccgtctcactgtt-3'). Nested amplification was achieved with primers AP2 (5'-tataggcctcgagcggcggc-3') and PBI121LB-WP2 (5'-aagaaaaaccaccagctac-3'). Nested PCR products were verified by agarose gel electrophoresis, and directly sequenced by the University of North Carolina Lineberger Comprehensive Cancer Center DNA sequencing facility using primer PBI121LB-WP2. Genomic integration sites were identified by BLAST (Altschul *et al.*, 1990) at the SIGNAL (<http://signal.salk.edu>) and NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>) websites.

Analysis of the Salk Institute's SIGNAL project data

All comparisons to the Salk Institute's SIGNAL project T-DNA integrations are based on integration data available in the supplemental data of Alonso *et al.* (2003). The data available in the SIGNAL database accessible at <http://signal.salk.edu> contain artifacts resulting from errors in sequencing, sample contamination, and difficulties in analyzing integrations into repeat-containing genomic regions. The SIGNAL project integration data available in the supplemental material of Alonso *et al.* (2003) has been filtered to remove integrations that are likely artifacts, and are therefore a more appropriate resource to use.

Acknowledgements

We thank Jose Alonso and Anna Stepanova for assistance in replicating SIGnAL project experimental procedures. We thank Carletha Blanding for assistance in performing the PCR screens. We also thank Chris Halweg, Anton Calloway, Arthur Weissinger, Niki Robertson, Mark Conkling, and Bill Thompson for helpful discussions. KEF was supported by a GAANN fellowship (Department of Education) and grants from Vector Tobacco and the USDA.

Supplementary Material

The following material is available from <http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2312/TPJ2312sm.htm>

Table S1 Integration sites of a sample of PCR-identified and kanamycin-selected lines

Table S2 Genetic analysis of T-DNA segregation in select PCR-identified T₂ lines

Table S3 GUS expression in kanamycin-sensitive and -resistant lines identified by PCR

References

- Allen, G.C., Spiker, S. and Thompson, W.F. (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. *Plant Mol. Biol.* **43**, 361–376.
- Alonso, J.M., Stepanova, A.N., Leisse, T.J. *et al.* (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science*, **301**, 653–657.
- Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.
- Aufsatz, W., Mette, M.F., van der Winden, J., Matzke, A.J. and Matzke, M. (2002) RNA-directed DNA methylation in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **99** (Suppl. 4), 16499–16506.
- Azpiroz-Leehan, R. and Feldmann, K.A. (1997) T-DNA insertion mutagenesis in *Arabidopsis*: going back and forth. *Trends Genet.* **13**, 152–156.
- Barakat, A., Carels, N. and Bernardi, G. (1997) The distribution of genes in the genomes of Gramineae. *Proc. Natl Acad. Sci. USA*, **94**, 6857–6861.
- Barakat, A., Gallois, P., Raynal, M., Mestre-Ortega, D., Sallaud, C., Guiderdoni, E., Delseny, M. and Bernardi, G. (2000) The distribution of T-DNA in the genomes of transgenic Arabidopsis and rice. *FEBS Lett.* **471**, 161–164.
- Bechtold, N., Jolivet, S., Voisin, R. and Pelletier, G. (2003) The endosperm and the embryo of *Arabidopsis thaliana* are independently transformed through infiltration by *Agrobacterium tumefaciens*. *Transgenic Res.* **12**, 509–517.
- Bode, J., Benham, C., Knopp, A. and Mielke, C. (2000) Transcriptional augmentation: modulation of gene expression by scaffold/matrix-attached regions (S/MAR elements). *Crit. Rev. Eukaryot. Gene Exp.* **10**, 73–90.
- Brunaud, V., Balzergue, S., Dubreucq, B. *et al.* (2002) T-DNA integration into the *Arabidopsis* genome depends on sequences of pre-insertion sites. *EMBO Rep.* **3**, 1152–1157.
- Chen, S., Jin, W., Wang, M., Zhang, F., Zhou, J., Jia, Q., Wu, Y., Liu, F. and Wu, P. (2003) Distribution and characterization of over 1000 T-DNA tags in rice genome. *Plant J.* **36**, 105–113.
- Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.
- Copenhaver, G.P. and Pikaard, C.S. (1996) RFLP and physical mapping with an rDNA-specific endonuclease reveals that nucleolus organizer regions of *Arabidopsis thaliana* adjoin the telomeres on chromosomes 2 and 4. *Plant J.* **9**, 259–272.
- Cottage, A., Yang, A.P., Maunders, H., de Lacy, R.C. and Ramsay, N.A. (2001) Identification of DNA sequences flanking T-DNA insertions by PCR-walking. *Plant Mol. Biol. Rep.* **19**, 321–327.
- Cubero, J., Martinez, M.C., Llop, P. and Lopez, M.M. (1999) A simple and efficient PCR method for the detection of *Agrobacterium tumefaciens* in plant tumours. *J. Appl. Microbiol.* **86**, 591–602.
- De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. (1998) *Agrobacterium tumefaciens* transformation and cotransformation frequencies of *Arabidopsis thaliana* root explants and tobacco protoplasts. *Mol. Plant Microbe Int.* **11**, 449–457.
- De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. (1999) The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. *Plant J.* **20**, 295–304.
- De Buck, S., Van Montagu, M. and Depicker, A. (2001) Transgene silencing of invertedly repeated transgenes is released upon deletion of one of the transgenes involved. *Plant Mol. Biol.* **46**, 433–445.
- Dietz, A., Kay, V., Schlake, T., Landsmann, J. and Bode, J. (1994) A plant scaffold attached region detected close to a T-DNA integration site is active in mammalian cells. *Nucleic Acids Res.* **22**, 2744–2751.
- Dominguez, A., Fagoaga, C., Navarro, L., Moreno, P. and Pena, L. (2002) Regeneration of transgenic citrus plants under non selective conditions results in high-frequency recovery of plants with silenced transgenes. *Mol. Genet. Genomics*, **267**, 544–556.
- Elmayan, T. and Vaucheret, H. (1996) Expression of single copies of a strongly expressed 35S transgene can be silenced post-transcriptionally. *Plant J.* **9**, 787–797.
- Emani, C., Sunilkumar, G. and Rathore, K.S. (2002) Transgene silencing and reactivation in sorghum. *Plant Sci.* **162**, 181–192.
- Feldmann, K.A. (1991) T-DNA insertion mutagenesis in *Arabidopsis* – mutational spectrum. *Plant J.* **1**, 71–82.
- Fobert, P.R., Miki, B.L. and Iyer, V.N. (1991) Detection of gene regulatory signals in plants revealed by T-DNA-mediated fusions. *Plant Mol. Biol.* **17**, 837–851.
- Forsbach, A., Schubert, D., Lechtenberg, B., Gils, M. and Schmidt, R. (2003) A comprehensive characterization of single-copy T-DNA insertions in the *Arabidopsis thaliana* genome. *Plant Mol. Biol.* **52**, 161–176.
- Franz, P., De Jong, J.H., Lysak, M., Castiglione, M.R. and Schubert, I. (2002) Interphase chromosomes in *Arabidopsis* are organized as well defined chromocenters from which euchromatin loops emanate. *Proc. Natl Acad. Sci. USA*, **99**, 14584–14589.
- Gelvin, S.B. (2003) Improving plant genetic engineering by manipulating the host. *Trends Biotechnol.* **21**, 95–98.
- Gheysen, G., Herman, L., Breyne, P., Gielen, J., Vanmontagu, M. and Depicker, A. (1990) Cloning and sequence-analysis of truncated T-DNA inserts from *Nicotiana tabacum*. *Gene*, **94**, 155–163.
- Han, K.H., Ma, C.P. and Strauss, S.H. (1997) Matrix attachment regions (MARs) enhance transformation frequency and transgene expression in poplar. *Transgenic Res.* **6**, 415–420.
- Herman, L., Jacobs, A., Van Montagu, M. and Depicker, A. (1990) Plant chromosome/marker gene fusion assay for study of normal and truncated T-DNA integration events. *Mol. Gen. Genet.* **224**, 248–256.
- van Holde, K. (1989) *Chromatin*. New York: Springer-Verlag.
- Hui, E.K., Wang, P.C. and Lo, S.J. (1998) Strategies for cloning unknown cellular flanking DNA sequences from foreign integrants. *Cell Mol. Life Sci.* **54**, 1403–1411.

- Ichikawa, T., Nakazawa, M., Kawashima, M. *et al.* (2003) Sequence database of 1172 T-DNA insertion sites in *Arabidopsis* activation-tagging lines that showed phenotypes in T₁ generation. *Plant J.* **36**, 421–429.
- Janakiraman, V., Steinau, M., McCoy, S.B. and Trick, H.N. (2002) Recent advances in wheat transformation. *In Vitro Cell. Dev. Biol.-Plant*, **38**, 404–414.
- Jeddeloh, J.A., Bender, J. and Richards, E.J. (1998) The DNA methylation locus DDM1 is required for maintenance of gene silencing in *Arabidopsis*. *Genes Dev.* **12**, 1714–1725.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.
- Koncz, C. and Schell, J. (1986) The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by a novel type of *Agrobacterium* binary vector. *Mol. Gen. Genet.* **204**, 383–396.
- Koncz, C., Martini, N., Mayerhofer, R., Koncz-Kalman, Z., Korber, H., Redei, G.P. and Schell, J. (1989) High-frequency T-DNA-mediated gene tagging in plants. *Proc. Natl Acad. Sci. USA*, **86**, 8467–8471.
- Krysan, P.J., Young, J.C. and Sussman, M.R. (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell*, **11**, 2283–2290.
- Krysan, P.J., Young, J.C., Jester, P.J., Monson, S., Copenhaver, G., Preuss, D. and Sussman, M.R. (2002) Characterization of T-DNA insertion sites in *Arabidopsis thaliana* and the implications for saturation mutagenesis. *OMICS*, **6**, 163–174.
- Lindsey, K., Wei, W., Clarke, M.C., McArdle, H.F., Rooke, L.M. and Topping, J.F. (1993) Tagging genomic sequences that direct transgene expression by activation of a promoter trap in plants. *Transgenic Res.* **2**, 33–47.
- Ma, C. and Mitra, A. (2002) Intrinsic direct repeats generate consistent post-transcriptional gene silencing in tobacco. *Plant J.* **31**, 37–49.
- Makarevitch, I., Svitashv, S.K. and Somers, D.A. (2003) Complete sequence analysis of transgene loci from plants transformed via microprojectile bombardment. *Plant Mol. Biol.* **52**, 421–432.
- Mitsuhara, I., Shirasawa-Seo, N., Iwai, T., Nakamura, S., Honkura, R. and Ohashi, Y. (2002) Release from post-transcriptional gene silencing by cell proliferation in transgenic tobacco plants: possible mechanism for noninheritance of the silencing. *Genetics*, **160**, 343–352.
- Mlynarova, L., Loonen, A., Mietkiewska, E., Jansen, R.C. and Nap, J.P. (2002) Assembly of two transgenes in an artificial chromatin domain gives highly coordinated expression in tobacco. *Genetics*, **160**, 727–740.
- Pan, X., Liu, H., Clarke, J., Jones, J., Bevan, M. and Stein, L. (2003) ATIDB: *Arabidopsis thaliana* insertion database. *Nucleic Acids Res.* **31**, 1245–1251.
- Peach, C. and Velten, J. (1991) Transgene expression variability (position effect) of CAT and GUS reporter genes driven by linked divergent T-DNA promoters. *Plant Mol. Biol.* **17**, 49–60.
- Permingeat, H.R., Alvarez, M.L., Cervigni, G.D., Ravizzini, R.A. and Vallejos, R.H. (2003) Stable wheat transformation obtained without selectable markers. *Plant Mol. Biol.* **52**, 415–419.
- Qin, H., Dong, Y. and von Arnim, A.G. (2003) Epigenetic interactions between *Arabidopsis* transgenes: characterization in light of transgene integration sites. *Plant Mol. Biol.* **52**, 217–231.
- Rios, G., Lossow, A., Hertel, B. *et al.* (2002) Rapid identification of *Arabidopsis* insertion mutants by non-radioactive detection of T-DNA tagged genes. *Plant J.* **32**, 243–253.
- Roseman, R.R., Johnson, E.A., Rodesch, C.K., Bjerke, M., Nagoshi, R.N. and Geyer, P.K. (1995) A P element containing suppressor of hairy-wing binding regions has novel properties for mutagenesis in *Drosophila melanogaster*. *Genetics*, **141**, 1061–1074.
- Rosso, M.G., Li, Y., Strizhov, N., Reiss, B., Dekker, K. and Weisshaar, B. (2003) An *Arabidopsis thaliana* T-DNA mutagenized population (GABI-Kat) for flanking sequence tag-based reverse genetics. *Plant Mol. Biol.* **53**, 247–259.
- Sawasaki, T., Takahashi, M., Goshima, N. and Morikawa, H. (1998) Structures of transgene loci in transgenic *Arabidopsis* plants obtained by particle bombardment: junction regions can bind to nuclear matrices. *Gene*, **218**, 27–35.
- Sessions, A., Burke, E., Presting, G. *et al.* (2002) A high-throughput *Arabidopsis* reverse genetics system. *Plant Cell*, **14**, 2985–2994.
- Shimizu, K., Takahashi, M., Goshima, N., Kawakami, S., Irifune, K. and Morikawa, H. (2001) Presence of an SAR-like sequence in junction regions between introduced transgene and genomic DNA of cultured tobacco cells: its effect on transformation frequency. *Plant J.* **26**, 375–384.
- Siebert, P.D., Chenchik, A., Kellogg, D.E., Lukyanov, K.A. and Lukyanov, S.A. (1995) An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res.* **23**, 1087–1088.
- Szabados, L., Kovacs, I., Oberschall, A. *et al.* (2002) Distribution of 1000 sequenced T-DNA tags in the *Arabidopsis* genome. *Plant J.* **32**, 233–242.
- Szittyá, G., Silhavy, D., Molnar, A., Havelda, Z., Lovas, A., Lakatos, L., Banfalvi, Z. and Burgyan, J. (2003) Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *EMBO J.* **22**, 633–640.
- Takano, M., Egawa, H., Ikeda, J.E. and Wakasa, K. (1997) The structures of integration sites in transgenic rice. *Plant J.* **11**, 353–361.
- Thomas, C.M., Jones, D.A., English, J.J., Carroll, B.J., Bennetzen, J.L., Harrison, K., Burbidge, A., Bishop, G.J. and Jones, J.D. (1994) Analysis of the chromosomal distribution of transposon-carrying T-DNAs in tomato using the inverse polymerase chain reaction. *Mol. Gen. Genet.* **242**, 573–585.
- Tinland, B. (1996) The integration of T-DNA into plant genomes. *Trends Plant Sci.* **1**, 178–184.
- Topping, J.F. and Lindsey, K. (1995) Insertional mutagenesis and promoter trapping in plants for the isolation of genes and the study of development. *Transgenic Res.* **4**, 291–305.
- Topping, J.F., Wei, W. and Lindsey, K. (1991) Functional tagging of regulatory elements in the plant genome. *Development*, **112**, 1009–1019.
- Ülker, B., Weissinger, A.K. and Spiker, S. (2002) E. coli chromosomal DNA in a transgene locus created by microprojectile bombardment in tobacco. *Transgenic Res.* **11**, 311–313.
- de Vetten, N., Wolters, A.M., Raemakers, K., van der Meer, I., ter Stege, R., Heeres, E., Heeres, P. and Visser, R. (2003) A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nat. Biotechnol.* **21**, 439–442.
- Wang, Z.Y., Hopkins, A. and Mian, R. (2001) Forage and turf grass biotechnology. *Crit. Rev. Plant Sci.* **20**, 573–619.
- Wassenegger, M., Heimes, S., Riedel, L. and Sanger, H.L. (1994) RNA-directed de novo methylation of genomic sequences in plants. *Cell*, **76**, 567–576.
- Wenck, A., Czako, M., Kanevski, I. and Marton, L. (1997) Frequent collinear long transfer of DNA inclusive of the whole binary vector during *Agrobacterium*-mediated transformation. *Plant Mol. Biol.* **34**, 913–922.
- Wolfe, A.P. (1998) *Chromatin: Structure and Function*, 3rd edn. San Diego: Academic Press.
- Ye, G., Stone, D., Pang, S., Creely, W., Gonzalez, K. and Hinchee, M. (1999) *Arabidopsis* ovule is the target for *Agrobacterium* in planta vacuum infiltration transformation. *Plant J.* **19**, 249–257.

Supplemental material for "Identification of *Arabidopsis thaliana* transformants without selection reveals a high occurrence of silenced T-DNA integrations"

Quantitative Measurement of GUS Activity

GUS activity was measured by monitoring cleavage of the β -glucuronidase substrate 4-methylumbelliferyl β -D-glucuronide (MUG) (Jefferson et al., 1987; Gallagher, 1992). The assay was adapted so that large numbers of samples could be assayed and measured in a 96-well plate format. Tissues were collected into microcentrifuge tubes containing ~200 μ l of 1mm glass beads and frozen in liquid nitrogen. Tissues were homogenized using a dental amalgamator. Samples were removed from liquid nitrogen, immediately placed in the amalgamator, and homogenized for 5sec. After homogenization, 1ml GUS Extraction Buffer [150mM Sodium Phosphate pH 7.0, 10mM EDTA, 10mM β -mercaptoethanol, 0.1% Triton X-100, 0.1% sarcosyl, 140 μ M PMSF] was added, and samples were mixed by an additional 5sec in the amalgamator. Debris was pelleted by centrifugation at 13000rpm in microcentrifuge for 15min at room temperature. For each sample, 200 μ l of supernatant was collected and kept on ice for use in MUG assays and for protein quantification.

The assay reaction consisted of 10 μ l sample extract and 130 μ l Assay Buffer [GUS Extraction Buffer containing 1.2mM 4-methylumbelliferyl β -D-glucuronide (MUG)(Sigma)]. The reaction was carried out in a dark 37°C water bath. After 20 minutes, 10 μ l of the reaction was transferred to 190 μ l Stop Buffer [200mM sodium carbonate] in an opaque 96-well plate. Repeated analyses demonstrated that the rates of MUG cleavage were linear for at least 20 minutes in the samples examined, therefore a single measurement of liberated MU at 20 minutes was an appropriate approach (Francis, 2004). Fluorescence was measured on a FLUO-star plate reader (BMG Labtechnologies Inc., Durham, NC) at 460nm when excited at 355nm. A standard curve corresponding to

50, 25, 5, 2.5, 0.5, 0.25, and 0 μ M 4-Methylumbelliferone (MU) was included with every plate and used to calculate the amount of liberated MU produced by each sample.

Values from the fluorescence assay were converted to moles of MU/minute, and then standardized by protein concentration to accommodate differences in tissue sampling and extraction. Protein concentrations were determined by methods described by Bradford (1976) and analyzed on a FLUO-star plate reader (BMG Labtechnologies Inc., Durham, NC) set to measure absorbance at 600nm. Final GUS activity values were recorded as pmole s MU/min/mg protein.

Table S-1. Integration sites of a sample of PCR-identified and kanamycin-selected lines.

PCR-Identified <i>nptII</i> -silencing ^a					
	Homology	Chromosome	Position ^b	Gene	Feature
S1-CG6	<i>A. thaliana</i>	4	+2587	At4g00890	
S3-EB4	<i>A. thaliana</i>	5	-147	At5g49550	
S3-GC3	<i>A. thaliana</i>	2/4			25s rDNA
S4-QB5	<i>A. thaliana</i>	1	-504	At1g54095	
S4-SA3	<i>A. thaliana</i>	1	-559	At1g11380	
S4-WE2	<i>A. thaliana</i>	1	+6568	At1g05570	
S7-BB1	<i>A. thaliana</i>	4	+1185	At4g05150	Centromere
S7-BD5	<i>A. thaliana</i>	1	+3942	At1g36035	
S7-HB5	<i>A. thaliana</i>	1	+74	At1g01490	
S7-JD6	<i>A. thaliana</i>	3	+211	At3g44150	
S7-KB2	<i>A. thaliana</i>	5	-26	At5g58270	
S7-LA6	<i>A. thaliana</i>	3	-1438	At3g29642	
S7-LD1	<i>A. thaliana</i>	3	-45	At3g14050	
S7-OE1	<i>A. thaliana</i>	2	-635	At2g44350	
PCR-Identified <i>nptII</i> -expressing ^a					
	Homology	Chromosome	Position	Gene	Feature
S3-AD1	<i>A. thaliana</i>	1	-711	At1g61820	
S3-CC5	<i>A. thaliana</i>	2	-1179	At2g02010	
S4-CE2	<i>A. thaliana</i>	3	-430	At3g10330	
S4-GB6	<i>A. thaliana</i>	4	-1262	At4g23690	
S4-YC1	<i>A. thaliana</i>	5	+187	At5g08100	
S7-AC5	<i>A. thaliana</i>	5	+4364	At5g37830	
S7-AC6	<i>A. thaliana</i>	1	+732	At1g64310	
S7-FB5	<i>A. thaliana</i>	5	-2094	At5g66880	
S7-GA3	<i>A. thaliana</i>	3	+2570	At3g15410	
S7-IC1	<i>A. thaliana</i>	5	+2515	At5g67610	
S7-NA5	<i>A. thaliana</i>	1	-1899	At1g29660	
S7-TE3	<i>A. thaliana</i>	5	-236	A15g53920	
Kanamycin-Selected <i>nptII</i> -expressing ^a					
	Homology	Chromosome	Position	Gene	Feature
S3-KR6	<i>A. thaliana</i>	5	+428	At5g41490	
S3-KR3	<i>A. thaliana</i>	1	-170	At1g77760	

^a *nptII*-silencing and *nptII*-expressing lines determined by T2 segregation on kanamycin.

^b position relative to predicted translational start site of nearest open reading frame.

Genetic Analysis of T-DNA Segregation in Select T2 Lines

We believe the evidence presented here supports the view that the high frequency of kanamycin sensitivity in the T2 progeny of PCR-identified lines is a result of transgene silencing. One other possibility is that some T1 PCR-identified lines were chimeric for the T-DNA. T2 seed from untransformed sectors of chimeric T1 plants would not be expected to demonstrate kanamycin tolerance, therefore the low frequency of kanamycin tolerance in some PCR-identified lines could also be explained by the existence of chimeric T1 plants producing untransformed T2 seed. Although some evidence exists in the literature that suggests *in planta* transformation can result in chimeric transformation events (Ye et al., 1999; Bechtold et al., 2003), strong evidence exists that *in planta* transformation occurs early in ovule development prior to megasporogenesis (Desfeux et al., 2000) and is therefore unlikely to result in chimeric T1 embryos. Nevertheless, to rule out chimerism and distorted segregation as an explanation for low ratios of kanamycin tolerance in T2 plants of some of the PCR-identified lines, we have examined samples of T2 lines for genetic segregation of T-DNA sequences.

Genomic DNA was individually isolated from 24 T2 seedlings from 11 PCR-identified lines demonstrating a range of kanamycin tolerance frequencies. A single kanamycin-selected line was also examined as a control. Each T2 seedling was then screened by PCR for the presence of T-DNA sequences using primers A and B. Amplification for an endogenous genomic control (primers DDM1up and DDM1dn was included in each reaction). A summary of the genetic segregation of T-DNA sequences in T2 seedlings is presented in Table S-2. All lines examined demonstrated segregation consistent with expected Mendelian frequencies for one or two independently segregating loci ($\alpha = 0.01$), suggesting that untransformed T2 seed from chimeric T1 plants is unlikely to be a major cause of the observed high frequency of kanamycin sensitivity in PCR-identified lines.

Table S-2. Genetic analysis of T-DNA segregation in select PCR-identified T2 lines.

Line	Freq. of T2 Kan. Res.	Freq. of T-DNA PCR-positive	single-locus p-value ^c	two-loci p-value ^c
S3-EB4	0.00 ^b	0.92	0.059	0.673
S7-BB1	0.00 ^b	0.67	0.346	
S7-OE1	0.00 ^b	0.96	0.018	0.673
S3-BE6	0.03 ^b	0.71	0.637	
S1-FD7	0.03 ^b	0.92	0.059	0.673
S7-LD1	0.08 ^b	0.92	0.059	0.673
S2-UB6	0.17 ^b	0.75	1.000	
S7-BD5	0.30 ^b	0.88	0.157	0.206
S4-QB5	0.44 ^b	0.71	0.637	
S1-CG6	0.49 ^b	0.67	0.346	
S4-HF6	0.75	0.71	0.637	
S4-KR5 ^a	0.88	0.88	0.157	0.206

^a Line S4 KR5 was identified by kanamycin-selection

^b T2 kanamycin resistance freqs. are significantly lower than the expected 3:1 ($\alpha = 0.01$)

^c p-value based on Chi Squared analysis

gusA expression in *nptII*-expressing and *nptII*-silencing lines

Data demonstrating a relationship between kanamycin resistance and *gusA* expression in the T2 generation of PCR-identified lines are presented in Table S-3. Kanamycin resistance is presented as the frequency of T2 seedlings demonstrating kanamycin resistance. Lines with kanamycin resistance frequencies significantly lower than 0.75 (as determined by a chi square test - see main body) were considered to be kanamycin sensitive. Gus activity (as determined by MUG assays) is presented as pmoles MU/min/mg protein.

Table S-3. Gus expression in kanamycin sensitive and resistant lines identified by PCR

<u>Kanamycin Sensitive Lines</u>			<u>Kanamycin Resistant Lines</u>		
Line	T2 Kan. Res.	GUS Activity	Line	T2 Kan. Res.	GUS Activity
S1-FF3	0.00	178.4	S1-JE1	0.57	102.9
S1-GD5	0.00	10.2	S3-CD1	0.61	203.4
S3-EB4	0.00	95.0	S3-OA4	0.63	6.2
S7-BB1	0.00	1.0	S3-CC5	0.64	19.3
S7-LA6	0.00	382.2	S1-GB6	0.64	20.9
S7-OE1	0.00	37.5	S2-CB6	0.65	319.5
S7-QA2	0.00	1.0	S2-XB5	0.67	154.5
S1-FD7	0.03	337.1	S7-OE4	0.67	14.7
S3-BE6	0.03	1.8	S1-IA1	0.68	103.0
S4-WE2	0.05	2.5	S7-FB5	0.69	332.2
S7-LD1	0.08	48.6	S2-EB6	0.69	120.4
S7-JD6	0.13	50.0	S4-YC4	0.69	56.4
S7-HB5	0.14	42.2	S1-CA4	0.71	343.1
S2-UB6	0.17	16.5	S2-CF4	0.71	130.2
S3-KE5	0.18	7.0	S3-AE2	0.71	491.1
S7-UF3	0.22	72.2	S2-SF4	0.71	93.7
S7-KB2	0.25	70.5	S2-UE1	0.71	51.3
S7-HE6	0.27	10.2	S2-YB5	0.71	214.0
S7-BD5	0.30	71.8	S7-BE1	0.74	132.4
S1-JB6	0.43	38.2	S1-JC7	0.74	358.4
S2-ZC6	0.43	152.2	S7-TB6	0.75	11.5
S1-JG2	0.44	9.6	S2-TA3	0.75	15.6
S4-QB5	0.44	62.4	S3-AD1	0.75	7.0
S4-SA3	0.48	55.9	S3-HF2	0.75	14.6
S1-CG6	0.49	49.6	S4-HF6	0.75	11.2
S3-GC3	0.53	92.6	S7-TE3	0.75	14.2
S4-QE2	0.56	90.6	S4-CE2	0.76	2.3
S3-GF6	0.57	5.4	S7-IC1	0.76	193.7
S2-MB3	0.57	31.4	S3-JF5	0.76	312.1
S7-RE1	0.57	26.9	S7-AD5	0.77	85.6
S7-ND4	0.58	120.8	S7-FD2	0.78	9.8
			S2-WF6	0.78	53.3
			S7-NA5	0.79	274.1
			S7-OD2	0.79	27.6
			S2-TC2	0.80	30.2
			S7-PA4	0.80	586.6
			S7-FC1	0.81	117.5
			S7-JB3	0.81	79.1
			S1-HD2	0.82	259.7
			S2-ZE5	0.82	195.3
			S1-GG5	0.83	75.9
			S2-LC4	0.83	427.8
			S1-JA4	0.86	57.5
			S2-GF2	0.86	18.1
			S2-AE6	0.90	139.4
			S3-EF5	0.92	33.8
			S4-AE3	0.92	161.0
			S4-GB6	0.92	71.8
			S7-GA3	0.94	73.6
			S7-AC5	0.95	130.2
			S4-EB6	0.96	165.1
			S4-PF6	0.96	76.9
			S4-HC1	1.00	135.0
Average	0.26	70.0	Average	0.77	134.6

REFERENCES

- Bechtold, N., Jolivet, S., Voisin, R. and Pelletier, G.** (2003) The endosperm and the embryo of *Arabidopsis thaliana* are independently transformed through infiltration by *Agrobacterium tumefaciens*. *Transgenic Res.* **12**, 509-517.
- Bradford, M.M.** (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Desfeux, C., Clough, S.J. and Bent, A.F.** (2000) Female reproductive tissues are the primary target of *Agrobacterium*-mediated transformation by the *Arabidopsis* floral-dip method. *Plant Physiol.* **123**, 895-904.
- Francis, K.E.** (2004) The Effects of T-DNA Integration Sites on Transgene Expression in *Arabidopsis* [dissertation]. North Carolina State University, Raleigh, NC.
- Gallagher, S.R.** (1992) Quantitation of GUS activity by fluorometry. In *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression* (SR Gallagher, ed). San Diego: Academic Press, pp. 47-59.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W.** (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901-3907.
- Ye, G., Stone, D., Pang, S., Creely, W., Gonzalez, K. and Hinchee, M.** (1999) *Arabidopsis* ovule is the target for *Agrobacterium in planta* infiltration transformation. *Plant J.* **19**, 249-257.