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The frequency of silencing in *Arabidopsis thaliana* varies highly between progeny of siblings and can be influenced by environmental factors

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Abstract

In a collection of 111 transgenic *Arabidopsis thaliana* lines, silencing of the *nptII* gene was observed in 62 (56%) of the lines and three distinct *nptII*-silencing phenotypes were identified. Two T-DNA constructs were used, which differed in distance and orientation of the marker gene relative to the border sequences. Comparison of the sets of lines generated with each vector, indicate that the T-DNA construct configuration influence the incidence of lines displaying silencing, as well as the distribution of silencing phenotypes. Twenty lines were investigated more thoroughly. The frequency of silencing varied between siblings in 19 lines, including three lines containing a single T-DNA copy. The last line showed 100% silencing. The *gus* gene present in both constructs could be expressed in the presence of a silenced *nptII* gene. Investigation of methylation at a single site in the *pnos* promoter revealed partial methylation in multi-copy lines, but no methylation in single-copy lines. For 16 lines, the overall frequencies of silencing differed significantly between control plants and plants exposed to temperature stress; in 11 of these lines at the 0.1% level. In several cases, the frequency of silencing in progeny of stress-treated plants was higher than for the control group, while other lines showed higher frequencies of kanamycin-resistant progeny for the stress-treated sibling plants.

Abbreviations: Ap^R – ampicillin resistant; Km^R – kanamycin resistant; Km^S – kanamycin sensitive; *nptII* – neomycin phosphotransferase II gene; *pnos* – nopaline synthetase promoter; *gus* – β-glucoronidase *uid* gene

Introduction

A number of factors have been identified which have an effect on the incidence of transgene silencing in plants, that is the copy number and chromosomal position (Linn et al., 1990; Hobbs et al., 1993), methylation pattern in the region of integration (Pröls & Meyer, 1992) and properties of the transgene sequence itself (Elomaa et al., 1995; Köhne et al., 1998). Some reports describe silencing of a single transgene copy (Meyer et al., 1993; Elmayan & Vaucheret, 1996), but reduced expression of transgenes is more often reported to be dependent on the presence of homologous sequences (reviewed by Matzke & Matzke 1998; Kooter et al., 1999). Two main mechanisms for homology-dependent gene silencing have been described: transcriptional transgene silencing (TGS) and post-transcriptional transgene silencing (PTGS). TGS is characterized by inactivated promoters, where the promoter sequence is methylated (reviewed by Kooter et al., 1999). PTGS on the other hand, involves formation of aberrant RNA molecules and induces silencing of all genes that produce RNA similar to the transgenic

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sequence. Methylation may also be associated with PTGS (reviewed by Fire, 1999).

Screening for silencing has been performed in small collections of transgenic *Arabidopsis*, tobacco and petunia lines. In two of the cases, the incidence of *trans*-inactivation was investigated following re-transformation (Matzke et al., 1989; Neuhuber et al., 1994). In these screens, silencing was identified in a few percent up to 50% of the lines tested (Matzke et al., 1989; Scheid et al., 1991; Kilby et al., 1992; Neuhuber et al., 1994; Van Blokland et al., 1994).

In a number of cases, environmental factors have been shown to modulate transgene activity. After a period of high light intensity and temperature, transgene silencing of the *A1* gene from maize inserted in petunia was observed in the field (Meyer et al., 1992). Other examples are the influence of high temperature on silencing of the *pat* transgene in *Medicago sativa* (Walter et al., 1992), the *Nia*, *nptII*, *luc* and *pat* transgenes in tobacco (Palauqui & Vaucheret 1995; Neumann et al., 1997; Conner et al., 1998; Köhne et al., 1998), and the *Ltp2-gus* transgene in rice (Morino et al., 1999). The different examples and the possible involvement of diverse mechanisms in environmental-induced transgene silencing, suggest that a better understanding of this phenomenon is desirable.

To investigate the incidence of silencing with different T-DNA configurations and under different environmental growth conditions, we generated a large collection of *Arabidopsis* lines. In the two vectors used, the reporter (*gus*) and selectable marker (*nptII*) genes are orientated differently in the T-DNA. The number of T-DNA loci in each of the 111 transgenic lines was determined through Mendelian segregation analyses of T2-seedlings grown on plates containing kanamycin (Km). *nptII* silencing was scored in the T3-generation by identifying seedlings with kanamycin-sensitive phenotypes and non-Mendelian segregation ratios.

In 20 lines T-DNA copy number and methylation status of the *Sac* II site of the *pnos* promoter driving *nptII* expression were investigated by Southern hybridization. These lines were also used to study the effect of controlled environmental stress. Since several studies have indicated that elevated temperature may increase the frequency of silencing, we chose 30°C as the basic stress condition. The C24 ecotype used in this study is able to withstand this temperature over longer periods. We report an elevated incidence of silencing after stress treatment for several lines. Lines in which the environmental stress did not influence the level of silencing, as well as lines where the stress treatment resulted in decreased silencing, were observed. Lines generated with either of the vectors were found in all the three classes, indicating that the configuration of the T-DNA did not influence the results.

The importance of T-DNA construct organization, genomic position, homozygosity and methylation for transgene silencing is discussed. In the majority of lines, the T-DNA(s) was inserted in a single locus. Therefore, a mechanism of environmental-influenced silencing dependent on the genomic position of the transgenes is also discussed.

Materials and methods

Plant transformation and transgenic constructs

Transgenic *Arabidopsis thaliana* plants (ecotype C24) were made using the transformation vectors pPCV002 35GUS (Koncz & Schell, 1986) and pKOH110 35SGUS (this study), containing the *gus* and *nptII* reporter genes (Figure 1). The pKOH110 35SGUS construct was generated by isolating *p35S-gus* from pPCV002 35SGUS using *Eco*R I and *Hind* III, and ligating the fragment into the corresponding sites of the pKOH110 vector (K.O. Holmström, pers.com). This vector was subsequently introduced into *Agrobacterium tumefaciens* (C58C1 rif^r) containing the non-oncogenic Ti-plasmid pGV2260 (Deblaere et al., 1985). Root-transformation was performed as previously described (Mandal et al., 1993).

Kanamycin resistance and GUS assay

For each of the transgenic lines generated, surface-sterilized T_2 seeds, obtained by self-fertilization of the primary transformants and T_1 plants, were plated on MS medium (Murashige & Skoog, 1962) supplemented with 2% sucrose (MS-2) and kanamycin (50 mg/l) to determine the segregation ratios of the *pnos-nptII* transgene. The plates were incubated in a growth chamber at 22°C with a light intensity of $100\,\mu E\,m^{-2}\,s^{-1}$ and 16 h daylength with 60% relative humidity. Segregation ratios of Km^R and Km^S phenotypes were used to divide the collection of the 111 transgenic lines into six categories depending on the number of transgenic loci and state of hemizygosity or homozygosity (Table 2). Single-locus lines

(A + B) were categorized based on 3:1 segregation ratios of T_1 and/or T_2 plants, while two-loci lines (C + D) were categorized based on 15:1 segregation ratios in T_1 and/or T_2. Some lines had 100% Km^R seedlings in T_1 and T_2 (E), indicating the presence of two or more loci, while others showed non-Mendelian ratios or a *nptII*-silencing phenotype in T_1 and/or T_2 (F). All segregation data were evaluated by χ^2 analysis and interpreted using standard statistical tables. χ^2 analysis was also performed to evaluate whether the percentage of lines displaying *nptII*-silencing in the different categories was different from the lines screened (Table 2).

Staining for β-glucoronidase activity was performed as described (Jefferson, 1989).

Stress treatments and screening for nptII-silencing

Eleven seedlings per transgenic line (germinated as described above) were replanted into pots after 4 weeks and kept at normal growth conditions for 3 days. Stress treatment was thereafter as follows: seven plants were subjected to 30°C day and night. Four of these plants were in addition sprayed with insecticide (Croneton, Bayer Norge A/S, Norway) twice, 1 week apart. Another four plants were given the same 30°C stress during the day, but the night temperature was lowered to 4°C. After 14 days, the plants were transferred to standard conditions (22°C). Thirteen Km^R T_2-plants per transgenic line were grown under standard conditions as control groups.

Half of the seeds collected from T_2-plants were subjected to a dormancy-breaking treatment (4 days at 37°C, over night at −80°C and 4 days at 4°C; Paul Grini, pers.com). Scoring of *nptII*-silencing was performed by plating dormancy-broken surface-sterilized T_3 seeds on MS-2 supplemented with Km and growing the seedling for four weeks. The scoring was repeated in the same way with the other half of the seeds, which was after-ripened at room temperature for at least 4 weeks and vernalized 1 week at 4°C prior to germination on Km-plates.

Statistic analysis

A 2×2 contingency table was used to evaluate if stress treatment affected the frequency of *nptII*-silencing. The number of green plants and plants with silencing phenotype was compared for the control versus stress groups as described (Bhattacharyya & Johnson, 1977). χ^2 statistics was compared with the χ^2 distribution with 1 degree of freedom.

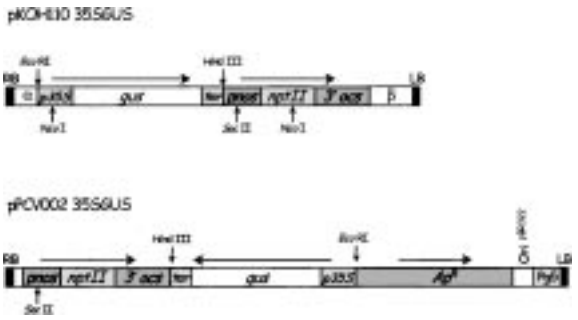

Figure 1. Schematic drawing of the T-DNA regions from the pKOH110 35SGUS and pPCV002 35SGUS constructs used for generating transgenic plants. BL, BR left and right T-DNA border. α, β T-DNA sequences. *p35S*, *pnos* 35S and *nos* promoters. *ter*, *3′ocs* polyadenylation signals. *gus* and *nptII*, β-glucoronidase and neomycin phosphotransferase gene, respectively. Ap^R Ampicillin resitance gene. Ori^{pBR322}, ori region from pBR322. Pg5, truncated promoter of T_L-DNA gene 5. Horizontal arrows on top of figure indicate direction of transcriptional units. Restriction sites for enzymes *Eco*R I, *Hind* III, *Sac* II and *Nco* I are shown.

Determination of T-DNA copy number and DNA methylation by Southern hybridization

DNA was extracted from rosette leaves as described by (Dellaporta et al., 1983) from four representative plants from each transgenic line, digested with restriction enzymes and used for Southern hybridization. *nptII* coding-region and *gus* coding-region probes were labeled by MSPL labeling (Espelund et al., 1990) of PCR-products generated by the *nptII*-primers 5′-GGCGATAGAAGGCGATG-3′ and 5′-CGCTTGGGTGGAGAGGC-3′, which anneal at positions 189 and 925, respectively (acc. no ISTN5X), and the *gus*-primers 5′-TGCGGTCACTCATTACGG-3′ and 3′-AGCGTAAGGGTAATGCGAGG-3′, which anneal at positions 330 and 1103, respectively (acc. no CV101TD).

Copy number was determined by hybridizing the same Southerns blots, containing DNA digested with *Hind* III or *Eco*R I, with both the *nptII* and the *gus* probe. Since each of these enzymes only have a single recognition site in each of the T-DNA constructs (Figure 1), the number of bands hybridizing to the probes indicate the number of integrated T-DNA copies. When two copies are integrated as inverted tandem repeats one of the probes will identify two hybridizing bands, and the other probe only one band, with either of the enzymes. Southern blots with DNA digested both with *Eco*R I and *Hind* III and hybridized to the *gus* probe, were used to control that the 35S-GUS fragment of the T-DNAs was present and of the expected size. Methylation in the *Sac* II recognition

site in the *pnos* promoter was investigated by digesting the DNA with a methylation-insensitive delimiting enzyme (*Nco* I for the pKOH110 35SGUS-lines, and *Hind* III for the pPCV002 35SGUS-lines) in addition to *Sac* II, followed by Southern hybridization with the *nptII*-probe.

DNA was usually digested O.N.. The activity of the restriction endonucleases used to investigate copy number and methylation was checked by adding 0.5 μg unmethylated ϕX174 or λ-DNA to 1/10 of the reaction mix. The presence of the expected fragments of restricted DNA in such control reactions upon agarose gel electrophoresis was used as an indicator of efficient and complete digestion by the various enzymes.

Results

Three nptII-silencing phenotypes were identified

Transforming *A. thaliana* C24 with the vectors pPCV002 35SGUS and pKOH110 35SGUS (Figure 1) resulted in 67 and 44 independently generated transformants, respectively. Four weeks after germination on medium with Km (see Materials and methods) silencing of *nptII* was scored for each line. Three different silencing phenotypes were identified (Figure 2). Type I (Figure 2B) was defined as white cotelydonous plants with leaf primordia, light green cotelydonous plants and white plants with 3–4 small leaves. Type II was defined as plants with white deformed leaves often with green spots (Figure 2B). Type III consisted of larger green plants, with spotted leaves (Figure 2B). Seedlings not containing the *nptII* gene grown in the presence of Km have a phenotype similar to the Type I phenotype. However, the spotted and deformed Type II and III phenotypes are never encountered in wild-type seedlings. In these two phenotypes we assume that *nptII* is silenced in the cells of the white parts of the seedling, encrippling normal leaf development in the presence of Km.

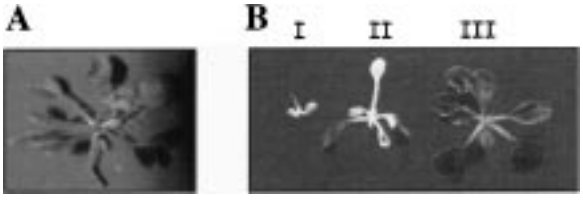

Figure 2. Silencing phenotypes. (A) Km^R phenotype. (B) *nptII*-silencing phenotypes. Type I, white cotyledonous plants or light green plants with maximum 3–4 leaves; Type II, white plants with deformed, pointed leaves often with green spots; Type III, larger green plants with spotted leaves. Depicted plants have all been grown for 4 weeks on MS-2 plants containing Kanamycin (50 mg/l).

For the pKOH110 35SGUS lines (K-lines), all categories of silencing phenotypes could be found within one line (Table 1). For the pPCV002 35SGUS lines (P-lines), all but one (line P4) displayed a characteristic silencing phenotype (Table 1). For four of the lines, the phenotypes differed between the control and the stressed groups (lines K3, K7, K8 and K10; Table 1).

NptII-silencing was found in more than half of the transgenic lines

Segregation analyses were performed on the T_1- and T_2-generations of the transgenic lines to determine the number of integration loci (see Materials and methods and Table 2). Silencing was scored in the T_3-generation, and observed in 56% of the 111 lines. A higher percentage of lines with *nptII*-silencing was found in K-lines (66%) than the P-lines (49%). Five of the K-lines (11%) displayed *nptII*-silencing in T_2, while this was found for 6% of the P-lines (Table 2).

Single-locus lines accounted for 45% of the lines displaying *nptII*-silencing, which is not significantly different from the percentage of single-locus lines screened (55%) (Table 2). However, the silenced lines of the homozygous single-locus B category represented a significantly smaller fraction of the total number of lines with silencing, than the B category of the total material (21% vs. 32%) (Table 2). Separate analyses of the P- and K-lines in the B category showed a significant under-representation of *nptII*-silenced lines generated with the pPCV002 35SGUS vector only (12% of the silenced lines vs. 27% of the material screened, Table 2). For all other categories, the fraction of lines displaying silencing was proportional to each category's representation in the total material screened (Table 2).

In a majority of lines, the frequency of nptII-silencing was significantly different between the stressed group and the control group

Eight P-lines (numbered P1-P8) and 12 K-lines (numbered K1-K12) displaying *nptII*-silencing were chosen for further studies. Eleven T_2 plants from each of the transgenic lines were temperature-stressed at 30°C during the rosette growth stage. Subsets of plants were in addition given cold nights or insecticide

Table 1. Identification of *nptII*-inactivation phenotypes

Line[a]	Inactivation phenotype	Comment
K-lines		
K1	Type I	
K2	All	
K3	All	Control: Type II, III; Stress: Type I, II
K4	Type I, II	One sibling with Type III
K5	All	Two siblings with Type III, the rest have Type I and II
K6	All	In two siblings all types are identified, the rest have Type III
K7	Type II & III	Control: 5 siblings with Type III and 7 with II and III; Stress: Type III
K8	All	Control: mainly Type I, Type III in one sibling; Stress: Type I and II
K9	All	
K10	Type II & III	Control: 5 siblings with Type I and II, 7 with Type III; Stress: Type III
K11	Type I	
K12	Type I & III	One sibling has mainly Type I
P-lines		
P1	Type III	
P2	Type II	
P3	Type II	
P4	Type I	One sibling with Type III
P5	Type II	
P6	Type I	
P7	Type I	
P8	Type III	

[a]The phenotypes are given for the twenty lines chosen for further studies.

spraying as these are conditions transgenic plants may encounter in the field (see Materials and methods). After 2 weeks with stress, plants were transferred to standard conditions (22°C), where they flowered and set seeds.

The frequency of *nptII*-silencing was determined as a percentage of *nptII*-silenced seedlings compared to the total number of seedlings. Between 1000 and 1300 seedlings, both from the stress-treated and from the control siblings of each line, were categorized as kanamycin-resistant or displaying *nptII*-silencing, and the overall silencing frequencies of the stress and control groups were calculated for each line (Figure 3). Since the extra stress treatments (4°C and insecticide, see Materials and methods) turned out to give no additional effects (data not shown) the stress-group was considered as one for the whole analysis.

Significant differences in overall frequency of silencing between the stress and control groups (as tested by a contingency chi-square analysis, see Materials and methods) were evident for all except four lines (K4, K8, P6 and P8; Figure 3). Four out of five lines with a low overall frequency of *nptII*-silencing (<10%) showed an elevated frequency in the stressed group compared to the control group (lines K1, K2, K6 and K12; Figure 3). This was also observed for several of the lines with a medium overall frequency (10–50%; lines K3, K9, P3, and P7, Figure 3), but not for any lines with more than 50% silencing. In six out of the eight lines with increased frequencies after stress treatment the changes were significant at the 0.1% level (HS in Figure 3). A significant decrease in overall silencing frequency, in five out of eight cases at the 0.1% level, was observed for one line with low (K11), and several lines with medium (lines K5, K7, K10, and P4) and high (lines P1, P2 and P5) overall frequency of silencing (Figure 3). Significant changes in the frequency of silencing could not be correlated to the T-DNA vector used.

The frequencies of silencing varied between progeny of siblings within most lines

For both the control and the stressed group we observed variation in silencing frequencies between pro-

Table 2. Identification of transgenic *Arabidopsis* lines with *nptII*-silencing

Category	Total					pKOH110 35SGUS					pPCV002 35SGUS				
	No. of lines (%)[a]	(%)[b]	*nptII*-silencing (%)[a]	(%)[b]	(%)[c]	No. of lines (%)[a]	(%)[b]	*nptII*–silencing (%)[a]	(%)[b]	(%)[c]	No. of lines (%)[a]	(%)[b]	*nptII*-silencing (%)[a]	(%)[b]	(%)[c]
A	26 (23.4)	} 55.0	15 (24.2)	} 45.2	57.7	12 (27.3)	} 65.9	8 (27.6)	} 58.6	66.6	14 (20.9)	} 47.8	7 (21.2)	} 33.3	50.0
B	35 (31.5)[d]		13 (21.0)[d]		37.1	17 (38.6)		9 (31.0)		52.9	18 (26.8)[e]		4 (12.1)[e]		22.2
C	9 (8.1)	} 18.0	6 (9.7)	} 21.0	66.6	4 (9.1)	} 13.6	4 (13.8)	} 17.2	100	5 (7.5)	} 20.9	2 (6.1)	} 24.3	40.0
D	11 (9.9)		7 (11.3)		63.6	2 (4.5)		1 (3.4)		50.0	9 (13.4)		6 (18.1)		66.6
E	21 (18.9)		12 (19.4)		57.1	4 (9.1)		2 (6.9)		50.0	17 (25.4)		10 (30.3)		58.8
F	9 (7.4)		9 (14.5)		100	5 (11.4)		5 (17.2)		100	4 (5.9)		4 (12.1)		100
Total	111 (100)		62 (100)		55.8	44 (100)		29 (100)		65.9	67 (100)		33 (100)		49.2

The transgenic lines were classified into six categories using the segregation data from T_1 and T_2. A: Single-locus lines, hemizygous in T_2. B: Single-locus homozygous lines. C: Two-loci lines, hemizygous in T_2. D: Two-loci homozygous lines. E: Homozygous lines, two or more loci. F: *nptII*-silencing in T_2. *nptII*-silencing was scored by plating T_3 seeds on Km selective medium. The number of pKOH110 35SGUS and pPCV002 35SGUS transgenic lines in each category is given, as well as the numbers in the total material. [a]Percentage of total. [b]Percentage combined for hemi and homozygous lines. [c]Percentage of *nptII*-silencing in each category. [d,e]Significantly different at the 5% level when tested with χ^2 analysis.

geny of different siblings (Figure 4; Table 3). The only exceptions were line P1 displaying 100% silencing in the control group, and line K8 with 100% silencing in all siblings. Most of the lines with low overall frequency of silencing had a small fraction of siblings displaying silencing (Table 3; Figure 4). In lines displaying a medium overall frequency of silencing, several had a high fraction of siblings displaying progeny with *nptII*-silencing (Table 3; Figure 4). The five high-silencing frequency lines showed silencing in progeny of all siblings, although the frequency varied between siblings for lines K4, P2 and P5 (Table 3).

Considering this high sibling variation, significant differences in silencing frequencies between control and stressed groups might be due to changes in the fraction of siblings with progeny displaying silencing. Alternatively, stress might change the fraction of siblings with progeny displaying high versus low frequencies of silencing. To test these hypotheses, for each line the fractions of siblings in the control and stress groups displaying silencing in their progeny was determined, as well as the fractions with low (<10%) and high (>10%) frequencies of silencing (Figure 4).

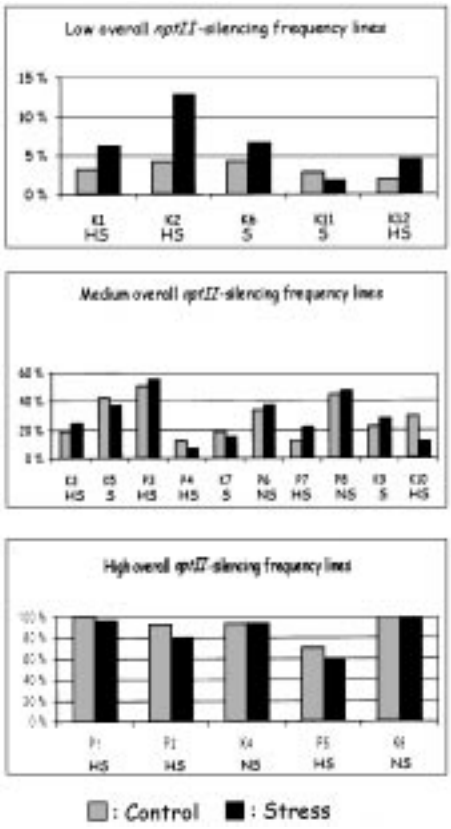

Figure 3. Analysis of the influence of stress on frequencies of *nptII*-silencing in 20 independent transgenic *Arabidopsis* lines. The figure shows the overall silencing frequency for 1000–1300 seedlings in control versus stress groups for each line, and is divided into three groups of overall silencing frequency (low <10%, medium between 10% and 50%, high >50%). The differences in overall silencing frequencies were evaluated using a contingency χ^2 analysis. The level of significance is given underneath the line number, with S, significant at the 5% level; HS, highly significant, at the 0.1% level; NS, not significant.

No clear correlation between stress-increased silencing and fraction of siblings with silenced seedlings was observed (Figure 4A). In three lines of this category (K1, K6, K12), an increase in the fraction of siblings displaying silencing was observed, while the opposite was observed for line K2 and K9. However, for the latter lines, there was an increase in the fraction of siblings showing high frequencies of silencing (Figure 4A). For the lines K3, P3 and P7, there was a shift from low to high frequency of silenced progeny per sibling.

Similar results were obtained for the group of lines where a decrease in the overall level of silencing was found in the stressed group (Figure 4B). Two lines (K11 and P4) had an increased fraction of siblings displaying silencing in the stressed group, but at the same time an increased fraction with low silencing levels. This shift towards lower frequencies of silenced progeny per sibling results in a lower overall silencing frequency compared to the unstressed controls. For the lines K7 and K10, the fraction of siblings with silenced progeny decreased in the stressed group, and at the same time, the fraction belonging to the high frequency group decreased. Among the lines with more than 50% overall frequency of silencing, the three lines (P1, P2 and P5) with lowered frequencies in the stressed group had reductions in the fractions of siblings for which 100% of the progeny displayed silencing (Table 3).

In the group of lines without significant differences in overall *nptII*-silencing frequency between the stressed and the control groups (K4, K8, P6 and P8), a high frequency of silenced progeny was observed for all siblings in both groups (Table 3).

Three out of 20 lines showing nptII-silencing harboured a single T-DNA copy

Transgene copy number was determined for silenced lines by Southern analysis. Genomic DNA was digested with *Eco*R I or *Hind* III to investigate the number of left and right borders integrated (see Materials and

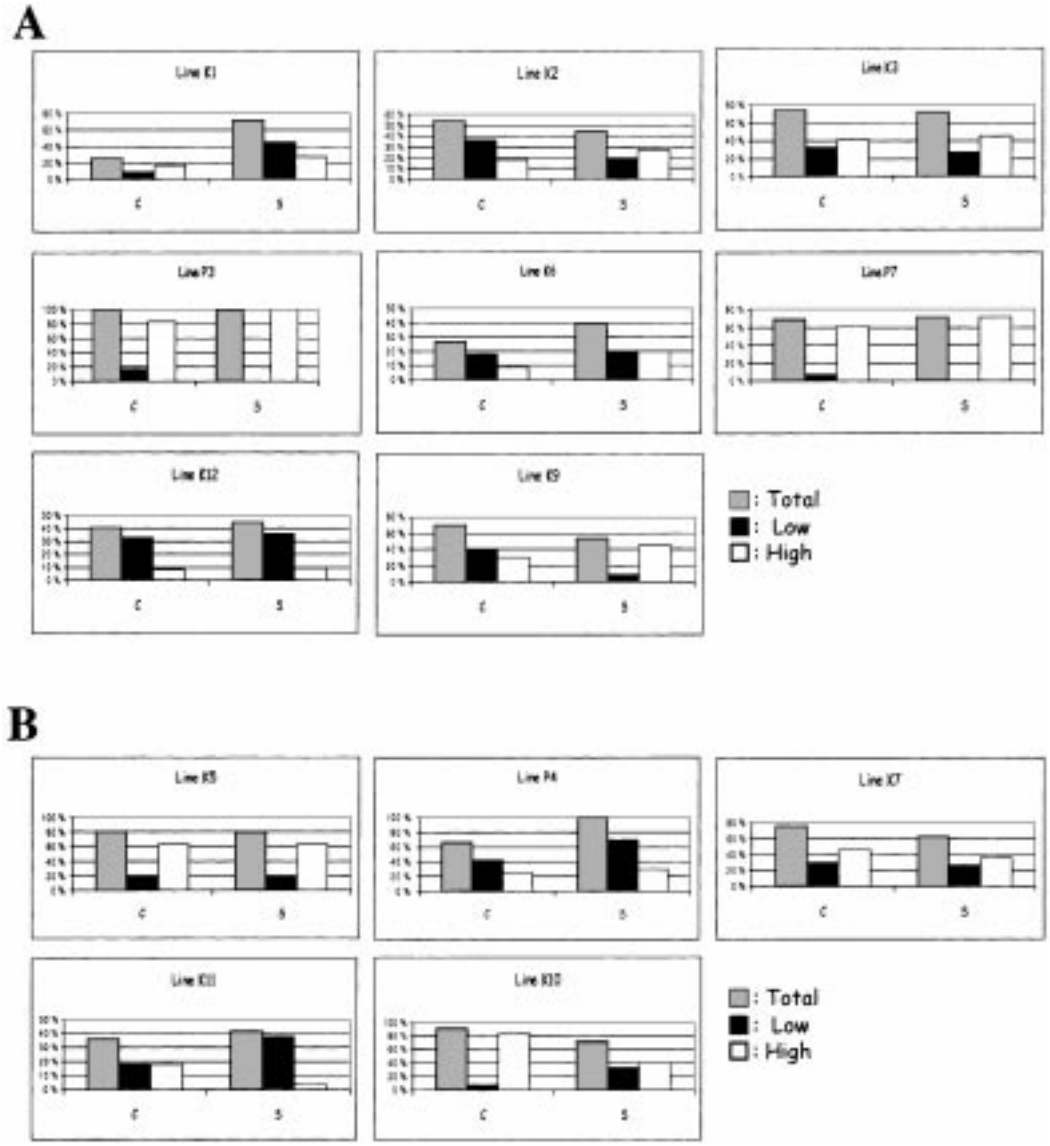

Figure 4. Fraction of siblings in control (C) and stress (S) groups with *nptII*-silencing in their progeny. The fractions of siblings with low (<10%) and high (>10%) silencing are also shown. (A) Lines with higher overall frequency of silencing in the stressed group. (B) Lines with decreased overall frequency of silencing in the stressed group. Data are not shown for lines where all siblings displayed a high frequency of *nptII*-silencing.

methods). The Southern blots were hybridized with a *nptII* and a *gus* coding-region probe. Seventeen of the 20 lines contained multiple T-DNA copies (Table 3).

Of the eight single-locus lines, two hemizygous lines (K11 and P4) showed a single hybridizing band and were thereby identified as single-copy. The remaining single-locus hemizygous and all the single-locus homozygous lines contained multiple T-DNA copies (Table 3). Two of them contained an inverted repeat of the transgene (lines K10 and K12, cf. Materials and methods).

For the seven lines displaying *nptII*-silencing in T_2 (category F), Southern analysis identified a single T-DNA copy in line P6; two copies inserted as inverted repeats in lines K5 and P8; and two tandem repeats, with a deleted *gus* gene in one of the copies, in line P7 (Table 3). The last three lines of this category contained four or more copies (Table 3).

Table 3. Characteristics of lines displaying *nptII*-silencing

Line	Variation in *nptII*-silencing frequency between siblings[a]		Overall silencing frequency[b]	Category [c]	Copy #	Organization
	Control	Stress				
Lines where frequency of silencing is lower in control than in stress groups						
K1	16.3–17.6	1.2–20.7	L	A	>2	nd
K2	3.2–13.8	3.9–61.7	L	B	2–4[e]	nd
K3	1.0–71.7	2.7–84.0	M	D	2	→, →
K6	1.1–42.5	3.9–32.8	L	E	2	→, →
K9	1.4–96.6	2.2–83.3	M	C	5[f]	nd
K12	1.1–12.7	1.6–36.9	L	A	2	→←
P3	2.4–100 [d]	10.5–100 [d]	M	A	5	nd
P7	0.9–22.7	12.7–77.8	M	F	2[g]	→→
Lines where frequency of silencing is comparable in control and stress groups						
K4	56.8–100 [d]	65.6–100 [d]	H	F	>4	nd
K8	100 [d]	100 [d]	H	F	>4	nd
P6	28.1–40.7 [d]	28.1–50.0 [d]	M	F	1	→
P8	34.2–52.4[d]	41.4–51.7[d]	M	F	2	→←
Lines where frequency of silencing is higher in control than stress groups						
K5	11.9–100	1.6–99.4	M	F	2[h]	→←
K7	0.9–62.1	1.1–39.5	M	B	4–5	nd
K10	2.4–38.7	2.0–53.8	M	B	2	→←
K11	0.7–4.2	0.73–1.9	L	A	1	→
P1	100 [d]	91.0–100 [d]	H	E	>2	nd
P2	62.5–100 [d]	26.2–100 [d]	H	F	>4[i]	nd
P4	1.7–100	1.7–18.9	L	A	1	→
P5	25.9–100[d]	30.7–78.4[d]	H	E	5–6[j]	nd

[a]The frequency of silencing is given for the siblings displaying silencing. [b]L: Low overall frequency of silencing <10%. M: Medium overall frequency of silencing between 10% and 50%. H: High overall frequency of silencing >50%. [c]Refer to Table 2. [d]All siblings within the line display silencing. [e]Fewer fragments in progeny from three siblings. [f]Extra fragments in progeny of seven siblings. [g]Contains one partial copy. [h]Extra copy in progeny of one sibling. [i]Fewer copies in progeny of one sibling. [j]Fewer fragments in progeny of two siblings.

Two lines had been categorized as two-loci by segregation analysis (Table 3). Line K3 was shown to contain two copies, whilst line K9 contained multiple copies. The Southern analysis confirmed the presence of two or more copies in the three lines (K6, P1 and P5) of category E (Table 3).

Homozygosity increased the probability of silencing in one line

Five of the lines studied more thoroughly had been identified as hemizygous in the T_2 generation by segregation analysis (lines K1, K9, K11, K12, P3 and P4) (Table 3). To investigate whether there was a correlation between homozygosity and silencing of the *nptII* gene, the frequencies of silencing in progeny of hemi and homozygous siblings of the T_3 generation were compared. Only in line K12 there was an over-representation of homozygous siblings displaying silencing, that is, 4/5 of the siblings with progeny displaying silencing were homozygous. In line P3, all the siblings displayed silencing, while for the rest of the hemizygous lines (lines K1, K11 and P4) silencing was proportionally represented between homozygous and hemizygous siblings (data not shown).

Methylation was observed in the Sac II *site of the pnos promoter in multi-copy, but not in single-copy lines*

We investigated if loss of kanamycin resistance was correlated with methylation of the *Sac* II site in the *pnos* promoter region, since this has been shown in other studies (Matzke et al., 1989; Kilby et al., 1992).

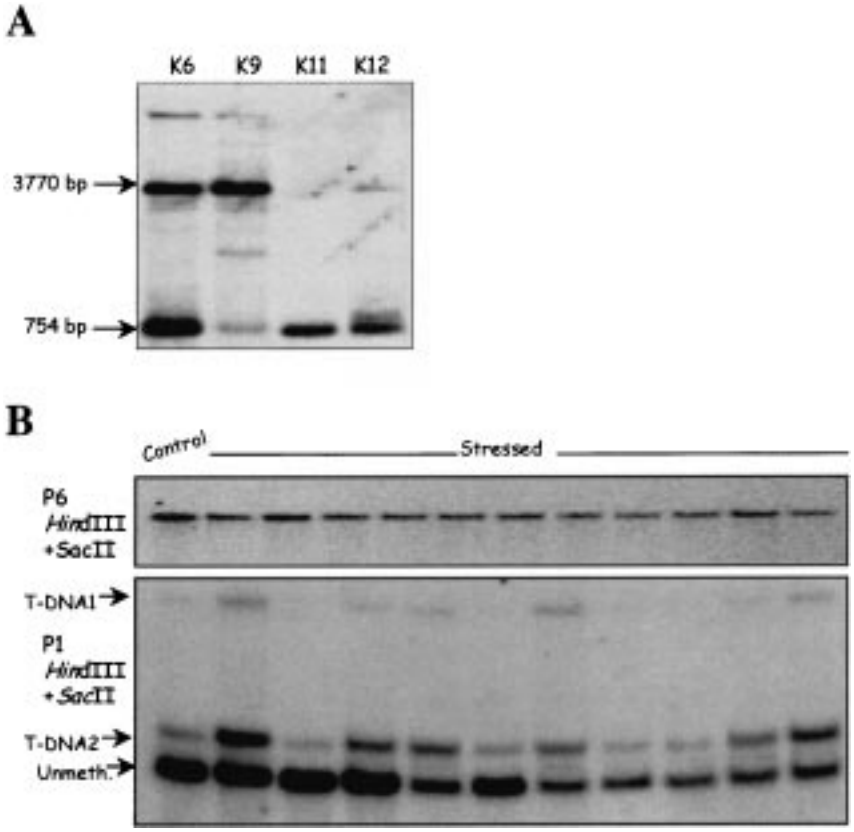

Figure 5. Methylation analysis of the *Sac* II site in the *pnos* promoter. (A) DNA from K-line plants was digested with the methylation insensitive enzyme *Nco* I and the methylation sensitive *Sac* II enzyme, and subjected to Southern hybridization using the coding region of *nptII* as a probe. *Nco* I has a recognition site in the 35S promoter and at the 3′ end of the *nptII* gene. Digestion with *Nco* I generated a 3770 bp fragment which hybridizes to the probe. Digestion with *Sac* II will divide this fragment, and generate a hybridizing fragment of 754 bp. The figure shows examples of different levels of methylation in different lines, for example almost complete methylation in line K9 and no methylation in line K11. (B) Digestion of DNA from P-lines with *Hind* III and complete digestion in the *Sac* II recognition site in the *pnos*-promoter by the methylation sensitive *Sac* II enzyme results in a hybridizing band of 1660 bp. In line P6, this is the only band found, indicating that this site is unmethylated in progeny of all siblings. The overall silencing frequency in this line was more than 30% (cf. Figure 2). In line P1, in addition to the 1660 bp band expected when the *Sac* II site is unmethylated (Unmeth.), two bands of variable intensities are seen. These bands (T-DNA1 and T-DNA2) represent fragments spanning each of the T-DNA copies from the internal *Hind* III site to a first *Hind* III site in the flanking plant DNA. Variation in relative hybridization labeling for the three bands reflects variations in methylation levels of the *Sac* II site. This variation was not correlated to *nptII*-silencing frequencies, which was between 91 and 100% per sibling.

In all the multi-copy K-lines, the *Sac* II site was partially methylated, while this site was unmethylated in the single-copy line K11 (examples shown in Figure 5A). In the P-lines, the *Sac* II site was partially methylated in five out of six multi-copy lines (line P1, P3, P5, P7 and P8; examples shown in Figure 5B). In the sixth (line P2), the site was fully methylated (data not shown). In contrast, the site was unmethylated in the single-copy lines P4 (data not shown) and P6 (Figure 5B).

In most cases, no differences were found in the methylation patterns in progeny of stressed siblings as compared to control sibling progeny, for example, this site is unmethylated both in control and in stressed progeny of line P6 irrespective of silencing frequency (Figure 5B). In some cases, however, variations in methylation levels were evident within a line, for example line P1 (Figure 5B). This variation was not correlated with silencing frequencies.

Variation in frequency of silencing was not correlated to rearranged transgenes

Southern analysis (see Materials and methods) with a *nptII* coding-region probe (Figure 6) and a *gus* coding-region probe (not shown) was performed on

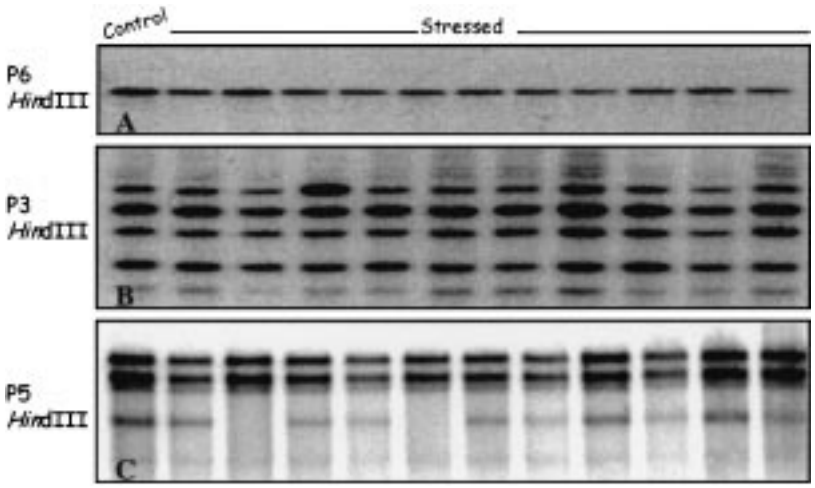

Figure 6. Analyses of transgene copy number by Southern hybridizations using a *nptII*-probe on progeny from one control sibling and 11 stress-treated siblings. (A) A single hybridizing band is indicative of one T-DNA copy in line P6. Hydridization with a *gus*-probe gave the same result (not shown). (B) The hybridization pattern in line P3 indicates the presence of at least five copies in progeny of all siblings. The hybridization patterns in all lanes are similar although the level of silencing in progeny of different siblings varied considerably (cf. Table 3). (C) The hybridization patterns in line P5 show that one copy is missing in the progeny of some siblings. Such variation in copy number could not be correlated with variations in silencing frequencies.

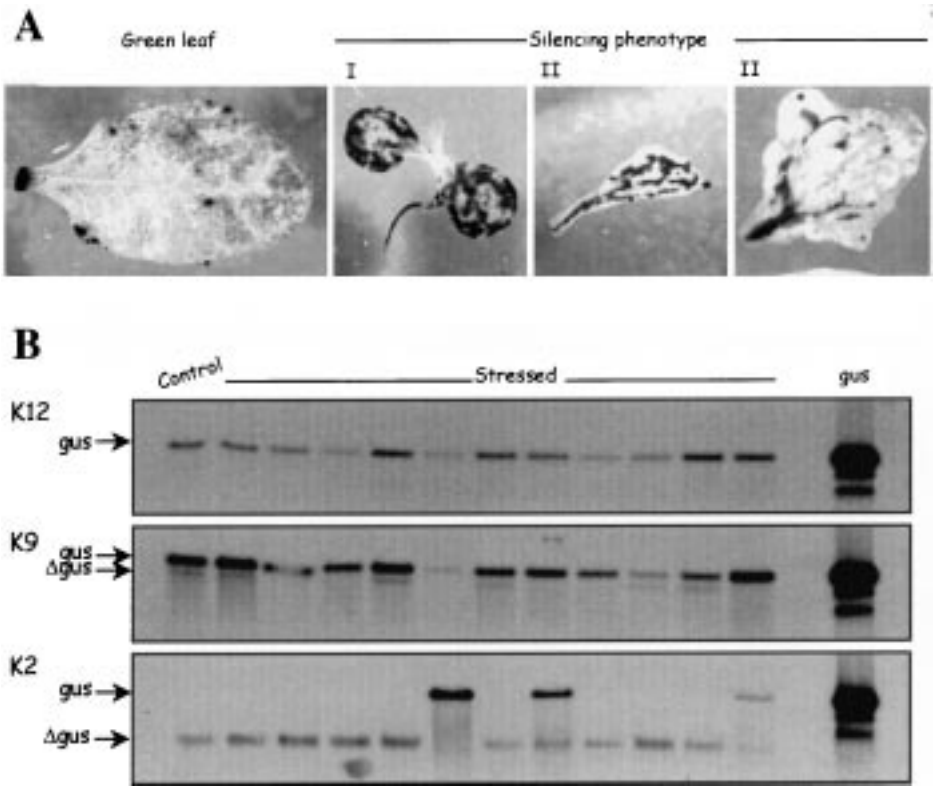

Figure 7. Gus analysis. (A) Gus staining of leaves from Km^R plant (Green leaf) and Km^S plants of different phenotype (Type I and Type II). (B) Integrity of gus gene. Genomic DNA was digested with *Eco*R I and *Hind* III, blotted and hybridized to a *gus* coding-region probe. A hybridizing band of 2714 bp (gus) was expected as seen for line K12. The smaller fragments (Δgus) in line K9 and K2 indicate deletions in the *gus* gene. As a control pKOH110 35SGUS plasmid digested with *Eco*R I and *Hind* III was included in each gel (lane labeled gus).

progeny of stress-treated plants to analyze whether the observed variations of *nptII*-silencing frequencies were associated with rearrangements of the transgenes. In the majority of lines (e.g. P6 and P3, Figure 6A and 6B), the same number of hybridizing bands were found in progeny of all siblings. Five lines (K2, K5, K9, P2 and P5) showed siblings with a changed hybridization pattern (Table 3; Figure 6C). However, no correlation was found between the silencing frequency and the rearranged transgenes, or between copy number and stress-influence on *nptII*-silencing.

Silencing of the pnos-nptII transgene and the p35S-gus transgene was not correlated

The transgenic lines displaying *nptII*-silencing were screened for GUS activity. This was undertaken in part to discriminate between the Type I silencing phenotype of plants displaying *nptII*-silencing and normal cotyledonous seedlings of untransformed plants. In the green plants tested, GUS activity, and a single *Eco*R I-*Hind* III restriction fragment of 2714 bp hybridizing to a *gus* probe, were observed in all lines except K2 and K9 (Figure 7). In line K9, two fragments were identified, of which one was of the expected size. For line K2, progeny of many siblings only had one smaller than expected fragment. This indicates that the lack of GUS-activity was due to a deletion and not to silencing of the *gus* gene.

For both vectors, GUS activity was observed in all the three *nptII*-silencing phenotypes (Figure 7A). Although it should be noted that not all cells express GUS at the same strong level, our results show that the two different transgenes are not necessarily silenced cooperatively. However, for some of the hemizygous lines, Type I silenced progeny of homozygous siblings did not stain for GUS activity at all (lines K1, K12, and P3), indicating complete silencing of the *gus* gene (data not shown).

Discussion

Transgenic Arabidopsis *displays a high incidence of silenced lines independent of T-DNA construct used*

As many as 56% of the 111 lines tested in our study show silencing. Others have reported a high incidence of silencing in *Arabidopsis*, petunia, tobacco and rice (Matzke et al., 1989; Kilby et al., 1992; Neuhuber et al., 1994; Van Blokland et al., 1994), stressing that transgene silencing is not a vector-, gene- or species-specific phenomenon. A general mechanism for scanning the genome of intrusive DNA, followed by modulation of this DNA, has thus been proposed for plants (Matzke et al., 1996).

The majority of the lines investigated in our study were multi-copy (Table 3). A high copy number may render the transgenic lines more susceptible to silencing, due to homology-dependent silencing mechanisms (Assaad et al., 1993; Matzke et al., 1994). However, our data show (see Table 3) that the presence of two or more copies need only result in a low level of silencing ($<$10%). In four of our lines (lines K5, K10, K12 and P8), the T-DNA was integrated as an inverted tandem repeat (IR). This situation is considered inherently prone to *de novo* methylation and silencing of both endogenous and exogenous genes (reviewed by Selker, 1999). Silencing in these lines is therefore not unexpected.

Cases describing silencing of single-copy transgenes are rare (Meyer et al., 1993; Elmayan & Vaucheret, 1996). Our results show that in *Arabidopsis*, the incidence of single-copy transgenes displaying silencing is high enough to be identified in a screen of just over 100 lines. Of the 20 lines investigated thoroughly in our study, three are single-copy (lines K11, P4 and P6). In addition, several lines displaying progressive silencing have been identified in another study using 11 single-copy homozygous lines (Meza et al., unpublished).

The orientation of the nptII gene may influence the incidence of silencing

A higher percentage of silencing was observed for the lines transformed with pKOH110 35SGUS compared to pPCV002 35SGUS, and in contrast to the P-lines, several silencing phenotypes were observed within each K-line. In addition to copy number, position and GC content of the T-DNA compared to the flanking DNA can be expected to influence transgene expression (reviewed by Matzke & Matzke, 1998). The CG content in our two vectors is the same ($\sim$49%), and the pattern of integration is likely to be similar for different T-DNA constructs. Therefore, differences in silencing frequencies and phenotypes for our K- and P-lines might be due to properties deviating between the two T-DNA constructs.

Bacterial sequences have been associated with reduced transgene expression both in mammals and plant lines (Iglesias et al., 1997). The pPCV002 35

SGUS T-DNA contain more prokaryotic DNA (note the Ap^R gene, Figure 1) than pKOH110 35SGUS. However, this does not lead to a higher incidence of silenced P-lines, and therefore seem to be of minor importance.

In pKOH110 35SGUS *pnos-nptII* is separated from the right border by 3000 bp, while close to this border in pPCV002 35SGUS (Figure 1). A direct influence by neighboring plant DNA on T-DNA reporter gene promoter activity near the right border has been suggested (Breyne et al., 1992). Thus, in the present study one could expect the influence of the neighboring DNA on the *nptII* gene to be higher in P- than in K-lines. On the contrary, the observed incidence of silencing was higher in K-lines. Possibly, in pKOH110 35SGUS where the 3′-end of the *nptII* gene is close to the LB, transcription from a plant promoter into the T-DNA from the left border could generate an antisense *nptII* transcript leading to gene silencing. Whether transcriptional interference and antisensing is a major cause of position effects needs further investigation.

Homozygosity influence silencing in certain lines only

Based on our original screening results, single-locus homozygous P-lines (category B) appeared to be more stable than any other category of P-lines and all categories of K-lines, in sharp contrast to earlier reported cases of homozygosity-promoted silencing (De Carvalho et al., 1992; Deborne et al., 1994; Dehio & Schell, 1994). However, Southern analysis revealed that three out of four P-lines showing silencing in the T_2 generation (category F), were single-locus (lines P6, P7 and P8). In line P7, less than 25% silenced progeny was observed per sibling, indicating that this is a homozygous line (Table 3). Thus, it is reasonable to assume that the observed under-representation of silenced single-locus homozygous P-lines in the T_3 generation, is due to silencing in the previous generation.

Only one line (K12) was identified with a statistical over-representation of silencing in the homozygous versus the hemizygous state. Our results therefore indicate that homozygosity-promoted silencing of certain transgene-loci and configurations is dependent on the genomic context.

Variation in frequency of silencing between progeny of siblings is not dependent on loci- or copy-number

Variations in the frequency of transgene silencing between different siblings of the same line have previously been shown in multi-copy lines of transgenic petunia, *Arabidopsis* and tobacco (Schmülling & Röhrig, 1995; Ulian et al., 1996; De Neve et al., 1999). Our results show that such variation can be found also in single-copy lines.

We assume that the epigenetic changes leading to *nptII*-silencing in seedlings, is a stochastic event taking place in cells of the sibling plants, and that these changes are transmitted to daughter cells generated by mitosis and later meiosis (Schmülling & Röhrig, 1995). Silencing is thereby transmitted to embryos resulting from self-pollination. It is unlikely that the epigenetic changes are initiated solely in each seed or seedling, since the frequency of silencing in progeny and the phenotype of silenced progeny is characteristic for the seedlings from each individual sibling plant. The fraction of siblings displaying silencing and the frequency of silencing in progeny from each sibling are likely to be dependent on the position and the number of cells in which the silencing event occurs.

In 12 out of 20 lines, the T-DNAs have been integrated in a single locus (Table 3). It is conceivable that the variable expression of the *nptII*-gene in these lines is due to constraints on transcription imposed by structural features in the flanking plant DNA or the T-DNA itself, for example chromatin configuration and/or DNA methylation (Pröls & Meyer 1992; Meyer et al., 1993; Ten Lohuis et al., 1995). Genomic position leading to hypermethylation and silencing has previously been shown for single-locus transgenes in petunia (Meyer & Heidmann, 1994). However, in our single-copy lines, methylation analysis of the unique *Sac* II site in *pnos* did not reveal methylation. In contrast, for our multi-copy lines as well as those investigated by others, methylation of the *Sac* II site seems to be correlated with a decrease in *nptII* gene expression (Matzke et al., 1989; Kilby et al., 1992; Matzke et al., 1993; Ulian et al., 1996). Still, we cannot exclude that methylation is involved in silencing of our single-copy transgenes, as the methylation level of the *Sac* II site may have been too low for detection in the assay used. Alternatively, other more critical sites may be methylated, as proposed by Kilby et al. (1992).

The two vectors used contain the *35Sgus*-gene in addition to the *pnos-nptII*-gene. If the position of the T-DNA in the genome can influence *nptII*-silencing, one might expect a coordinated silencing of *nptII* and *gus*, while we only have indications of *gus* silencing in a few of our lines. We cannot exclude, however, that the *35S*-promoter is less sensitive than the *pnos*-promoter to structural constraints.

Environmental stress can influence the frequency of silencing positively or negatively

In our study, stress was applied prior to flower development, indicating that stress applied during the rosette growth stage, or during the transition of the vegetative meristem to an inflorescence meristem, might result in altered frequencies of silencing. Stress seems to enable changes in the susceptibility to silencing at three different levels. First, stress can result in a change in the fraction of sibling plants giving rise to silenced progeny, for example, line K1. Second, the number of seeds of each plant where the transgene has been silenced can be influenced, probably due to a change of the number of cells with silenced transgene(s) within individual plants, for example, line P3. And third, the silencing phenotype can be changed. In seedlings of the white cotyledon phenotype (Type I), all transgene copies are likely to be completely silenced in all cells. In seedlings of the Type II and III phenotype, which develop beyond the cotyledon stage and may have green spotted, pointed leaves, transgenes are likely to be only partially silenced. In line K3, which show a significant increase in silencing after stress treatment, the seedlings of the stressed plants showed the Type I and II phenotypes, while the control seedling were of the II and III type. Conversely, line K10 show a significant reduction in *nptII*-silencing and at the same time all the silenced progeny from the stressed group had the Type III phenotype, while mainly Type I and II were found in the control group.

There are indications that chromatin remodeling can be used by plants in the perception of environmental signals (Meyer, 1999), and examples both of increased and reduced levels of methylation in response to stress (Burn et al., 1993; Kovarik et al., 1997). Therefore, we propose that in cases where silencing is modulated by environmental factors, the transgenes have been integrated into genomic regions that experience epigenetic alterations during stress-treatment, for example, changes in methylation patterns and/or chromatin conformations. The lack of detectable methylation of the *Sac* II-site of our single copy lines may indicate that the chromatin configuration is more important than the presence of methylation (Kooter et al., 1999). Our efforts are now aimed towards studies of the genomic sequences flanking the T-DNA in one-locus lines, some of which contain a single copy. Such information may be useful in the generation of transgenic lines in which a stable expression pattern is ensured.

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