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Transgene Silencing in Wheat Transformed with the WSMV-CP Gene

¹Zhiwu Li, ²Ying Liu and ³Philip H. Berger

¹Department of Plant Pathology, ²Department of Statistics, Kansas State University, Manhattan, KS 66506,

³Center for Plant Health Science and Technology, USDA, Raleigh, NC 27606, USA

Abstract: Wheat (*Triticum aestivum*) was co-transformed with the *bar* gene and Wheat Streak Mosaic Virus Coat Protein gene (WSMV-CP) by the *biolistic* method. Transgenic wheat carrying the WSMV-CP showed non-uniform segregation patterns due to transgene loss or silencing. Loss of transgene expression was observed at the T₁, T₂ or T₃ generations. Among these silenced lines, transgenic line 566B was chosen for detailed studies. Results indicated that all 566B T₁ plants containing the WSMV-CP expressed WSMV coat protein and all showed strong resistance to WSMV. While the WSMV-CP was carried through to the T₂ and T₃ generations, all transgenic plants in these generations showed transgene silencing. Expression of WSMV-CP could be restored, at least temporarily, in most of these silenced plants by treatment with 5-azacytidine (5-AzaC).

Key words: *Triticum aestivum*, Wheat Streak Mosaic Virus (WSMV), transgene silencing, 5-azacytidine

INTRODUCTION

Transgene silencing has been frequently found in plants produced using microprojectile bombardment, resulting in unpredictable transgene expression^[1]. Where transgene silencing is observed, the introduced traits usually cannot be transmitted to successive generations in a predictable manner^[2]. Several hypotheses have been proposed to explain the mechanism of transgene silencing at the transcriptional or post-transcriptional levels^[3]. The important factors associated with transcriptional gene silencing (TGS) are the number of transgene copies introduced into plants^[4], DNA methylation^[5] and integration sites such as those near repetitive DNA or heterochromatin regions^[6]. Additionally, these factors can interact or combine with each other to inactivate transcription. For example, multiple or repeated transgenes can induce methylation or heterochromatinization^[7]. Post-transcriptional Gene Silencing (PTGS) is a mechanism distinct from TGS. In PTGS, promoters are active and the genes transcribed, but mRNA fails to accumulate or is specifically degraded^[8]. The main characteristic of PTGS is that certain RNAs such as aberrant RNA, endogenous RNA or virus RNA that is homologous to transgenes can induce PTGS^[9]. While TGS and PTGS are different mechanisms of transgene silencing, they appear to be related. For example, DNA methylation, which is often associated with TGS^[10], can also be found associated with PTGS^[11].

We have successfully transformed into wheat the WSMV-CP, using commercially grown Pacific Northwest wheat cultivars, to obtain resistance to WSMV. Expression and translation of WSMV-CP transgenes was required for WSMV resistance in transgenic plants^[12]. An extensive investigation on the expression of foreign genes among our transgenic wheat plants, generated by the biolistic method, revealed that varied non-Mendelian segregation ratios of the virus resistance to susceptible phenotype frequently occurred, indicating transgene silencing. Despite the fact that it is still not clear how many mechanisms were involved in transgene silencing, certain factors such as DNA methylation and multiple copies of transgene have been frequently observed in association with TGS.

MATERIALS AND METHODS

Immature embryos of the winter wheat cultivar "Daws" and spring wheat cultivar "Centennial" were co-transformed by particle bombardment with pMASG1, which contains a WSMV-CP gene (Fig. 5) and pDPG165, which contains the *bar* gene and used as a select marker^[13]. Among 237 putative transformants, 68 lines that contained WSMV-CP were chosen to examine segregation of phenotype and transgene silencing. Seeds from T₀ plants (primary generation) containing the WSMV-CP were collected for progeny testing.

Corresponding Author: Zhiwu Li, Ph.D., 4024Throckmorton, Plant Sciences Center,
Kansas State University, Manhattan, KS. USA66506
Tel: 785-5322789 Fax: 785-5325692 E-mail: zwli@ksu.edu

Plant transformation: Seeds (12 days after pollination) were sterilized in 70% ethanol for 1 min, 10% bleach for 3 min and briefly washed in ddH₂O. Embryos were excised and placed on MS-basal media (Murashige and Skoog, Sigma-Aldrich Chem. Co., St. Louis, MO) containing 3% sucrose and 0.3% phytigel, pH 5.8. The embryos were incubated for four weeks in the dark at 27°C for calli development. Approximately 40 calli were used in the center of a culture plate each time for gene transformation by bombardment. Both pMASG1 and pDPG165 were dehydrated onto 1 µg gold particles at a 1:2 molar ratio (bar:WSMV-CP). Ten microliter of solution containing a total of 1 µg plasmid DNA was dehydrated onto a suspension screen then delivered into the calli using a biolistic PDS-1000/He delivery system (Bio-Rad, Hercules, CA). After bombardment, the plant recovery and expression of the transgenes and plant selection procedure were the same as described before^[12].

Polymerase chain reaction: DNA was extracted by “DNAeasy Plant Mini kit” according to the manufacturer’s instructions (Qiagen Inc., Valencia, CA). around 100 ng of DNA was used for PCR reaction. The sense-strand primer used for PCR to amplify WSMV-CP was 5’AAAGGGACGGGTGGT TCATT 3’ and the anti-sense primer was 5’ CTGGGCTCGTTGTGTTGATT 3’. PCR was performed using a PTC-220 Thermal Cycler (MJ. Research, Inc. Alameda, CA). Samples were denatured at 94°C for 3 min and then denatured, annealed and extended at 94, 59 and 72°C for 30, 35 and 60 sec, respectively, for 35 cycles. PCR products were analyzed by 1% agarose gel electrophoresis.

DNA extraction and Southern blot analysis: Plant DNA was extracted by CTAB method following Sambrook’s protocol^[14]. About 30 µg DNA extracted from wheat leaves as described above was digested with *Bam*H1, which *Bam*H1 has only one recognition site on transformation vector. The digested DNA was then separated by 0.9% agarose gel electrophoresis using a constant 30 V, overnight. Separated DNA fragments were denatured into single-stranded molecules by washing in alkaline buffer and then transferred onto a charged nylon membrane (Roche, Indianapolis, IN) following standard alkaline transfer procedures^[14]. A 1.2 kb fragment containing WSMV-CP sequence was labeled as a probe for chemiluminescent detection using the Roche labeling kit (Roche, Indianapolis, IN) according to the manufacturer’s instructions. Southern hybridization was carried out using the DIG High Prime DNA Labeling and Detection Kit II (Roche, Indianapolis, IN). The procedure followed the manufacturer’s instructions.

Phenotype analysis for virus resistance: Progeny from T₁ and T₂ generations of each line were planted in groups of 16 in the greenhouse around 25°C, 16 h photoperiod. Each plant was mechanically inoculated with WSMV-infected plant sap extracted with 25 mM sodium phosphate buffer, pH 7.0 at ratio of 3:1 (w/v) and 1% carborundum. Five non-transgenic “Daws” or “Centennial” plants were inoculated as positive controls. Five healthy control plants were mock-inoculated with distilled water. To determine virus susceptibility and symptom development, a ranking symptom severity index system was used: 0, no disease development; 1, disease spotting around the leaf margins; 2, mild inter-veinal clearing; 3, leaf chlorosis; 4, necrosis. Symptom indexes were scored 15-20 days after virus inoculation.

Enzyme-linked Immunosorbent Assay (ELISA): Wheat leaf tissue was ground in carbonate coating buffer (CCB)^[15]. Three aliquots of 100 µL each of diluted protein sample were applied to enzyme-linked immunosorbant assay (ELISA) plate wells, as three replications. The protein was allowed to bind to the plate overnight at 4°C; all the wells were washed three times with water and Tris-buffered saline containing 0.05% (v/v) TWEEN-20 (TBS-TWEEN). One hundred microliters of diluted rabbit anti-WSMV coat protein serum (1:2000 in PEP buffer (TBS-TWEEN containing 2% polyvinyl-pyrrolidone and 2% egg albumin) was added into each well and incubated for 4 h at 37°C. After the primary antibody incubation, the plate was washed three times with TBS-TWEEN. The secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (Pierce Biotech, Rockford, IL), diluted in PEP at 1:1000, was added to the plate, incubated for 4 h at 37°C and then washed three times with TBS-TWEEN. Finally, 100 µL of 1 mg per mL alkaline phosphatase substrate, diluted in diethanolamine substrate buffer^[15], was added. The plate was incubated for 40 to 60 min and the absorbance was read at 405 nm with an ELISA plate reader.

Western blot analysis: Leaf tissue 100 mg was ground into a fine powder in liquid nitrogen. Total protein was extracted in dissociation buffer (2% SDS, 2% 2-mercaptoethanol, 40% sucrose and 0.25 M Tris-HCl, pH 6.8). Five micrograms of extracted protein preparations were subjected to 12.5% SDS-PAGE gel electrophoresis and were subsequently transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) using semi-dry transfer system according to manufacturer’s specifications (Bio-Rad, Hercules, CA). The membranes were blocked for 1 h in 3% dry-milk

powder in a TBS-TWEEN buffer (TRIS-buffered saline containing 0.05% TWEEN-20). The blocked membranes were incubated overnight with primary rabbit anti-WSMV coat protein antibody (at 1:2,000 in TBS-TWEEN). Finally, the membranes were incubated with Goat-anti-rabbit alkaline phosphatase conjugate (Sigma, Louis, MO) (1:2,000 dilution in TBS). The membrane was developed using alkaline phosphatase substrate according the manufacturers' instructions (Promega, Inc. Madison, WI).

5-AzaC treatment: Seeds from plants showing silencing of the transgene and seed from non-transformed plants were sterilized by washing three times in ddH₂O, 70% ethanol 1 min, 10% bleach 3 min and washed again in sterilized ddH₂O two times. Sterilized seeds were germinated on MS media (Sigma-Aldrich Chem. Co., Louis, MO) with or without 75 mg L⁻¹ 5-AzaC (Sigma-Aldrich Chem. Co. Louis, MO). Germination was done in darkness until seedlings were at the 2-leaf stage. The seedlings were then transferred to light for 2-3 days until the leaves turned light green. Thereafter, plants were transplanted into soil (without 5-AzaC) and grown under greenhouse conditions for further analysis.

RESULTS

Non-uniform segregation patterns and transgene silencing: Transgene silencing was observed and frequently caused non-uniform segregation patterns of transgenic wheat carrying WSMV-CP. Segregation ratios of the WSMV resistance phenotype in T₁ to T₃ generation plant of several lines were variable and un-expectable (Table 1). For example in transgenic line 343I, segregation in the T₁ generation was 1:1.3 and 44% of the plants were resistant to WSMV. In the T₂ generation of 343I, 100% of the transgenic plants containing WSMV-CP showed virus resistance and a segregation ratio of 16:0. In the T₃ generation, the segregation ratio was 0:16 and all

Table 1: Segregation ratios and percent resistance of four representative transgenic lines through the T₃ generation

Line ¹	Generation	Resistant plants	Susceptible plants	Segregation ratio	Percent resistance
337A*	T ₁	5	11	1:2.2	31
	T ₂	9	7	1.3:1	56
	T ₃	11	5	2.2:1	69
343I*	T ₁	7	9	1:1.3	44
	T ₂	16	0	16:0	100
	T ₃	0	16	0:16	0
366D*	T ₁	10	6	1.1:3	63
	T ₂	5	11	1:2.2	31
	T ₃	0	16	0:16	0
566B	T ₁	11	5	2.2:1	69
	T ₂	0	16	0:16	0
	T ₃	0	16	0:16	0

¹All lines were tested on the basis of phenotype and PCR

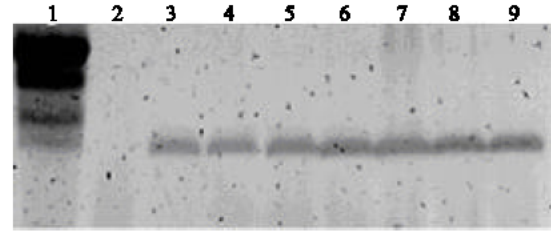


Fig. 1: Western blot analysis of 566B T₁ for WSMV coat protein expression. Lane 1, Protein marker; lane 2, infected control; lane 3, uninfected non-transformed control; lanes 4-10, plants of 566B T₁ generation containing the WSMV-CP gene

transgenic plants were susceptible to WSMV. This indicated that the WSMV-CP in T₂ generation had been silenced. In another line 566B, PCR (data not shown) and western blot results indicated that all the transgenic plants of the T₁ generation expressed the WSMV coat protein gene (Fig. 1). These transgenic plants showed a strong WSMV resistance phenotype (Fig. 2a). On the basis of ELISA, which was carried out 21 days after WSMV infection, the virus titer of transgenic plants was significantly lower than that of infected control plants (Fig. 3). However, in the next generation of 566B (T₂), all transgenic plants containing the transgene showed a susceptible phenotype after WSMV inoculation and transgenic plants showed typical WSMV symptoms (Fig. 2b). ELISA analysis indicated that the virus titer of transgenic plants was as high as that of infected control plants (Fig. 4). Further analysis by western blotting indicated that WSMV-CP transgenes in the T₂ generation were inactive; WSMV coat protein could not be detected in any T₂ transgenic plant before virus challenge (data not shown). Analysis of the T₃ generation of 566B by PCR, virus infection experiments and western blotting (data not shown) indicated that transgene silencing was transmitted to the next generation, since all transgenes of T₃ plants were still inactivated and none of these plants expressed coat protein. Virus inoculation experiments showed that all plants tested of the T₃ generation remained susceptible to WSMV infection.

Copy number of transgenes in transgenic plants of 566B T₁: The transgene copy number of 566B T₁ generation was analyzed by Southern analysis and the results (Fig. 5B) showed that more than one DNA fragment was observed in each genomic DNA sample of PCR positive plants. This result indicated that all of the 566B T₁ transgenic plants tested have multiple copies of WSMV-CP gene, with transgene copy number ranging from 3-6 copies.

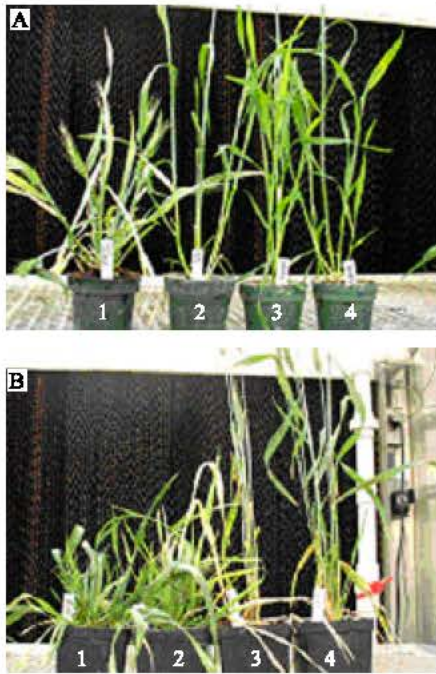


Fig. 2a: Transgenic plants of 566B T₁ generation inoculated with WSMV. 1, Non-transformed wheat inoculated with WSMV; 2, non-transformed wheat without WSMV inoculation; 3 and 4, transgenic plants of 566B T₁ inoculated with WSMV
 b: Transgenic plants of 566B T₂ generation inoculated with WSMV. 1 and 2, transgenic plants of 566B T₂ inoculated with WSMV; 3 and 4, non-transformed without WSMV inoculation

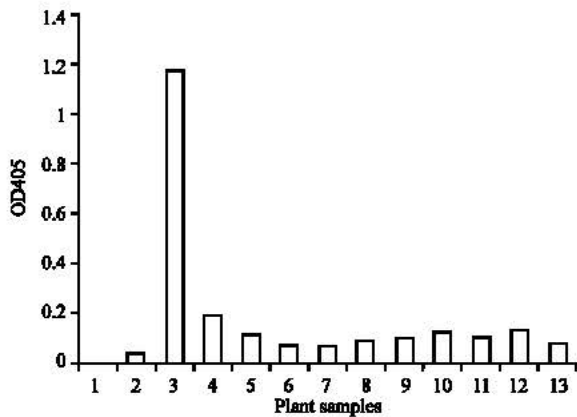


Fig. 3: ELISA analysis of 566B T₁ plants 21 d after inoculation with WSMV. 1, Blank control; 2, un-infected non-transformed control; 3, infected non-transformed control; 4-13, plants of 566B T₁ containing the WSMV-CP gene

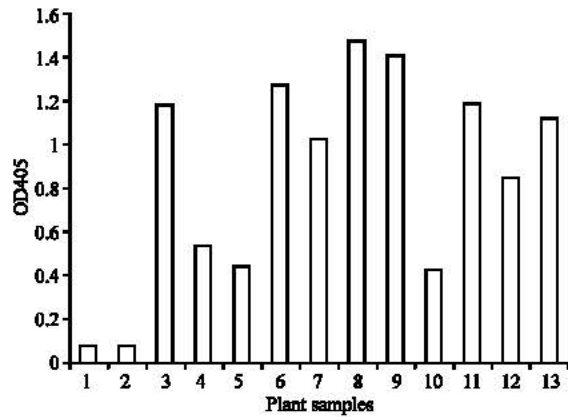


Fig. 4: LISA analysis of 566B T₂ generation. 1, Blank control; 2, un-infected non-transformed control; 3, infected non-transformed control; 4-13, plants of 566BT₂ containing the WSMV-CP gene

Reversal of transgene silencing: Transgenic seeds of 566B T₁ and 566B T₂ were germinated and cultured on media containing 75 mg L⁻¹ 5-AzaC for about 18 days. Five days after these plants were transplanted into soil without 5-AzaC, WSMV coat protein could be detected in these plants by western blot analysis (Fig. 6). No WSMV coat protein could be detected in any transgenic plant of 566B T₂ or T₃ that was germinated on media without 5-AzaC. Virus challenge experiments showed that 12 days after WSMV inoculation, transgenic plants treated with 5-AzaC had no disease symptoms. Transgenic plants without 5-AzaC treatment or non-transformed controls had typical symptoms of wheat streak mosaic disease (Fig. 7). ELISA analysis of these plants indicated that the expression of WSMV coat protein in the transgenic plants treated with 5-AzaC had significantly lower titer of WSMV, but transgenic plants not treated with 5-AzaC had titers similar to infected control plants.

DISCUSSION

WSMV-CP transgenic wheat lines of the T₁, T₂ or T₃ generations showed evidence of gene silencing. Genetic and molecular analyses of a typical transgene silencing line (566B) suggested one or more silencing mechanisms starting in T₂ generation or late T₁ generation. Where 100% of the plants of 566B T₂ were silenced and this silencing was stable and meiotically transmitted to the subsequent generation. It is interesting that transgenes were active in the first generation, but silenced in subsequent generations. This may indicate that some

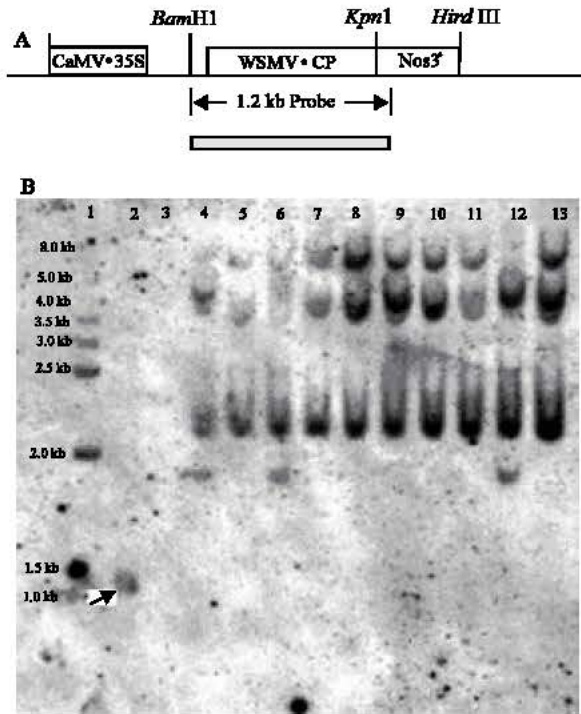


Fig. 5: Southern blot showing transgenic plants of 566B T₁ containing multiple copies of WSMV-CP. (A) graphic representation of putative transgene insertion fragment (not to scale). The 1.2 kb fragment containing WSMV-CP sequence (indicated by solid box) used as specific probe is from a *Bam*H1 and *Kpn*I digest. (B) Genomic DNA was digested by *Bam*H1 and the blot hybridized with a 1.2 kb WSMV-CP specific probe. Lane 1, 1.0 kb DNA ladder; lane 2, transformation construct digested with *Bam*H1/*Kpn*I. Arrow indicates location of expected 1.2 kb fragment. lane 3, non-transformed control. Lanes 4-13, DNA from putative transgenic wheat plants of 566B T₁

transgene silencing factors, such as the interaction between multiple transgene copies, or an increasing level of DNA methylation by some unknown mechanism(s), may have occurred and accumulated during plant development, resulting in cause transgene silencing.

DNA methylation has been observed in some transgene silencing events. DNA methylation can prevent some transcriptional factors from recognizing the DNA helix structure^[10]. Thus, the transcriptional machinery may lose its ability to function. On the other hand, some specific transcriptional repressors could recognize the methylated DNA sequences and reduce the ability of transcription^[6]. In this report, another transgene silencing recovery related to DNA methylation was found when

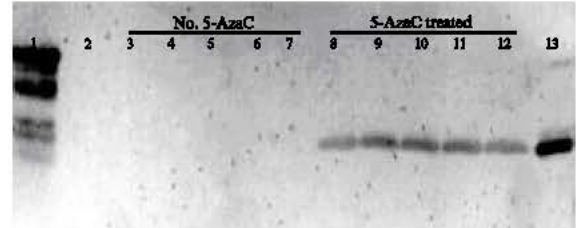


Fig. 6: Western blot analysis of 5-AzaC-treated 566B T₂ transgenic plants (5 days after transplanting into soil). Lane 1. non-transformed wheat inoculated with WSMV; lane 2, non-transformed wheat, not inoculated; lanes 3-7, transgenic plants of 566B T₂ without 5-AzaC-treatment, not inoculated; lanes 8-12, transgenic plants treated with 5-AzaC, not inoculated; lane 14, purified WSMV

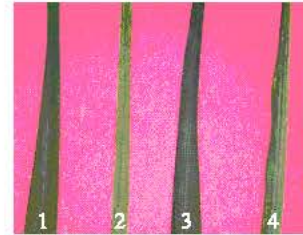


Fig. 7: Recovery of WSMV resistance by 5-AzaC treatment in WSMV-CP (silenced) wheat. (1) leaf from a non-transformed plant treated with 5-AzaC without virus inoculation; (2) leaf from a non-transformed plant treated with 5-AzaC inoculated with WSMV; (3) leaf from a transgenic plant of 566B T₂ treated with 5-AzaC and inoculated with WSMV; (4) leaves from a transgenic plant of 566B T₂ without 5-AzaC treatment and inoculated with WSMV

transgene silenced plants were treated with the demethylating reagent 5-AzaC.

5-AzaC is known to be an effective demethylating reagent. 5-AzaC results in demethylation of DNA directly by incorporation of this analogue in place of cytosine during DNA replication^[17] and may also indirectly inhibit the action of methyltransferases^[18]. Reversal of transgene silencing by 5-AzaC has been reported for tobacco^[19], sorghum^[20] and rice^[1,21]. This report shows that transgene silencing events in wheat could also be reversed by 5-AzaC treatments. Reversal of transgene silencing among 566B T₂ and T₃ by 5-AzaC indicated that DNA methylation was involved in this silencing, at the transcriptional level. Since transgenic plants of 566B T₁ expressed coat protein, transgene methylation of line 566B T₂ was likely induced by some *de novo* methylation

mechanism(s), which may stimulate the activation of DNA methyltransferase around or at transgene sequences, resulting in hypermethylation of promoter and/or coding regions.

In plants, DNA methylation is important for developmentally regulated gene expression. The total level of DNA methylation increases during plant development from the seedling stage to seed stage^[16] and DNA methylation levels in seeds is the highest of all plant development stages^[22]. It is possible that transgene methylation of 566B T₂ was induced in the late stage of the T₁ generation by the methylation regulatory mechanism that operates during plant development. Patterns of methylation of transgenes would be maintained in the T₂ generation and transmitted to the next generation by DNA half-conservation replication and the action of methyltransferase^[23].

Multiple copies of transgenes may trigger DNA methylation and cause transgene silencing^[24]. The distribution of methyltransferase, which carries out DNA methylation, is not random, where most methylated residues occur within repetitive DNA^[7]. Repeated sequences may provide an important signal for increasing DNA methylation^[25]. In this report, all transgenic plants of 566B T₁ generation contained multiple copies of the transgene. While the relationship between multiple copies of transgenes and DNA methylation is in need of further study, it is possible that transgene repeats functioned as signals to trigger *de novo* DNA methylation in the successive generation, perhaps beginning at the late stage of the T₁ generation or early T₂ generation.

REFERENCES

1. Kumpatla, S.P., W. Teng, W.G. Buchholz and T.C. Hall, 1997. Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. *Plant Physiol.*, 115: 361-373.
2. Depicker, A. and M. Van Montagu, 1997. Post transcriptional gene silencing in plants. *Curr. Opin. Cell Biol.*, 9: 373-82.
3. Fagard, M. and H. Vaucheret, 2000. Transgene silencing in plants: How many mechanism? *Annu. Rev. Plant Physiol. Plant Mol.*, 51: 167-194.
4. De Buck, S., M. Van Montagu and A. Depicker, 2001. Transgene silencing of invertedly repeated transgenes is relieved upon deletion of one of the transgenes involved. *Plant Mol. Biol.*, 46: 433-45.
5. Cho, J.H., H. Kimura, T. Minami, J. Ohgane, N. Hattori, S. Tanaka and K. Shiota, 2001. DNA methylation regulates placental lactogen I gene expression. *Endocrinology*, 142: 3389-3396.
6. Petersen, K., R. Leah, S. Knudsen and V. Cameron-Mills, 2002. Matrix attachment regions (MARs) enhance transformation frequencies and reduce variance of transgene expression in barley. *Plant Mol. Biol.*, 49: 45-58.
7. Bennetzen, J.L., P. SanMiguel, C.N. Liu, M. Chen, A. Tikhonov, A. Costa de Oliveira, Y.K. Jin, Z. Avramova, S.S. Woo, H. Zhang and R.A. Wing, 1996. The Hybaid Lecture. Microcollinearity and segmental duplication in the evolution of grass nuclear genomes. *Symp. Soc. Exp. Biol.*, 50: 1-3.
8. Hammond, S.M., A.A. Caudy and G.J. Hannon, 2001. Post-transcriptional gene silencing by double-stranded RNA. *Natl. Rev. Genet.*, 2: 110-119.
9. Ratcliff, F.G., S.A. MacFarlane and D.C. Baulcombe, 1999. Gene silencing without DNA: RNA-mediated cross-protection between viruses. *Plant Cell*, 11: 1207-1216.
10. Kass, S.U., N. Landsberger and A.P. Wolffe, 1997. How does DNA methylation repress transcription? *Trends Genet.*, 13: 444-449.
11. Smith, H.A., S.L. Swaney, T.D. Parks, E.A. Wernsman and W.G. Dougherty, 1994. Transgenic plant virus resistance mediated by untranslatable sense RNAs: Expression, regulation and fate of nonessential RNAs. *Plant Cell.*, 6: 1441-1453.
12. Hansen, J., P.J. Shiel, P. McCarthy, P.H. Berger and R.S. Zemetra, 1998. Transformation of Soft White Winter Wheat (*Triticum aestivum*) for Virus Resistance. In: *Proc 9th Inter. Wheat Genet. Symp.* (Slinkard, A.E., Ed.). University Extension Press, Saskatoon, Saskatchewan, Canada, 3: 186-188.
13. Spencer, T.M., W.J. Gordon-Kamm, R.J. Daines, W.G. Start and P.G. Lemaux, 1990. Bialophos selection of stable transformants from maize cell culture. *Theor. Applied Genet.*, 79: 625-631.
14. Sambrook, J. and D.W. Russell, 2001. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
15. Clark, M.F., R.M. Lister and M. Bar-Joseph, 1986. ELISA technique. *Methods Enzymol.*, 118: 742-66.
16. Meehan, R.R., J.D. Lewis and A.P. Bird, 1992. Characterization of MeCP2, a vertebrate DNA binding protein with affinity for methylated DNA. *Nucl. Acids. Res.*, 20: 5085-5092.
17. Jones, P.A., 1985. Altering gene expression with 5-azacytidine. *Cell*, 40: 485-486.
18. Bouchard, J. and R.L. Momparler, 1983. Incorporation of 5-Aza-2'-deoxycytidine-5'-triphosphate into DNA. Interactions with mammalian DNA polymerase alpha and DNA methylase. *Mol. Pharmacol.*, 24: 109-114.

19. Palmgren, G., O. Mattson and F.T. Okkels, 1993. Treatment of *Agrobacterium* or leaf disks with 5-azacytidine increases transgene expression in tobacco. *Plant Mol. Biol.*, 21: 429-435.
20. Emani, C., G. Sunilkumar and K.S. Rathore, 2002. Transgene silencing and re-activation in sorghum. *Plant Sci.*, 162: 181-192.
21. Kloti, A., X. He, I. Potrykus, T. Hohn and J. Futterer, 2002. Tissue-specific silencing of a transgene in rice. *Proc. Natl. Acad. Sci. USA.*, 99: 10881-10886.
22. Messeguer, R., M.W. Ganai, J.C. Steffens and S.D. Tanksley, 1991. Characterization of the level, target sites and inheritance of cytosine methylation in tomato nuclear DNA. *Plant Mol. Biol.*, 16: 753-770.
23. Wigler, M., D. Levy and M. Perucho, 1981. The somatic replication of DNA methylation. *Cell*, 24: 33-40.
24. Vaucheret, H., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J.B. Morel, P. Mourrain, J.C. Palauqui and S. Vernhettes, 1998. Transgene-induced gene silencing in plants. *Plant J.*, 16: 651-659.
25. Goyon, C., T.I.V. Nogueira and G. Faugeron, 1994. Perpetuation of cytosine methylation in *Ascobolus immersus* implies a novel type of maintenance methylase. *J. Mol. Biol.*, 240: 42-51.