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E. C. Ulian · J. M. Magill · C. W. Magill · R. H. Smith

DNA methylation and expression of NPT II in transgenic petunias and progeny

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Abstract The expression and inheritance of the NPT II (neomycin phosphotransferase II) gene was studied in four transgenic petunia (*Petunia hybrida* Vilm.) plants and their progeny. The four transgenic plants, each of which had more than one site of insertion, were different from each other in the level of foreign gene expression. Transmission of one or more NPT II alleles to progeny as deteceted by DNA hybridization did not lead to consistant or predictable patterns of NPT II expression. All transgenic plants and their progeny displaying NPT II enzyme activity contained unmethylated *Sst*II (methylation-sensitive restriction enzyme) sites in the nopaline synthase (NOS) promoter (controlling NPT II gene transcription); whereas, 13 of 17 plants which contained the NPT II gene and which showed no NPT II activity had methylated *Sst*II sites. Two progeny of 1 transgenic plant appeared to have some unmethylated *Sst*II sites, but no NPT II enzyme activity was found in leaf tissue samples. DNA methylation of the *Sst*II site in the NOS promoter is strongly correlated with a decrease in NPT II gene expression in transgenic petunia plants and their progeny. However, DNA methylation alone could not account for the variability seen in NPT II gene expression.

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E. C. Ulian
Caixa Postal 162, Centro de Tecnologia Copersucar, 1340 Piracicaba SP, Brazil

J. M. Magill · C. W. Magill
Department of Plant Pathology and Microbiology, Texas A&M University, College Station, TX 77843, USA

R. H. Smith (✉)
Department of Soil&Crop Sciences, Texas A&M University, College Station, TX 77843, USA

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Introduction

The understanding of how transformed plants incorporate foreign DNA and how that DNA is expressed in subsequent generations is of paramount importance for genetic engineering of agronomically important crop plants. DNA methylation is one control mechanism by which gene expression may be regulated in plant cells. Many reports show that foreign DNA introduced into cells often becomes highly methylated and that the foreign genes are not expressed. Hepburn et al. (1983) demonstrated a decreased expression of foreign DNA which had been introduced into plants through *Agrobacterium*. In that study, tumors caused by an *Agrobacterium* strain containing the opine genes did not produce the opines. When treated with 5-azacytidine (an inhibitor of DNA methylation) these tumor lines began synthesizing opines. Comparable observations were subsequently made by Amasino et al. (1984) who observed that the phenotypic variation expressed by *Agrobacterium*–transformed tumor lines was due to the methylation of different regions in the T-DNA (transforming DNA). Hershkovitz et al. (1990) reported that in vitro methylation of the plasmid used to introduce the bacterial CAT (chloramphenicol acetyltransferase) gene into petunia protoplasts resulted in complete inhibition of CAT gene expression in the transformed cells.

Transgenic *Arabidopsis* demonstrated progressive loss of kanamycin resistance over four generations of inbreeding; this loss of resistance was associated with methylation of the nopaline synthase, (NOS) promoter (Kilby et al. 1992). Recently, Ottaviani et al. (1993) reported the absence of GUS (β-glucuronidase) expression in potato with multiple copies of the GUS gene due to methylation of the promoter and coding region.

Multiple insertions of a foreign gene often occur during the transformation of cells, and copies generally become methylated. Linn et al. (1990) showed that in petunia plants transformed with a *Zea mays* gene single-copy plants usually showed continuous expression of pigment; whereas, multiple-copy integrants generally exhibited methylation of all of the promoters integrated. Also, the presence of one copy of a gene can affect the methylation and expression of a second copy inserted into the genome. Matzke et al. (1989) sequentially transformed tobacco plants with two distinct T-DNAs encoding different selection and screening markers. Genetic analysis of the progeny of doubly transformed plants showed that the genes from the first transformation event (T-DNA I) were often not expressed when the promoter regions of these genes were methylated. However, in plants where backcrossing or self-fertilization produced progeny containing only T-DNA I, the genes were expressed, and the promoters were unmethylated. The conclusion was that the introduction of the second T-DNA affected the expression of genes in an unlinked T-DNA already present in the genome. In a subsequent paper, Matzke and Matzke (1991) reported that the extent of the methylation of the NOS promoter in these transformed plants decreased in successive generations with a concomitant increase in the strength of the kanamycin-resistant phenotype, presumably due to the increased expression of the NPT II (neomycin phosphotransferase II) gene under NOS promoter control. Hobbs et al. (1990) also reported a low level of GUS expression in tobacco plants to be associated with integration of multiple gene copies as well as methylation of the DNA. More recently, Hobbs et al. (1993) have shown that the presence of inverted repeats integrated at one site led to low levels of GUS expression, even affecting other unlinked GUS insertions.

In the study presented here, four primary transgenic petunia plants and their progeny were selected to examine NPT II gene activity and methylation at an *Sst*II (CCGCGG) site of the NOS promoter. These four plants varied from each other in the expression of the NPT II gene and in the methylation of the NOS promoter.

Materials and methods

Explant source

Seeds of a commercial petunia (*Petunia hybrida* Vilm.) variety, 'Rose Flash'(F_1 hybrid: 'Single Grandiflora' × 'Deep Rose'), were provided by the Ball Seed Company, Chicago, III. The seeds were germinated under sterile conditions. The shoot tips were excised and transformed using *Agrobacterium* as described previously (Ulian et al. 1988, 1994). None of the primary transformants (which had been selected on 200 mg/l kanamycin in vitro) were chimeric for GUS expression based on random leaf tissue sampling of individual plants.

Bacterial strain and plasmids

The binary vector pRGUS2 was constructed by Dr. T. Thomas (Biology Department, Texas A&M University). It contains a chimeric marker gene composed of the regulatory sequences of the NOS gene and the coding sequence of a bacterial NPT II gene which confers resistance to kanamycin. It also contains the UGS reporter gene (Fig. 1).

The vector was prepared by cloning a *Bam*HI to *Sst*II restriction fragment containing the GUS coding region into the polylinker site of pROK2, an expression vector derived from pROK1 by the insertion of a polylinker. This placed the GUS gene between the CaMV 35S promoter and the NOS polyadenylation signal in the T-DNA. The pRGUS2 plasmid was conjugated from *E. coli* strain HB101 into the avirulent *A. tumefaciens* strain LBA 4404 as described (Simpson et al. 1986). Bacteria growth and maintenance was performed as previously described (Ulian et al. 1988).

Double digests of this T-DNA with *Hin*dIII and *Sst*II yields a 2.1-kb fragment if neither site is methylated (Fig.1).

Detection of DNA methylation

Plant DNA was extracted from 1 g fresh leaf tissue utilizing the method of Dellaporta et al. (1985), and stored at −20 °C until use. Leaf samples representing more than ten leaves from each plant collected at random were pooled. DNA samples (3 µg) were digested with either *Hin*dIII or *Hin*dIII plus *Sst*II (Gibco-BRL, New York) for 16–18 h according to the manufacturer's instructions. *Hin*dIII cleaves AAGCTT sequences while *Sst*II cleaves CCGCGG sequences, but only if the internal C is unmethylated. The DNA fragments were subsequently separated overnight on a 0.8% agarose gel at 30 V and transferred to Genescreen Plus (NEN Research Products, Boston) according to the manufacturer's instructions.

Hybridization and rehybridization of DNA

The membrane was prehybridized at 42 °C overnight in 30 ml of solution containing 50% formamide, 1% SDS, 1 *M* NaCl, and 10% dextran sulfate. The radioactive probe solution (50 µl) was added to 500 µl of a 10 mg/ml solution of denatured herring sperm DNA and injected into the bag using a 1-ml hypodermic syringe and needle. The small hole was heat-sealed, and the hybridization was performed overnight at 42 °C. The membrane was washed as follows: (1) Two 5-min washes in 2 × SSC and 1% SDS at 65 °C, (2) Two 30-min washes in a solution containing 2 × SSC and 1% SDS at 65 °C and (3) Two 30-min washes in 0.1 × SSC at room temperature. All washes were performed with constant agitation. After washing, the membrane was placed in a plastic bag, sealed and exposed to Kodak X-OMAT autoradiographic film.

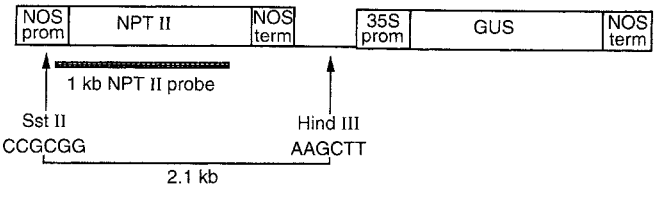
Production of probe

The NPT II probe was prepared as described in Ulian et al. (1994).

Determination of NPT II activity in transformed plants

Twenty mg of a 1-g tissue sample representing over ten leaves was collected from each plant and immediately extracted in 200 µl of ice

Fig. 1 A simplified map of the region of the pGUS2 plasmid containing the NPTGUS chimeric construct. The 2.1-kb fragment resulting from a *Hin*dIII –*Sst*II digestion as well as the 1-kbNPTII probe region are shown in detail



cold GUS lysis buffer (Jefferson 1987) of the following composition: 50m*M* $NaPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10mM Na_2 EDTA, 0.1% sodium lauryl sarcosine, and 0.1% Triton X-100. The homogenates were centrifuged at 4 °C for 15 min in a microfuge, and the supernatant transferred to fresh tubes and stored at −80 °C. The total protein of each sample was determined by the Bradford method.

NPT enzyme activity was analyzed following the method of Staebell et al. (1990). Final results are shown as an average of three assay values normalized for total protein content, establishing enzyme activity as picograms NPT II/h per microgram total protein. There was no significant variation in the level of expression of NPT II activity over several months' sampling of an individual plant.

Results and discussion

Four plants (G09, B111, RC1, G81) expressing different gene product activity were selected to study the relationship of methylation at the *Sst*II site in the NOS promoter to the expression and inheritance of the NPT II gene. Plant G09 was selected because it gave consistently high levels of NPT II activity, and plant B111, because it had a consistently lower level of NPT II activity. Although plant RC1 did not show NPT II activity it was selected because several of its progeny did. Plant G81 was selected because it expressed the NPT II gene in culture (resistance to kanamycin) and in the leaves of the vegetative plant, but as a flowering plant NPT II activity in leaf tissue could not be detected.

Each of these primary transformed plants was selfed and backcrossed. In all of the backcrosses, pollen from the transgenic (NPT II^+) plant was used to fertilize the NPT II^- parent. All progeny were screened for kanamycin resistance during seed germination and for NPT II enzyme activity in leaf tissue after 30 days. The results of screening seedlings for kanamycin resistance are shown in Table 1. The ratios observed from selfs and testcrosses clearly deviate from those expected for simple Mendelian inheritance patterns (Table 1). In no case does the expected number of kanamycin-resistant plants approach the expected number for a single expressed gene insert.

Genomic DNA was isolated from each of the 4 primary transformed plants and from 5 progeny chosen at random (Figs. 2–5). DNA was digested with *Hin*dIII alone and in double digests (HS) with both *Hin*dIII and *Sst*II. Southern analyses of all DNA digests with *Hin*dIII alone resulted in DNA fragments significantly larger than 2.1 kb when hybridized with the 1-kb probe (Fig. 1). In double digests, the DNA from several plants yielded 2.1-kb fragments, indicating that the *Sst*II sites in the NOS promoter were unmethylated. Of the primary transformed plants, only G09 had an unmethylated *Sst*II site in a NOS promoter (Fig. 5). DNA double digests (HS) of primary transgenic plants G81 (Fig. 3) and RC1 (Fig. 2) didn't show the 2.1-kb band, indicating that the *Sst*II site in the NOS promoters of these plants was methylated.

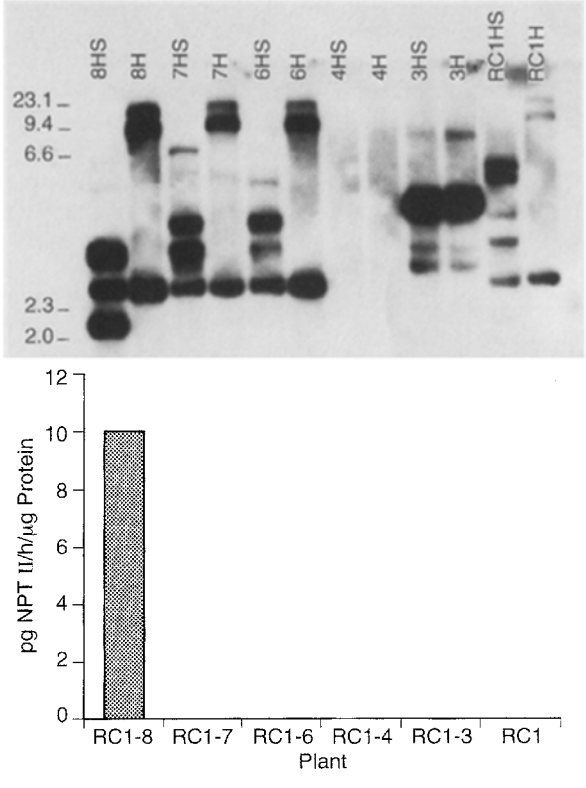
In each transformant examined, there is more than one site of insertion. Southern analysis of *Hin*dIII digests (Figs. 2–5) revealed that each of the primary transformants have $NPTII^+$–hybridizing sequences present at two or three sites in the genome. Though only 5 progeny of each self-fertilized R_0 plant were examined using Southern analysis, most of the NPT II DNA-hybridizing fragments appear to segregate independently, thus minimizing the likelihood that larger bands are simply products of incomplete *Hin*dIII digestions.

Table 1 Segregation of kanamycin resistance in progeny from selfs and testcrosses

Plant		Self Observed		Testcross Observed	
	Number of copies	NPT II^+	NPT II^-	NPT II^+	NPT II^-
RC1	3	24	108	0	90
G81	3[a]	0	90	0	90
B111	2	0(2)[b]	100[b]	0	100
G09	3	73	57	49	68

[a] Possibly two insertion sites, one with tandem copies
[b] Two of the 100 progeny showed NPT II^+ activity in leaf samples after 30 days

Fig. 2 A comparison of the phenotypic expression of the NPT II gene in plant RC1 and its progeny, and the methylation status of the *Sst*II site in the NOS promoter of these plants. The NPT II values are pg/h per microgram protein. Plant genomic DNA was digested with either *Hin*dIII (H) or *Hin*dIII plus *Sst*II (*HS*) and analyzed by Southern hybridization. Values represent an average of three independent assays



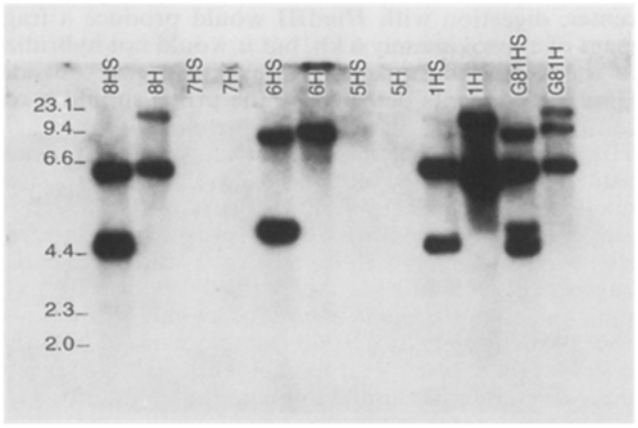
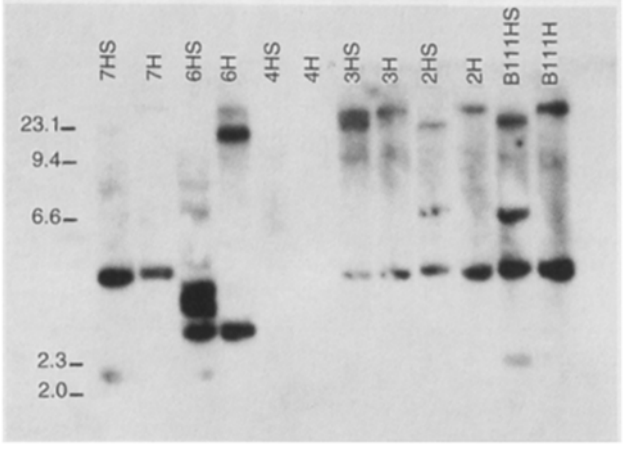
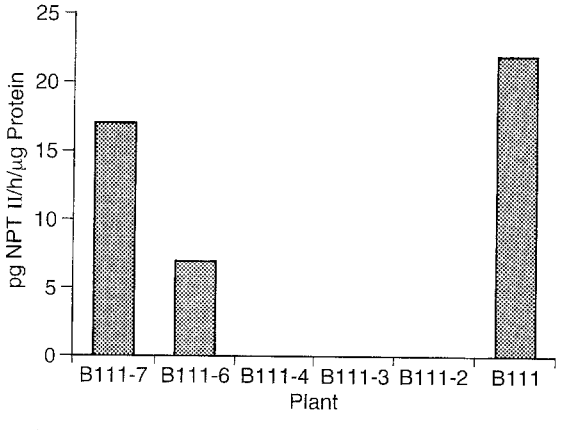
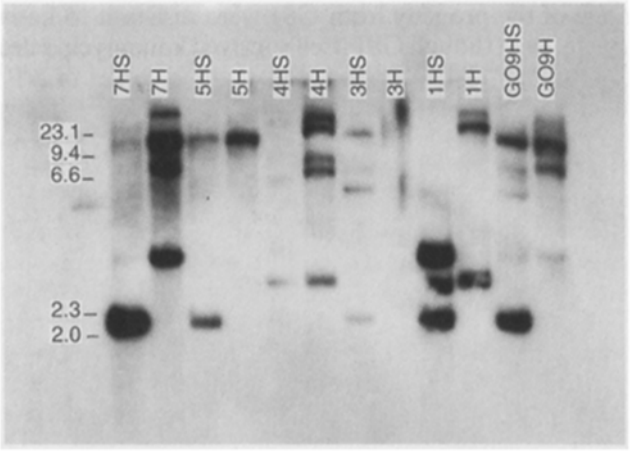


Fig. 3 Methylation status of the *Sst*II site in the NOS promoter of plant G81 and its progeny. Since none of the plants expressed NPT II activity, no NPT II values are shown. Plant genomic DNA was digested with either *Hin*dIII (H) or *Hin*dIII plus *Sst*II (HS) and analyzed by Southern hybridization.

Fig. 4 A comparison of the phenotypic expression of the NPTII gene in plant B111 and its progeny and the methylation status of the *Sst*II site in the NOS promoter of these plants. The NPT II values are pg/h per microgram protein. Plant genomic DNA was digested with either *Hin*dIII (H) or *Hin*dIII plus *Sst*II(*HS*) and analyzed by Southern hybridization. Values represent an average of three independent assays







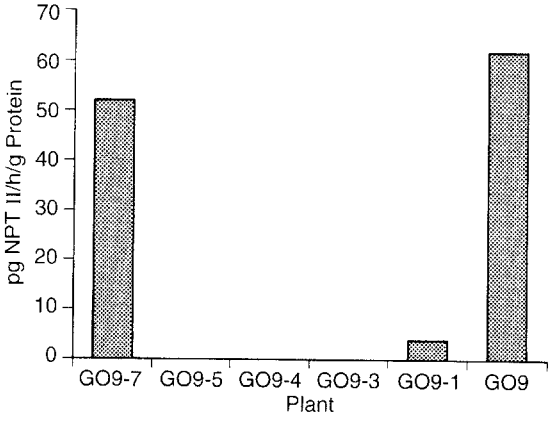


Fig. 5 A comparison of the phenotypic expression of the NPT II gene in plant GO9 and its progeny and the methylation status of the *Sst*II site in the NOS promoter of these plants. The NPT II values are pg/h per microgram protein. Plant genomic DNA was digested with either *Hin*dIII (H) or *Hin*dIII plus *Sst*II (*HS*) and analyzed by Southern hybridization. Values represent an average of three independent assays

Insertions into R_o plant genomes are not completely stable. One of the 5 progeny examined from self-fertilizations of 3 of the primary transformants had kanamycin-hybridizing *Hin*dIII fragments that differed in size from those in the parent. Progeny number 3 from RC1, progeny number 6 from B111, and progeny number 1 from GO9 each gave anomalous bands; whereas, each of the other progeny had 0,1,2, or 3 hybridizing fragments of the same size as those detected in the respective parent. Thus, in most progeny the fragments detected were inherited as expected for RFLPs (restriction fragment length polymorphisms) from a hemizygous parent.

NPT II expression (kanamycin resistance) does not follow simple modes of inheritance. The 4 R_o plants examined in this study were selected because they gave different levels or patterns of expression of the transformed gene. However, in none of the plants was a simple one gene pattern of inheritance detected among the progeny. The maximum frequency of kanamycin-resistant progeny (56%) was obtained from plant G09, the plant selected for a high level of expression (60 pg NPT II/h per microgram protein). At the other extreme,

none of the progeny from G81 were resistant to kanamycin, even though G81 itself survived kanamycin selection in vitro and contains at least three copies of NPT II-hybridizing sequences. If it is assumed that an expressed NPT II$^+$ allele is dominant, selfs for a plant heterozygous for a single functional allele would be predicted to produce 75% resistant progeny. If it is assumed that any one of '*n*' NPT II$^+$ alleles that become integrated into the host genome confers resistance when expressed, then only $(1/4)^n$ of the F_2 progeny and $(1/2)^n$ of the testcross progeny would be expected to be susceptible. This also is clearly not the case.

More complex models of regulation in other transgenic plants are not sufficient to explain the observed results. Since integration of multiple copies of the GUS gene into tobacco genomes lead to lower expression than a single insertion (Hobbs et al. 1990), the integration of two or three copies into each plant in this study may explain why so few of the plants examined were resistant to kanamycin. However, this model is not completely compatible with the results. First, the hypothesis could be applied only to progeny and not to the original plants, which were grown in the presence of kanamycin. There are several proven examples in fungi where the introduction of a duplicate copy or copies leads to the inactivation of both repeats (repeats-induced point mutations or RIPPING), and this occurs only if meiosis is allowed to proceed (Selker and Garrett 1988). The same process has been suggested to lead to sufficient differences in the repeats of higher eukaryotes to prevent ectopic recombination between non-allelic repeats and thus to provide stability to the genome. However, the patterns of NPT II$^+$ expression seen in this study are not fully consistent with such activity, since examples can be seen in specific progeny which disagree. For example, progeny number 7 of G09 inherited all three NPT II$^+$ RFLPs present in the parental line and has high activity; whereas, progeny number 5 inherited only one and has no activity.

Recently, the lack of GUS expression in tobacco plants in which more than a single copy was inserted has been linked to the integration of inverted repeats. Hobbs et al. (1993) found that inverted repeats (resulting from Ti plasmid T-DNA integration at one site) led to low levels of expression from all *uidA* alleles present. Application of this model to the current study can be addressed on a case-by-case basis. If an inverted repeat occurred with two copies of the NPTII$^+$ locus together in the center, digestion with *Hin*dIII would produce a 5-kb fragment detectable by the kanamycin probe. Without methylation, this arrangement would produce two 2.1-kb fragments upon double digestion with *Hin*dIII and *Sst*II. Hybridizing fragments of any other size would have to originate from other integration sites. The smaller *Hin*dIII fragment in B111 is about 5 kb, but its presence does not necessarily cause inactivity of NPTII$^+$, and it is not reduced by double digestion with *Sst*II. For inverted repeats integrated in the opposite orientation, i.e., with two GUS alleles together in the center, digestion with *Hin*dIII would produce a fragment of approximately 6 kb, but it would not hybridize to the kanamycin probe. However, the two 'outside' *Hin*dIII fragments detected by the probe should show complete linkage.

The one case where hybridization patterns are compatible with this alternative is G81. The largest and smallest *Hin*dIII digest fragments are both present in progeny 1 and 8 or both absent (progeny 5, 6, and 7). However, progeny 6 does have the middle *Hin*dIII fragment of G81, and it is only partially digested by *Sst*II and then to a fragment of approximately 5 kb. Since none of these progeny have detectable levels of NPTII$^+$ in leaf assays, it seems very unlikely that inverted repeats alone are the basis for inactivity.

Methylation of the *Sst*II sites appears to be correlated with NPTII expression but can not explain all of the anomalous results. Goring et al. (1991) also observed a lack of gene expression in transgenic tobacco which was not due to methylation of the NOS promoter. Other regulatory mechanisms involved in foreign gene expression include gene suppression (with increased copy number) and position effects (Linn et al. 1990; Matzke et al. 1991).

Many studies have shown that the activity of foreign genes in transformed plants can be decreased by methylation of regulatory elements (Hobbs et al. 1990; Kilby et al. 1992; Linn et al. 1990; Matzke and Matzke 1991; Matzke et al. 1989; Ottaviani et al. 1993; Weber et al. 1990) as does this study. But this study also shows that the inheritance and the gene expression of foreign genes in the progeny of stably transformed plants is difficult to predict. The development of better constructs with regulatory sequences specific to the species under study may be of great value in developing transgenic crop plants.

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