Plant Molecular Biology **43:** 323–346, 2000.
M.A. Matzke and A.J.M. Matzke (Eds.), Plant Gene Silencing.
© 2000 *Kluwer Academic Publishers. Printed in the Netherlands.*

Transgene silencing in monocots

Lakshminarayan M. Iyer[1], Siva P. Kumpatla[2], Mahesh B. Chandrasekharan[1] and Timothy C. Hall[1,*]

[1]*Institute of Developmental and Molecular Biology, Texas A&M University, College Station, TX 77843-3155, USA (* author for correspondence; e-mail: tim@idmb.tamu.edu);* [2]*Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268, USA*

Key words: genome surveillance processes, heterochromatinization, methylation, post-transcriptional gene silencing, transgene structure

Abstract

Plant gene silencing was originally thought to be a quirk of transformation procedures, but is now recognized to be a facet of vitally important gene regulatory systems, present in all organisms. Monocot plants, especially the grasses, play a foremost role in the agricultural economy of all nations, and their biotechnological manipulation offers great potential for both developed and developing countries. Here, we review reported instances of transgene silencing in monocots and relate the processes of transcriptional and post-transcriptional gene silencing (TGS, PTGS) in perspective to the rapidly burgeoning knowledge of these phenomena in many organisms. Recent findings include the involvement of an RNA-dependent RNA polymerase and a nuclease in PTGS systems and the close relationship between methylation and chromatin structure in TGS events.

Introduction

In the excitement of using transgenic approaches to learn how genes work, it is often forgotten that many, probably most, genes are silent for much of a plant's lifetime. Thus, in concert with factor arrays that activate genes, it is likely that complex groups of elements combine with chromatin architecture (and possibly DNA topology; Bi and Broach, 1997) to suppress expression. Thus, while the basic structure of a gene – promoter, coding region, terminator – is simple, regulation of expression is modulated by factor interactions, epigenetic events, chromatin structure and in many other ways. It is, therefore, not surprising that many transgenes do not express as expected. Originally, this was explained in an ill-defined manner as reflecting chromosomal location, but the elegant early findings of transgene silencing in doubly transformed tobacco by Matzke *et al.* (1989) led to the recognition that transformation could itself result in epigenetic modification of expression. More recently, the connection between genome methylation and recruitment of factors (Nan *et al.*, 1998) that can in turn conscript histone deacetylases (Wade *et al.*, 1998), leading to chromatin condensation, has been recognized as another fundamental aspect of transcriptional gene silencing (TGS).

Transgene cosuppression, another landmark discovery, resulted from the expression of a chalcone synthase transgene in petunia that was expected to yield more intensely pigmented flowers, but instead yielded white flowers (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Cosuppression, along with various forms of viral gene silencing (Lindbo *et al.*, 1993; English *et al.*, 1996; Baulcombe, 1999), is now recognized as a post-transcriptional gene silencing (PTGS) event (van Blokland *et al.*, 1994). The recent finding that plant RNA-dependent RNA polymerase (RdRP) is involved in PTGS provides insight into the way in which sequence specificity is attained and has also revealed links with gene silencing phenomena in other organisms, such as quelling in *Neurospora crassa* (Cogoni and Macino, 1999a) and RNAi in *Caenorhabditis elegans* (Fire, 1999).

The various TGS and PTGS mechanisms did not evolve in response to modern genetic engineering. Rather, they are components of powerful and complex systems that are involved both in normal gene regulation processes and in protection from extragenomic and intragenomic parasites (Bestor, 1998; Kumpatla *et al.*, 1998; Matzke and Matzke, 1998b). Nevertheless, when foreign genetic information is introduced into the genome, it is likely to be perceived by these surveillance processes as alien and is likely to be functionally inactivated or eliminated. TGS and PTGS are often perceived as entirely distinct processes and, functionally, blocking of expression is indeed a different event than is the modulation of an already expressed transcript (Meyer, 1996). However, silencing of a specific transgene can result from a combination of both TGS and PTGS effects.

Although early reports on gene silencing in plants dealt with dicots, it is apparent that transgenes in monocots are equally susceptible to silencing processes. In this review, we have attempted to relate silencing of monocots to current concepts in gene silencing. Questions that arise include whether specific genomes are more susceptible to silencing than others and, if so, whether monocot and dicot genomes are similarly susceptible to various silencing strategies, and whether the method of transformation (direct DNA transfer or *Agrobacterium*-mediated transfer) affects the frequency and nature of silencing. Other considerations are the stochastic nature of silencing in which genetically identical siblings exhibit differences in silencing or reactivation, and the inheritance of silencing or of expression (including recovered expression) in progeny.

Reported instances of transgene silencing in monocots

Rice

We have considered rice (*Oryza sativa*) first in the list of specific examples because, at this time, cases of monocot transgene silencing are most thoroughly documented in this species. In addition to being a primary human food resource in many nations and an important commercial cereal in developed countries, its small genome makes it an excellent monocot model for genomic sequencing and analysis (Shimamoto, 1995). Rice was the first monocot crop plant for which routine molecular transformation was established and several different approaches have been used. These include electroporation (Zhang *et al.*, 1988), polyethylene glycol-mediated transformation of protoplasts (Li *et al.*, 1990), particle bombardment of regenerable tissues (Cao *et al.*, 1992) and, more recently, *Agrobacterium*-mediated transformation of embryogenic rice calli (Dong *et al.*, 1996; Hiei *et al.*, 1994). Transgene silencing has been reported from both laboratory experiments and early field trials, albeit at various frequencies (see Table 1).

Cases of transcriptional gene silencing in rice

Detailed analyses of the silencing of a *Bt* gene (*Btt cryIIIA*) transcribed from a 35S promoter and a bialaphos resistance gene (*bar*) driven by a maize ubiquitin promoter (*Ubi1*) have been described in rice transformed using bombardment of embryogenic calli (Kumpatla *et al.*, 1997; Kumpatla and Hall, 1998a, b). An aberrant segregation pattern for both *cryIIIA* expression and *bar* resistance was observed in progeny as a result of silencing. Silencing of the *bar* gene was observed in homozygous R_1 individuals and shown by nuclear run-on assays to be at the transcriptional level and was correlated with methylation of the *Ubi1* promoter. Genomic analysis of the transformants revealed multiple (>10) full-length and partial copies of the *bar* gene construct, which was present as a complex transgene locus with rearrangements typical of transformants derived by the biolistic procedure (Kumpatla and Hall, 1999). Reactivation of BAR expression in progeny seedlings with 5-azacytidine (azaC) was closely correlated with the restoration of the unmethylated (*Hpa*II) restriction pattern (Kumpatla *et al.*, 1997). In a further study of these reactivated plants, transgene silencing recurred 25–50 days after azaC treatment in all except one plant (Kumpatla and Hall, 1998a). The expressing lines often showed instability of transgene expression in the R_2 and R_3 progeny. Analysis of R_3 progeny from five expressing R_2 lines showed recurrence of silencing in the progeny of three of five lines, with frequencies of up to 40% (Kumpatla and Hall, 1998b). A general increase in methylation of the *Ubi1* promoter was observed in the R_2 and R_3 progeny and, once a line was silenced, the silencing was meiotically stable. A stochastic process for the activation of promoter methylation was invoked, with methylation of key cytosine residues being seen as the triggering event.

Silencing of chitinase and hygromycin phosphotransferase (*hpt*) genes has been reported in transgenic rice plants derived from PEG-mediated transformation of rice protoplasts (Chareonpornwattana *et al.*, 1999).

Table 1. Survey of recorded instances of gene silencing in monocots.

Monocot	Promoter	Coding region	Copy number if determined	Transformation method	TGS/ PTGS	Comments	References
Rice						diploid; $n = 12$	
	35S	BAR-nos	>10	PEG	ND	2/17 plants derived from the same callus showed silencing of the *bar* gene and showed multicopy transgene inserts and silencing	Rathore *et al.*, 1993
	35S	NPTII	4–12	PEG	ND	Five R_2 plants were checked for *nptII* resistance and none expressed the gene. The transgenes were typically multicopy	Schuh *et al.*, 1993
	35S 35S RTBV	NPTII GUS GUS	>10	PEG	ND	Non-Mendelian inheritance of expression and stochastic induction of silencing in T_3 progeny	Peng *et al.*, 1995
	35S A ssu	GUS-nos GUS	1–>10	Biolistics	ND	As the number of *gus* transgenes exceeded 10, frequency of expression of *gus* in hygromycin-resistant individuals decreased from 7/16 to 1/8	Cooley *et al.*, 1995
	35S 35S	HPT-nos GUS-nos	1–5	Biolistics	ND	Non-Mendelian inheritance of both the *hpt* and the *gus* gene was observed in one of the plants	Qu *et al.*, 1996
	Act1-act1	BAR-nos	ND	*Agrobacterium*	ND	One line showed silencing of bar in the R_2 generation and there was increased methylation of the gene insert	Park *et al.*, 1996
	35S-adh1 35S-amv	BAR-nos GUS-spA	1–5 1–5	Biolistics	TGS?	6 of 9 plants that had both *bar* and *gus* gene were silenced for either the *bar* or the *gus* gene	Oard *et al.*, 1996
	35S-adh1 35S-amv	BAR GUS	2–7	Biolistics	ND	See text	Kohli *et al.*, 1999a
	Wx	Wx	1–4	Electroporation	TGS and PTGS	See text	Itoh *et al.*, 1997
	Ubi1 35S	BAR CRYIIIA	>10	Biolistics	TGS	See text	Kumpatla *et al.*, 1997, 1998; Kumpatla and Hall, 1998a, b, 1999
	Ubi1	RF2a	ND	Biolistics	PTGS	See text	Yin *et al.*, 1997
	35S	GUS	ND	*Agrobacterium*	PTGS	See text; note the experiments were only done in rice calli	Waterhouse *et al.*, 1998

Table 1 continued.

Monocot	Promoter	Coding region	Copy number if determined	Transformation method	TGS/ PTGS	Comments	References
	Ubi1 35S Ubi1 35S	CRY1Ac-nos CRY2A-nos GNA-nos HPT-nos	1–15	Biolistics	ND	Non-mendelian inheritance and silencing were observed for some transformants and plants showing 15 copies showed silencing	Maqbool and Christou, 1999
	35S	RYMVCP-nos	2	Biolistics	PTGS	See text	Pinto *et al.*, 1999
	LTP	GUS-nos	4–12	PEG	ND	See text	Morino *et al.*, 1999
	35S 35S	Rice CHN HPT	5	PEG	TGS	See text	Chareonpornwattana *et al.*, 1999
Barley						diploid; $n = 7$	
	Ubi1	BAR-nos	1–>20	Biolistic	ND	See text	Wan and Lemaux, 1994
Maize						segmental allotetraploid; $n = 10$	
	35S	NPTII-ocs	4	Electroporation of immature zygotic embryos	ND	Neomycin sensitivity in two plants and complex inserts in several of the tested plants	D'Halluin *et al.*, 1992
	35S Adh1 35S MFS 14P Zm 13P 35S	NPTII GUS BAR GUS GUS PAT	1–10	Biolistics	ND	An extensive analysis of the expression of over 112 transgenic lines; see text	Register *et al.*, 1994
	35S 35S-int	BAR-nos GUS-nos		*Agrobacterium*	ND	Aberrant segregation ratios were reported in the R_1 generation of a few plants	Ishida *et al.*, 1996
	Act1 35S	GUS-nos BAR-nos	>1	Biolistic	ND	T_1 plants coexpressing the *bar* and *gus* genes showed aberrant segregation and coexpression of the genes in T_2 progeny derived by self- or cross-fertilization	Zhang *et al.*, 1996
Oats						allohexaploid; $n = 3x = 21$	
	35S-Adh1 Adh1-int	BAR-nos GUS-nos	1–15	Biolistic	ND	See text	Pawlowski *et al.*, 1998
	Act1 Ubi1	HPT-nos GUS-nos	1–>10	Biolistic	ND	Of 7 T_0 lines that expressed GUS, progeny of 4 lines showed a non-Mendelian inheritance of GUS expression while 1 line showed silencing of *gus* in all the T_1 progeny.	Cho *et al.*, 1999

Table 1 continued.

Monocot	Promoter	Coding region	Copy number if determined	Transformation method	TGS/ PTGS	Comments	References
Pearl millet						diploid; $n = 7$	
	35S	HPH-35St	1–5	Biolistic	ND	See text	Lambé *et al.*, 1995
	35S	GUS-ocs					
Ryegrass						(annual) diploid; $n = 7$	
	Orys1	as- LOLP5	ND	Biolistic	PTGS?	See text	Bhalla *et al.*, 1999
Sugarcane						polyploid $x = 8$–10; $n = 32$–40	
	Ubi1	uSMV-CP	4–10	Biolistic	PTGS	See text	Ingelbrecht *et al.*, 1999
Wheat						allohexaploid; $n = 3x = 21$	
	HMWGS	chimeric	5–6	Biolistic	ND	See text	Blechl and Anderson, 1996
		HMWGS	2–10	Biolistic	ND	12 T_0 plants that expressed both the *bar* and the chitinase transgenes showed silencing of chitinase in most of the T_1 progeny expressing *bar*	Chen *et al.*, 1998
	35S	CHN					
	Ubi1	BAR					
	HMWGSP	HMWGS	3–50	Biolistic	ND	See text	Alvarez *et al.*, 2000

Promoters	Coding sequences
35S, cauliflower mosaic virus 35S promoter	Bar/PAT, bialophos resistance / phosphinothricin acetyltransferase
35S-adh1, 35S promoter with the maize 5′ Adh1 intron	CHN, rice chitinase
35S-amv, 35S promoter with an alfalfa mosaic virus leader sequence	Cry1Ac, *Bacillus thuringiensis* cry1Ac toxin
Act-act1, Actin 1 promoter with actin intron	Cry2A, *Bacillus thuringiensis* cry2A toxin
HMWGS, high-molecular-weight glutenin subunit promoter	CryIIIA, *Bacillus thuringiensis* cryIIIA toxin
LTP, barley lipid transfer protein promoter	GUS, β-glucuronidase
MFS 14P, tapetal-specific maize promoter	GNA, snowdrop lectin
Orys-1, *Oryza sativa* (rice) pollen-specific promoter	HMWGS, high-molecular-weight glutenin subunit protein
RTBV, rice tungro bacilliform virus promoter	chimeric HMWGS, a chimeric HMWGS protein
Ubi/Ubi1, maize ubiquitin promoter	HPT, hygromycin phosphotransferase
Wx, rice granule-bound starch synthase promoter	as-LOLP5, antisense ryegrass pollen-specific p5 protein
Zm 13P, pollen-specific maize promoter	NPTII, neomycin phosphotransferase II
	RF2a, bZIP domain containing transcription factor in rice
	rice CHN, rice chitinase
	uSMV-CP, untranslatable sorghum mosaic virus coat protein
	nos, nopaline synthase terminator
	ocs, octopine synthase terminator
	35St, 35S polyadenylation end
	spa, poly(A) site from soybean ssu gene

Other abbreviations

n, haploid chromosome number; ND, not determined; PEG, protoplast transformation via polyethylene glycol; x, basic chromosome number

Both genes were expressed from the 35S promoter. The frequency of silencing was similar in plants derived from either a homozygous or hemizygous parent and its onset occurred some 3 weeks after germination. Silencing of both the *chitinase* and *hpt* transgenes was observed in about 23% of the progeny of one homozygous line (bearing 5 transgene copies) in the T_3 and T_4 generations. Once the silent phenotype was established, it was stable and meiotically heritable. The silencing of *chitinase* was shown by nuclear run-on experiments to be TGS in the lines that were studied, but the endogenous *chitinase* was not silenced.

Kohli *et al.* (1999a) reported the biolistic transformation of rice with constructs containing *gus*, *hpt* and *bar* coding regions, each driven by a 35S promoter. Of 500 plants, twelve independent transformants which expressed all genes in the R_0 generation were selected for further study. The transgene was typically found to be inserted at one locus and was present in 1–9 copies of rearranged or truncated sequence. Expression instability occurred at the R_1 or R_2 generation, with at least four of the 12 lines showing silencing. The authors found no obvious correlation of silencing with the transgene copy number as silencing was observed in plants harboring as few as 2 and as many as 7 copies. Both TGS and PTGS mechanisms were considered, but no conclusive molecular evidence was presented. However, promoter methylation (and, hence, TGS) was implicated since 5-azacytidine reactivation was observed in several experiments.

Cases of post-transcriptional gene silencing in rice

Silencing of a *gus* gene driven by a barley aleurone-specific lipid transfer protein (*ltp*) promoter (and a *nos* terminator) was reported in transgenic rice plants derived by protoplast electroporation (Morino *et al.*, 1999). The authors observed recurrent stochastic triggering of *gus* silencing in R_2, R_3, R_4 and R_5 progeny of expressing plants. Interestingly, differences were observed in the frequencies of silencing based on the environmental growth conditions. The *ltp-gus* plants typically had from 4 to 12 copies of the insert and at least two loci were implicated in the triggering of silencing. One was a rearranged locus, which yielded a complex RNA transcript containing both sense and antisense *gus* sequences. As this fragment was observed in several silenced transgenic plants, the authors concluded, albeit without nuclear run-on data, that the silencing was post-transcriptional. This aberrant RNA was hypothesized to interact with the full-length *gus* RNA present at another locus and to cause PTGS.

Recent exciting studies in *Caenorhabditis elegans* have shown that double-stranded (ds) RNA species can trigger a high level of silencing that specifically targets homologous sequences (Fire *et al.*, 1998; Fire, 1999). This dsRNA-mediated silencing is much more effective than that of antisense RNA and appears to function through a cosuppression mechanism (Montgomery and Fire, 1998). Waterhouse *et al.* (1998) applied a similar strategy in rice where they supertransformed calli from GUS-expressing transgenic rice plants with a construct encoding an RNA containing both the sense and antisense GUS sequence. GUS expression was silenced with a frequency of greater than 90%, confirming that this approach is very effective in plants.

RNA-mediated virus resistance was obtained by biolistic transformation of African varieties of commercial rice with a viral RNA-dependent RNA polymerase (RdRP) of rice yellow mottle virus (RYMV) expressed from a 35S promoter (Pinto *et al.*, 1999). A very high frequency of virus-resistant plants (12 of 14) was obtained. The resistance was shown to be mediated by PTGS as it coincided with low steady-state levels of transgene RNA with no difference in transcription rates (as shown by nuclear run-on assays), a situation found for several cases of virus resistance (Lindbo *et al.*, 1993). Analysis of the transgene inserts of a resistant line revealed two complex cosegregating transgene loci. The authors ascribed the observed high frequency of PTGS induction to the complex transgene rearrangement generated during particle bombardment. This line was resistant to several natural strains of the virus and this was attributed to a region of the viruses which shared greater than 90% nucleotide sequence identity with the transgene.

It appears that rice tungro bacilliform virus (RTBV) exploits RF2a, a host bZIP transcription factor that is critical for leaf tissue differentiation and vascular development. Expression of a sense *RF2a* gene from the *Ubi1* promoter in biolistically transformed rice plants resulted in sense suppression of RF2a (Yin *et al.*, 1997). The phenotype of these plants resembled that derived from antisense suppression of the corresponding endogenous gene in transgenic rice plants.

An unusual type of silencing was reported in rice plants transformed with a rice granule-bound starch synthase gene (*waxy*, *Wx*) (Itoh *et al.*, 1997). Transformation of rice with the *Wx* gene (promoter and coding sequence) by electroporation of embryogenic protoplasts resulted in two types of transformants. One

(Type I) showed silencing of Wx expression in all pollen grains of R_0 plants (50% were silenced for both the endogenous gene and the hemizygous transgene) and Type II transformants showed silencing of both the *Wx* endogenous gene and transgene in 50% of the pollen. Although the haploid pollen suppressed starch synthase activity, there was no cosuppression of the gene in the triploid endosperm of any of the R_1 progeny, indicating that tissue specificity and possibly modified ploidy levels were involved in the silencing process. However, the pollen-silencing phenotype was transmitted to some of the progeny after selfing or outcrossing (with wild-type rice), reminiscent of a paramutation-like effect. There was no obvious relationship between transgene copy number and the two pollen phenotypes and silencing was not observed for transformations into a mutant (*wx*) background. Although the mechanism (TGS or PTGS) of silencing was not elucidated, a paramutation-like effect was suspected.

Barley

While attempts to establish a routine transformation system in barley (*Hordeum vulgare*) have been slow relative to other cereals, direct DNA delivery (PEG-mediated DNA uptake in protoplasts) gave promising results (Lazerri *et al.*, 1991). Wan and Lemaux (1994) described the generation of large numbers of independently transformed fertile barley plants by particle bombardment of immature embryo-derived callus with pAHC25 (that expresses a *bar* and a *gus* gene, each under a maize *Ubi1* promoter). Some 35 T_0 plants were recovered from 18 transgenic calli and segregation data based on bialaphos resistance tests on 9 plants (representing 8 callus lines) showed that the *bar* gene was inherited at a single locus in these examples. However, several T_1 lines were identified that had an intact *bar* insert but were sensitive to the herbicide, revealing the occurrence of silencing.

Maize

Given the economic significance of maize (*Zea mays*), it is not surprising that there are many reports of transformation of several cultivars. However, there are very few reports of studies on transgene silencing in this plant and it is likely that a vast store of unpublished information on transgene structure, inheritance and expression exists.

Klein *et al.* (1990) studied several stably transformed maize calluses for GUS expression. While there was no correlation between the number of intact copies of the *gus* gene and expression, low levels of GUS expression were found to be correlated with methylation of the coding region. Register *et al.* (1994) reported a detailed analysis of transformation and transgene expression for maize plants from 112 calluses (obtained from embryogenic suspension cultures of A188×B73) independently transformed with pBARGUS, a construct carrying both *bar* and *gus* genes (Fromm *et al.*, 1990). Other gene constructs used for transformation included a *bar* or *pat* gene transcribed from a 35S promoter and a *gus* (referred to as *uidA* in many publications) gene transcribed from several promoters (*Adh1*, *MFS 14P* and *Zm 1 P*). As found for rice and other plants transformed by a particle bombardment procedure, the transgene was predominantly integrated at one locus, but was typically present in multiple copies and frequently rearranged, with relatively few of the plants containing an unrearranged unselected sequence (only 27% having an intact, unrearranged GUS sequence compared with 54% having an intact BAR sequence). Plants regenerated from the same callus line showed various expression levels for either or both the selection marker (*bar*) and the unselected marker (*gus*). For example, of 41 transformation events, >75% of the plants regenerated from 17 calluses expressed GUS but 13 calluses yielded GUS expression in 25% or less of the regenerated plants. Coexpression of *bar* and *gus* was not observed in plants that had the potential to express both genes and 32% of the plants expressed only one transgene at a detectable level. Silencing was also observed in the T_2 progeny of T_1 plants expressing the transgene. In 5 of 14 lines possessing a single locus, silencing was observed in many or all plants in the T_2 generation. The mechanism of silencing was not elucidated.

Oat

Oat (*Avena sativa* L) is amenable to transformation by particle bombardment of embryogenic tissue (Somers *et al.*, 1992). Pawlowski *et al.* (1998) described transgene silencing of the *bar* gene transcribed from a 35S promoter and a *gus* gene transcribed from an *alcohol dehydrogenase* (*Adh1*) promoter. In the T_0 generation, 19 (of 23) independent transformants showed phosphinothricin (PPT) resistance (due to the expression of the *bar* gene), and 18 of these contained full-length *bar* and *gus* genes. Molecular analyses of these plants revealed complex transgene integration

patterns, typical of biolostic procedures, with copy numbers ranging from 1 to 11. A majority of the plants had single-locus insertions. In the T_1 generation, only 5 of 16 lines analyzed consistently showed cosegregation of PPT resistance with *bar*, while the remaining 11 showed a high frequency of silencing in progeny bearing the transgene locus. In progeny from 13 lines of 21 T_0 GUS-expressing lines, 6 showed GUS staining while the remaining 7 did not show GUS staining, even though they possessed a full-length *gus* insert.

Pearl millet

In a long-term study of the expression and inheritance of *gus* and *hph* expression in transgenic pearl millet (*Pennisetum glaucum*) callus lines, Lambé *et al.* (1995) described the progressive silencing of *gus* expression. The transgenic lines were obtained by cobombardment of embryogenic calli or cell suspensions with *gus* and *hpt*, both under the control of a 35S promoter. A progressive silencing of *gus* expression was observed in which 9 of 14 callus lines that expressed both HPT and GUS 3 months after bombardment, showed no staining after 12 months and all lines were silenced after 18 months. However, HPT was expressed in all callus lines for the duration of the experiment. In azaC reactivation studies, barring one all the calluses showed GUS expression after 2 weeks of treatment. Isoschizomer analysis revealed a greater methylation of *gus* coding sequences compared with *hpt*, implicating silencing by methylation in these lines. Similar observations in the progressive silencing of callus lines have been made in wheat (Müller *et al.*, 1996) and perennial ryegrass (van der Maas *et al.*, 1994).

Ryegrass

In a recent report (Bhalla *et al.*, 1999), an antisense RNA strategy was used to reduce the amount of LOL P5, a major allergenic protein in ryegrass (*Lolium rigidum* L.) pollen. Transgenic ryegrass expressing the antisense *lol p5* gene driven by a pollen-specific *Ory s1* promoter was generated by particle bombardment of embryogenic calli. A great reduction in LOL P5 protein was observed and the pollen was hypoallergenic. The authors state that silencing correlated with expression of the antisense transgene. This suggests that it was not typical PTGS as this should lead to degradation of the antisense transcript. However, since both sense and antisense transcripts for LOL P5 would be present, it is tempting to think that dsRNA may have been formed and actively involved in the observed silencing. It will be interesting to learn more about the silencing mechanism in this important example of a positive application of gene silencing.

Sugarcane

Sugarcane (*Saccharum* spp.) is a monocot of great economic importance. It has a complex genome and commercially used varieties are polyploids. A detailed study on RNA-mediated virus resistance in transgenic sugarcane by Ingelbrecht *et al.* (1999) demonstrated the presence and the conserved features of PTGS in a monocot polyploid genome. RNA-mediated virus resistance was observed in transgenic plants (obtained by particle bombardment of embryogenic callus cultures), expressing an untranslatable form of the sorghum mosaic virus potyvirus coat protein from the maize *ubiquitin* promoter. Virus resistance was typically associated with transgenic plants showing low steady-state levels of various sizes of transgene RNA. Resistant plants typically had 4 to 10 copies of the transgene. PTGS-inducing plants actively transcribed the untranslatable coat protein gene and a clear correlation between induction of coding sequence methylation and virus resistance could be demonstrated in most cases. Shoots derived by vegetative propagation maintained the PTGS silencing phenotype, demonstrating mitotic stability.

Wheat

Wheat (*Triticum aestivum*) is an allohexaploid consisting of three genomes (A, B and D). The high-molecular-weight (HMW) seed glutenin subunit proteins (HMW-GS), important for dough elasticity, are encoded by two linked genes (*x* and *y*). Only 3–5 of the 6 potential HMW subunits are expressed at any given time, indicative of a natural gene silencing process in these plants. Increasing the HMW-GS content by transgenic methodologies is of significant commercial interest (Alvarez *et al.*, 2000).

The wheat cultivar Federal (which expresses subunits 1Ax2, 1Dx5, 1Bx7, 1By9 and 1Dy10) was cotransformed with HMW-GS genes 1Ax1 and 1Dx5 and a selectable *bar* marker gene by particle bombardment of immature embryos (Alvarez *et al.*, 2000). Two of the six lines (A and B) that were transformed for the 1Dx5 subunit expressed the transgene in the T_0 and T_1 generations, resulting in an increase in the overall amount of glutenins in the endosperm. Very interestingly, lines C and D (transformed for 1Ax1) that

contained some 3 copies of the transgene were found to express the transgene but silenced expression of the endogenous1Ax2 gene. Lines E (transformed for 1Ax1) and F (transformed for both 1Ax1 and 1Dx5) had 20–50 copies of the transgene and, surprisingly, while the transgenes E and F were expressed or only partially silenced, the endogenous HMW-GS were all silenced. Line E showed stable silencing of all the genes encoding HMW-GS in T_1 and T_2 progeny while line F showed reactivation of expression in the T_1 and T_2 progeny. In line F, 97 of 102 T_1 progeny showed silencing. Further analysis of 12 of these lines revealed that 5 showed activation of expression of the endogenous genes in the T_3 seeds.

In another report on silencing in wheat, Blechl and Anderson (1996) cotransformed wheat (cv. Bobwhite) by particle bombardment of immature embryos with the *bar* gene transcribed from a *ubiquitin* promoter and a chimeric high-molecular-weight glutelin construct that was composed of the *Dy10* promoter, 5′-untranslated region and 145 N-terminal amino acids from the Dy10 subunit of HMW-GS and 719 C-terminal amino acids, poly(A) signal and terminator from the Dx5 sequence. The chimeric protein was distinguishable by its size in the endosperm where it is normally expressed. One of the lines, which had 5–6 copies of the transgene, showed low expression of both the transgene and a 70% reduction in expression of the endogenous HMW-GS. While this is indicative of cosuppression, the mechanism was not elucidated.

Other monocots

Transformation of several other monocots has been reported but, other than a brief mention of failure to obtain GUS expression in some *Lilium longiflorum* plants transformed by particle bombardment (Watad *et al.*, 1998), no cases of silencing have been documented thus far. These include: asparagus (Hernalsteens *et al.*, 1984), banana (Sagi *et al.*, 1995; Schenk *et al.*, 1999), sorghum (Battraw and Hall, 1991; Casas *et al.*, 1993) and tritordeum (Barcelo *et al.*, 1994) and several orchids (Chen *et al.*, 1997; Kamo *et al.*, 1995). For a comprehensive list of monocots that have been transformed, see Vain *et al.* (1995).

Transgene structure

A high incidence of silencing has been observed in many organisms when the transgene is rearranged or when multiple copies are present as tandem or interspersed direct or inverted repeats (Assaad *et al.*, 1993; Rossignol and Faugeron, 1994; Dorer and Henikoff, 1997; Garrick *et al.*, 1998). In general, direct DNA transfer methods more frequently result in the insertion of multiple copies and complex rearranged transgenes than does *Agrobacterium*-mediated transformation (Hiei *et al.*, 1994; Cheng *et al.*, 1998; and personal observations). Repeat structures have been shown to incite both TGS and PTGS (Stam *et al.*, 1998). This relationship between transgene structure and silencing was well illustrated by a study of over 139 independent petunias transformed with the chalcone synthase gene in which all plants containing inverted repeats showed cosuppression patterns indicative of PTGS and those containing disperse repeats gave patterns suggestive of both PTGS and TGS (Jorgensen *et al.*, 1996). Vector sequences and AT/GC-richness of the region (isochore) surrounding the transgene insertion can also influence expression (Kumpatla *et al.*, 1998; Jakowitsch *et al.*, 1999).

Repeat sequences can attract *de novo* methyltransferases (Malagnac *et al.*, 1997) that in turn modify the chromatin architecture of the surrounding region. Methylation of the promoter region typically gives rise to TGS (Kumpatla *et al.*, 1997) whereas coding region methylation may incite PTGS, which is thought to result in the formation of aberrant RNA transcripts (English *et al.*, 1996; Hohn *et al.*, 1996; Jones *et al.*, 1998). Repeats aided by transgene rearrangements can cause unintended transcription of promoter or coding sequences, both of which have been demonstrated (Mette *et al.*, 1999) or proposed (Flavell, 1994; Montgomery and Fire, 1998) to cause silencing. That the presence of multiple copies provides additional opportunities for ectopic interactions between transgene sequences has also been proposed to cause activation of silencing (Kumpatla *et al.*, 1998).

Several studies on direct DNA transfer methods have reported concatenate formation and integration of transgenes predominantly at one locus, identified by Mendelian segregation. There appears to be little difference in frequency of insertion or rearrangements of genes when co-bombarded on different plasmids or as a cointegrate plasmid (Battraw and Hall, 1990) and transgenes typically integrate at one locus even if they are present on different plasmids during co-inoculation (Pawlowski and Somers, 1996). A study based on restriction fragment analysis of the structure of over 16 transformants in oat (derived by biolistic transformation) revealed that the inserts were

rearranged and had host DNA interspersed in the complex transgene locus (Pawlowski and Somers, 1998). The transgenes were silenced in most of the plants even though many possessed a potentially functional insert. Another study of biolistic-mediated transgene integration into rice (Kohli *et al.*, 1998) interpreted the single locus as being a cluster of multiple integration events. A recent study by Svitashev *et al.* (2000) showed the interspersion of transgene DNA with host DNA by *in situ* hybridization analyses on transgenic oat obtained by biolistic transformation. Rearrangement of transgene inserts were observed even when only one or two copies of the transgene are present (Kohli *et al.*, 1998), resulting in the formation of direct and inverted repeats that contained vector sequences dispersed within the three-gene cointegrate unit. Takano *et al.* (1997) describe the transgene configuration of two loci in rice plants transformed with a construct that contained an *hpt* and a *luciferase* (*luc*) gene. One insert was composed of two inverted fragments separated by plasmid sequences. In the other insert, the *luc* gene was deleted and the *hpt* gene was present in a tail-to-tail inverted configuration. The junction sequences of these inserts revealed duplications of host sequences, the presence of microhomologies between the plasmid sequence and the host genome, and one of the inserts possibly had a retrotransposon inserted prior to transgene integration, indicating that once a site in the genome is suitably exposed for integration events, it becomes a hotspot for the subsequent insertion of extraneous DNA. Morino *et al.* (1999) have described a complex rearranged locus that yielded a transcript containing both sense and antisense *gus* sequences in plants that silenced *gus*. Kumpatla and Hall (1999) described the presence of several rearranged insert configurations in rice that led to the transcription of vector sequences and of antisense RNA, all of which probably contribute to reduced transgene expression. Additional studies (Kumpatla, 1997) also describe the possible presence of repeat-induced point mutations in a process similar to that observed in *Neurospora* (Selker, 1997). Analysis of the breakpoints in the rearranged insert led to the proposal that the 35S promoter sequence is susceptible to fragmentation, a conclusion also reached by Kohli *et al.* (1999b). In studies on the chromosomal locations of an *Adh-1*/*gus* transgene in several oat transformants, Svitashev *et al.* (2000) found that chromosomal aberrations were frequently associated with integration sites and suggested that the process of biolistic transgene integration may lead to host DNA breakage.

The data summarized above point to multicopy, complex, inserts as being abundant during transformation processes and often being the major culprits in gene silencing. However, we have studied some 20 independent *Agrobacterium*-transformed rice plants that contain a single, intact copy of a construct that contains a *gus* reporter driven by a rice root-specific gene *RCg2* (Xu *et al.*, 1995) promoter that is flanked by *hpt* and *bar* genes. Interestingly, although *gus* expression is silenced the flanking genes remain functional. Thus, as has been seen in other cases (Meyer and Heidmann, 1994), single-copy, intact transgene inserts are also subject to silencing. How and why specific sequences are targeted for silencing are focal questions for current research.

Genome surveillance processes

The concept that transgene silencing processes reflect activities of normal genome functions, mentioned in the Introduction, is elaborated in Figures 1 and 2. While the events depicted by the highlighted layers in Figure 1 presumably arose in response to individual situations and stimuli, each contributes to the overall integrity of the genome and represents a potential barrier to the insertion of functional transgenes. Although not related to the vertebrate immune system in any true sense, these silencing defense ramparts are analogous in that they represent both general and specific defense systems that counter invasion of the organism.

An initial layer of defense against intrusive DNA is the action of enzymes that participate in host replication and repair machinery; these include DNases, polymerases, recombinases and ligases. These enzymes can cause scrambling (breaking, concatenations, degradation, rearrangements and sequence alterations) of the DNA sequence prior to the integration process (Figure 1a). Although detailed analyses of transgene structure remain few in number (especially for monocots), existing reports (Kumpatla *et al.*, 1997; Takano *et al.*, 1997; Pawlowski and Somers, 1998; Kohli *et al.*, 1998, 1999b; Kumpatla and Hall, 1999) make it evident that permutation, scrambling and degradation of transgene sequences frequently occurs both prior to integration and during the integration process (see also the more detailed discussion in the section on transgene structure). That these rearrangements reflect metabolic processes is supported by the

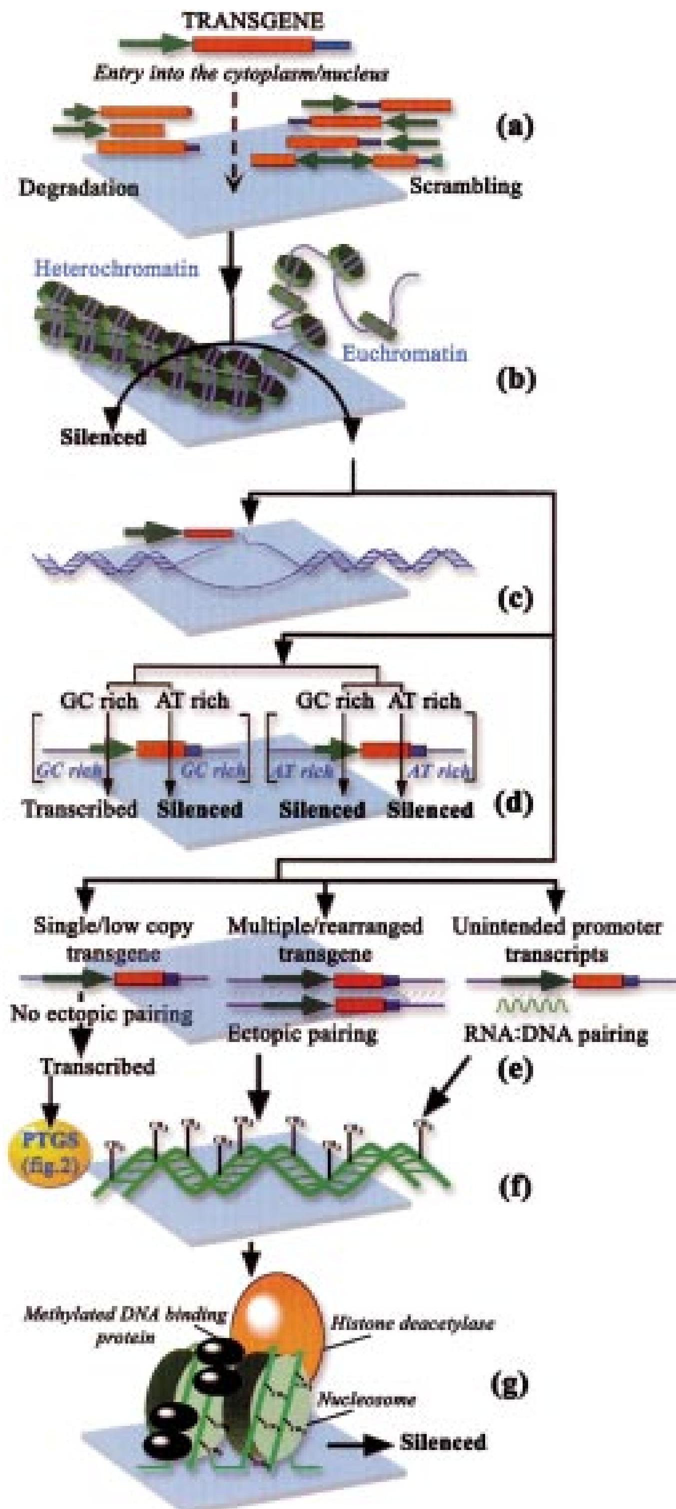

Figure 1. Genome surveillance and transcriptional gene silencing processes. The cell is envisioned as having several barriers at which invasive DNA is detected and potentially inactivated. a. Incoming DNA is subjected to various enzymatic activities that degrade or scramble the original transgene and vector sequences. b. Inside the nucleus, DNA can be integrated into heterochromatin (and typically silenced) or euchromatin. c. Integration intermediates may be recognized by methyltransferases or other surveillance enzymes. d. The inserted sequence may lie within a compatible or an incompatible sequence (isochore) region. e. Sequence similarity of multiple inserts or between the insert and endogenous sequence(s) can lead to ectopic pairing (DNA-DNA pairing); DNA-RNA pairing can also occur. f. DNA-DNA or DNA-RNA pairing can signal cytosine methylation. g. Methylated DNA serves as a signal for recruitment of methylated DNA-binding proteins and subsequently heterochromatinization (see text).

fact that elution and characterization of DNA from particles used for bombardment revealed no rearrangements (W.G. Buchholz, G.J.N Rao and T.C. Hall, unpublished observations), but plasmid-plasmid recombination has been detected for integrated DNA following co-bombardment (Kumpatla and Hall, 1999). Studies of transgene inserts generated by different transformation methodologies reveal that all methods can give rise to complex inserts, although it seems that direct DNA transfer methods have an especially high proclivity for generating complex rearranged transgenes as compared to *Agrobacterium*-mediated transformation, presumably because the virD2 and virE2 proteins associated with the T-complex provide protection prior to integration (Rossi *et al.*, 1996).

After insertion of transgene DNA, further rearrangements or eliminations may occur, for example by duplication or deletion during meiosis in combination with altered ploidy (polyploidy or aneuploidy), which seems to frequently occur during plant growth (Matzke *et al.*, 1999). However, personal observations (G.J.N. Rao, S.P. Kumpatla and T.C. Hall, unpublished) suggest that gross transgene rearrangements are rare beyond the R_1 generation.

Although currently an active topic of research, relatively little is known about the processes by which transgene DNA is integrated into the plant genome. The process of integration is probably facilitated at active replication forks (see Transgene structure) and regions of repair. A recent report on integration in murine fibroblast cells (Dellaire and Chartrand, 1998) following direct DNA transfer revealed that insertions were found to occur at random (i.e. in both euchromatin and heterochromatin) and were facilitated by double-stranded breaks. As noted above, Svitashev *et al.* (2000) have recently provided sound evidence that fracture of host DNA during biolistic transformation, and combination with transgene DNA by break repair, can give rise to interspersed DNA repeats. However, for *Agrobacterium*-mediated transfer, interaction between the virulence proteins associated with the T-DNA and host factors (including histone H2A) may also play a role in the insertion (Bundock *et al.*, 1995; Sonti *et al.*, 1995; Salomon and Puchta, 1998; Mysore *et al.*, 2000). Illegitimate recombination has been proposed to be the major mechanism by which transgene integration is brought about in both direct DNA and *Agrobacterium*-mediated transformation methods (De Buck *et al.*, 1999). In this process, DNA microhomologies are thought to contribute to recombinational insertion into a locus (Kohli *et al.*, 1999b). Most characterized transgene inserts are probably in transcriptionally active euchromatic regions because inserts within heterochromatin are immediately silenced and hence are not recovered during selection. Thus, heterochromatin (Figure 1b) provides broad protection against expression as well as insertion of intrusive DNA, including transgenes. The actual integration step (Figure 1c) may be one that is particularly susceptible to genomic surveillance processes as it is known that the cruciform integration structure is a preferred target for DNA methyltransferases (Bestor and Tycko, 1996; Bestor, 1998) that may mark the transgene for heterochromatinization and inactivation (Figure 1c). While it is debatable if DNA methylation *per se* can inhibit transcription, recent work has established that certain proteins specifically bind to methylated DNA (such as MeCP2) and recruit histone deacetylase which stimulates heterochromatin formation and hence silencing (Ng *et al.*, 1999; Wade *et al.*, 1999). As discussed below, upon integration, several alternative processes may interact with the transgene resulting in TGS or PTGS. Thus, TGS is portrayed in Figure 1 as a serial array of defenses, and this may indeed reflect the barriers encountered by intrusive DNA. However, once integrated, the intrusive DNA may be susceptible to many of the same or similar barriers but they will not necessarily have the same linearity or hierarchy of action.

Transcriptional gene silencing

Several parallels have been drawn between plant TGS and paramutation in plants, nucleolar dominance, position-effect variegation (PEV) in *Drosophila*, mating type and telomeric silencing in yeast, methylation induced premeiotically (MIP) in *Ascobolus* and repeat-induced point mutation (RIP) in *Neurospora* (Rossignol and Faugeron, 1994), and genomic imprinting in vertebrates and plants. Transcriptional transgene silencing is broadly classified into two types, *cis*-inactivation and *trans*-inactivation (Vaucheret, 1993; Matzke and Matzke, 1995).

Cis-inactivation can result from the insertion of multiple, rearranged copies of a transgene at a single locus or in the presence of closely linked copies of transgene by attracting TGS surveillance systems (like methylation or heterochromatin-forming proteins) that suppress transcription of promoters. It can also result from direct integration of transgene(s) into heterochromatin regions (often characterized as position effect variegation, PEV). *Cis*-inactivation

can also ensue from isochore incompatibility of the transgene sequence with the surrounding genome sequence (Matzke and Matzke, 1998a) (Figure 1d). The isochore concept (Bernardi, 1995) postulates that genomes contain very large regions of relatively AT- or GC-rich sequences and organize their genes within certain isochore regions. This concept has been explored in the monocot maize, where it was found that most protein-encoding genes are present in isochores covering an extremely narrow (1–2%) GC range that represents only 10–20% of the genome (Carels *et al.*, 1995). Interestingly, the multicopy genes encoding zeins, the major seed storage protein of *Zea mays*, are in a separate narrow isochore and it is tempting to speculate on the possibility that this feature may attenuate silencing of this gene family. As we have discussed previously (Kumpatla *et al.*, 1998), observations from isochore studies suggest that inserted sequences such as transposons are unstable in genomic environments that do not match their AT/GC composition.

Only a few reports have investigated the nature of sequences flanking transgenes. However, expressing transgenes in tobacco were associated with AT-rich sequences or enhancers, some of which were matrix attachment regions (MARs) whereas bacterial backbone vector sequences flanked non-expressing transgenes (Iglesias *et al.*, 1997; Jakowitsch *et al.*, 1999). AT-rich regions were also found to flank two expressing rice transgenes (Takano *et al.*, 1997), and Dong *et al.* (1996) found in rice that an expressing 35S-*gus* transgene was inserted within one of two copies of an endogenous gene.

Trans-inactivation occurs when one transgene locus (that is itself silent) exerts a dominant repressive effect on other loci (which may be linked) that typically include sequence homologies in promoter regions. Sequences as short as 90 bp have been shown to be sufficient to mediate silencing (Vaucheret, 1993). *Trans*-inactivation thus requires interaction of the silencing locus with the target sequence. This has been proposed to be typically caused by ectopic DNA-DNA pairing between the loci, resulting in a transfer of the silenced state from one locus to another (Figure 1e) (either by transfer of repressive chromatin states to targets or *de novo* methylation of target sequences). Since accurate pairing of like DNA sequences is intrinsic to vital cellular processes such as meiosis, it should not be surprising that effective systems exist within the genome to mediate recognition of identical sequences. The inverted-repeat conformation has been recognized in several cases of transgene silencing and seems to be especially susceptible to methylation for both TGS and PTGS (Stam *et al.*, 1998; Luff *et al.*, 1999; Melquist *et al.*, 1999). Alternatively, RNA-DNA pairing has also been shown to cause methylation of the homologous sequence and aberrant promoter transcripts have been shown to cause RNA-directed DNA methylation and silencing (Mette *et al.*, 1999) (Figure 1e). Thus, the unintended transcription of transgene promoters can result in silencing of the promoter and related sequences.

Methylation, heterochromatinization and transcriptional gene silencing

Transgene TGS is almost always associated with promoter methylation (Bestor and Tycko, 1996) and both symmetric and asymmetric methylation of cytosine residues is known to occur. In studies on monocots, methylation-associated transgene TGS has been rigorously documented for rice bearing a complex transgene insert that included multiple repeat elements (Kumpatla *et al.*, 1997; Kumpatla and Hall, 1999). Moreover, unlike PTGS, the epigenetic (methylated) and repressive state is maintained in progeny and, hence, meiotically. The repression of methylated promoters probably results from recruitment of chromatin modifying factors (such as histone deacetylases) and remodeling factors (such as SNF2 helicases) through methylated DNA-binding proteins (such as MeCP2) that prevent access of DNA to the transcription machinery, yielding a heterochromatin-like promoter status (Bird and Wolffe, 1999; Wolffe and Matzke, 1999). The transgene architecture, copy number and genomic position play an important role in determining whether a promoter sequence will be methylated and repressed. Nevertheless, even when the transgene insert is present in multiple (perhaps rearranged) copies and contains repeat sequences, many studies have reported expression. This implies that the induction of TGS is a multicomponent process.

Studies in *Drosophila* show that enhancer sequences (and consequently the proteins that bind to them), oppose heterochromatinization of a DNA sequence (Francastel *et al.*, 1999). Many transcription factors that bind or are associated with enhancer sequences recruit chromatin remodeling factors and/or modifying factors (like histone acetylases) to activate transcription. Similarly, locus control regions (LCRs), matrix and scaffold attachment regions (MARs and SARs) and associated proteins may reduce position effect variegation (PEV) and heterochromatinization

by organizing DNA into distinct structural domains (van der Geest *et al.*, 1994; Festenstein *et al.*, 1996; Goossens *et al.*, 1999). Boundary elements and insulators of enhancers (some of which may be present within MARs or SARs) have also been shown to insulate sequences from heterochromatinization and reduce PEV (Sun and Elgin, 1999). In contrast, Polycomb recognition elements (PREs) in *Drosophila* are known to attract Polycomb-group (PcG) protein complexes that induce the formation of higher-order repressive chromatin structures (heterochromatinization) and are involved in heritable maintenance of silencing during development (Paro *et al.*, 1998).

Thus, it can be imagined that the fate of transgene expression is an outcome of the contest for chromatin dominance that reflects the relative concentration of factors that cause heterochromatinization and those that oppose it in a given transgene environment. The nature of the promoter sequence in combination with surrounding sequences is probably why different transgenic promoters vary in the extent of TGS *in cis* (by PEV-like effects) or *in trans* (by paramutation-like effects; Neuhuber *et al.*, 1994; Jakowitsch *et al.*, 1999).

Until recently, chromatin has been seen as a rather uninteresting, ubiquitous, suppressor of gene expression. It is now clear that chromatin remodeling participates in gene-specific regulation as well as in more global control of expression. Indeed, an emerging insight is that both higher-order chromatin structure (Sun and Elgin, 1999) and, possibly, the structure of the nucleus itself participates in regulation of expression. In the future, it will be instructive to explore the impact of transgene insertion on the innate organization of this repository of genetic information. Several pieces of evidence (Wolffe and Matzke, 1999) now point to the role of chromatin as being the main effector of silencing (Figure 1f). In at least two reports of transgene silencing in plants, the silenced transgenes were found to be in an altered or condensed chromatin configuration (Ye and Signer, 1996; van Blokland *et al.*, 1997). In *Drosophila* (an organism lacking methylation as a major defense mechanism), silencing of transgene arrays was associated with heterochromatin formation and correlated with binding of the heterochromatin-associated heterochromatin protein-1 (HP-1, which has two chromo domains) (Koonin *et al.*, 1995; Fanti *et al.*, 1998). Overexpression of the mouse orthologue of HP-1 (M31) in mouse cell lines affected PEV of transgene expression depending on its chromosomal context (Festenstein *et al.*, 1999). Transgene *trans*-silencing caused by multiple transgene repeats in *Drosophila* was shown to act *in trans* and was reversed by PcG proteins, Polycomb (a single chromo domain-containing protein) and Polycomb-like, which are also involved in chromatin-mediated control of *Drosophila* homeotic genes (Pal-Bhadra *et al.*, 1997, 1999). These findings suggest that many aspects of silencing processes (*cis* and *trans* inactivation) can function independently of methylation and, indeed, methylation does not seem to be involved in some cases of paramutation in plants (Hollick *et al.*, 1997).

Post-transcriptional gene silencing

PTGS in plants is analogous to RNA interference (RNAi), first identified in *C. elegans* (Fire *et al.*, 1998), but now known to function in several other animals, including insects (Kennerdell and Carthew, 1998), vertebrates (Bahramian and Zarbl, 1999) and cnidarians (Lohmann *et al.*, 1999). PTGS is known as quelling in *Neurospora* (Cogoni and Macino, 1999a). It is now widely accepted that PTGS evolved as a defense system to counter viruses and transposable elements (Baulcombe, 1996; Montgomery *et al.*, 1998). The few existing reports of PTGS in monocots (Table 1) suggest that it probably operates through mechanisms similar to those observed in dicots since shared features include the existence and triggering of coding region methylation (Ingelbrecht *et al.*, 1994, 1999), mitotic stability (Guo *et al.*, 1999; Ingelbrecht *et al.*, 1999), the induction of virus recovery in transgenic plants (Ingelbrecht *et al.*, 1999; Pinto *et al.*, 1999), and the ability to cosuppress endogenous genes (Yin *et al.*, 1997). This is not surprising as many of the host genes that are responsible for PTGS-like processes in *C. elegans* (RNAi) and *Neurospora* (quelling) are conserved in plants, suggesting that several features of PTGS are common between widely diverged groups of eukarya (Cogoni and Macino, 1999a, b; Ketting *et al.*, 1999; Tabara *et al.*, 1999). However, studies on viral proteins that reverse PTGS reveal that, in plants, host-pathogen interactions have also contributed to the evolution of PTGS (Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Voinnet *et al.*, 1999). Thus, differences in features of PTGS between plants, if any, will be dependent on their evolutionary history, including pathogen interactions (Voinnet *et al.*, 1999).

Data from cosuppression, virus resistance and virus-induced gene silencing demonstrate that PTGS has nuclear and cytoplasmic components (Figure 2)

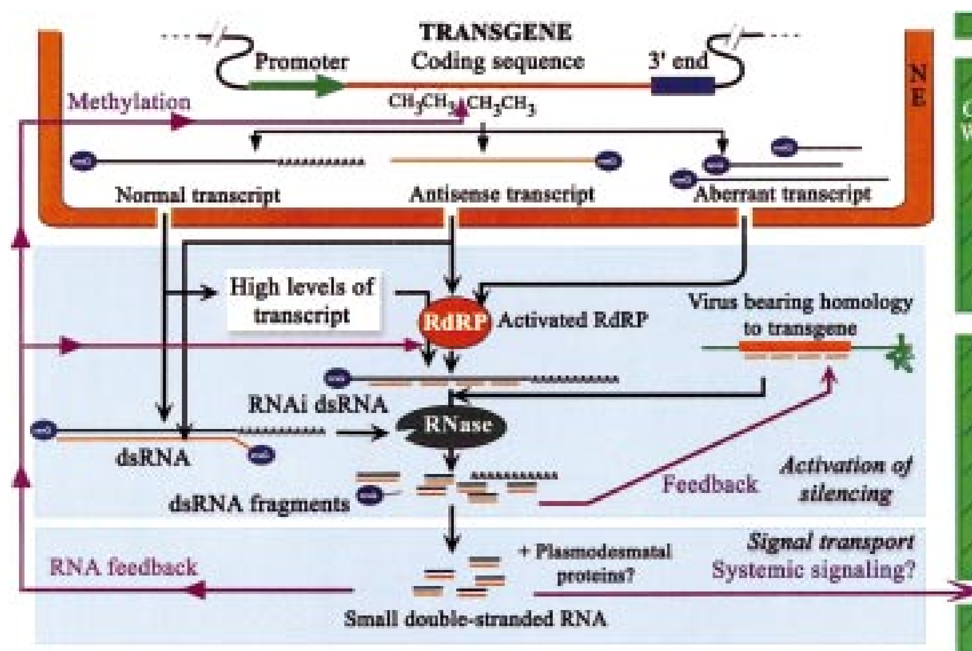

Figure 2. Post-transcriptional gene silencing events are depicted for both the nucleus (bound by the nuclear envelope, NE) and the cytoplasm (bound by the cell wall, CW). As described in the text, host RNA-dependent RNA polymerase (RdRP) and RNase are seen as central players in PTGS events. RNAi dsRNA refers to a dsRNA intermediate that is a substrate for PTGS-specific degradation.

and it is highly probable that cross-talk exists between processes within these compartments (English *et al.*, 1996; Sijen *et al.*, 1996; Jones *et al.*, 1998; Voinnet *et al.*, 1998). It also appears that the cytoplasmic processes can be induced in the absence of a genomic contribution. For example, PTGS can be induced as a consequence of virus infection in the absence of a transgene (Ratcliff *et al.*, 1997, 1999), leading to recovery of the plant from infection.

Nuclear events

Although there are reports of PTGS induction with single-copy inserts, the presence of inverted repeats and multiple copies of transgenes are typically associated with silencing (Jorgensen *et al.*, 1996). In general, PTGS is correlated with active transcription of the transgene, and transcriptional silencing of the transgene has been shown to reverse PTGS (English *et al.*, 1997; Que and Jorgensen, 1998).

The nature of RNAs that incite PTGS is not well understood. It is believed that either ectopic DNA-DNA or DNA-RNA pairing, or the formation (intended or unintended) of antisense transcripts that give rise to dsRNA from cryptic promoters 3′ to the transgene insert, results in the formation of aberrant RNA transcripts (which include RNAs lacking polyadenylation, or short polyadenylated RNAs, generated as a result of incomplete transcription) that activate silencing (Baulcombe and English, 1996; Depicker and Van Montagu, 1997; Metzlaff *et al.*, 1997; Montgomery and Fire, 1998; Que and Jorgensen, 1998; Stam *et al.*, 1998; Wassenegger and Pélissier, 1998). Alternatively, high levels of transcription, giving rise to accumulation of normal transcripts that exceeds a 'threshold' level, have been proposed to activate silencing (Lindbo *et al.*, 1993). Thus, as shown in Figure 2, normal transcripts, antisense transcripts and aberrant transcripts can all give rise to PTGS.

Several studies on PTGS have reported preferential methylation of cytosines in the coding region (Lindbo *et al.*, 1993; Ingelbrecht *et al.*, 1994, 1999; Jones *et al.*, 1998), contrasting with the preferential methylation of the promoter in TGS (Kumpatla *et al.*, 1997). Coding region methylation may help in maintaining gene silencing by inciting the formation of aberrant transcripts (Jones *et al.*, 1999). If this is the case, then (as discussed above) the density of coding region methylation may underlie the stochastic nature of PTGS induction among individual transformants carrying the same insert or within different tissues of a single transformant.

Cytoplasmic events
Upon the entry of PTGS-eliciting RNA into the cytoplasm, its degradation and that of any homologous RNAs is postulated to ensue and several reports have shown the presence of either degradation intermediates or aberrant transcripts (Goodwin *et al.*, 1996; van Eldik *et al.*, 1998). A critical question is how these RNAs are distinguished from normal cellular RNAs and targeted for elimination. As shown in Figure 2, an emerging insight is that host RdRP and RNase functions are central to these surveillance events. The cloning of a plant host RdRP that is normally induced during virus infection (Schiebel *et al.*, 1998), and the finding that mutations in QDE1 protein from *Neurospora* (which contains an RdRP-domain homologous to that of the plant sequence) reversed quelling (Cogoni and Macino, 1999a), have firmly confirmed the role and importance of host RdRP in PTGS (Wassenegger and Pélissier, 1998). The dsRNA formed through RdRP activity presumably serves as a target for RNase, providing the basis for sequence specificity of degradation. Small (ca. 25 nt) fragments of antisense orientation to the elicitor RNA have been observed in all studied cases of PTGS in plants (Hamilton and Baulcombe, 1999), but it is still not certain if host RdRP is involved in their synthesis.

An analysis of the phyletic distribution of RdRP in the non-redundant database reveals that it is present throughout the crown group of eukarya and has several paralogues in plants and *C. elegans* (Schiebel *et al.*, 1993; Cogoni and Macino, 1999a). Plant RdRPs can be classified into two groups: those that possess only an RdRP domain and those that have both an RdRP domain and an RNA recognition motif (RRM) domain (L. Aravind, personal communication). The RRM is present in several RNA-binding proteins, such as splicing factors (e.g. hnRNPA1) and RNA processing proteins (e.g. polypyrimidine tract-binding protein) and may contribute to the sequence specificity of this process (Birney *et al.*, 1993; Siomi and Dreyfuss, 1997). Studies on the activity, distribution, expression and role of these RdRPs are likely to reveal additional features of PTGS that may be manipulated for efficient transgene expression.

Formation of complementary RNA by RdRP activity appears to be followed by the degradation of dsRNAs by a RNase which may be constitutive or specific to PTGS (Lindbo *et al.*, 1993; Baulcombe, 1996). However, as regional double-strandedness is a common feature of all RNA molecules, it is unclear how degradation of specific RNAs is brought about during PTGS. One possibility is that the dsRNAs that are targeted are not continuous but comprise the template RNA plus multiple short complementary sequences (synthesized by RdRP), each with its own 5′-triphosphate and free 3′-OH end, that provides a different motif than that of native dsRNA regions (Figure 2; compare RNAi with dsRNA and viral dsRNA). A candidate nuclease for degradation of the targeted dsRNAs was identified as RNAi mutant *mut-7* in *C. elegans* (Ketting *et al.*, 1999). Since the RNaseD domain in this protein (which has both RNase and DNase activity) is also present in many proteins of all organisms, it is reasonable to suggest that the plant orthologue of MUT7 may be involved in degrading dsRNA generated during PTGS.

The aberrant transcripts generated in silenced plants are proposed to incite RdRP activity, leading to the formation of small (ca. 25 nt) dsRNAs that (as shown in Figure 2) may feed back to the nucleus and cause DNA methylation of the coding sequence or may propagate systemically through the phloem by an unknown mechanism similar to that used by viruses and viroids (Palauqui *et al.*, 1997; Voinnet *et al.*, 1998; Jones *et al.*, 1999), triggering widespread PTGS.

Mutants that increase or decrease the severity of PTGS have been isolated in *Arabidopsis* but these have not been cloned (Elmayan *et al.*, 1998; Morel and Vaucheret, 2000), and (as mentioned above) some viral proteins have been shown to be capable of reversing PTGS (Anandalakshmi *et al.*, 1998; Beclin *et al.*, 1998; Brigneti *et al.*, 1998; Kasschau and Carrington, 1998; Marathe *et al.*, 2000). Both DNA and RNA viruses, and viruses that affect monocots and dicots have been shown to possess these proteins. How these proteins reverse silencing remains to be determined, but the patterns of reversal (Voinnet *et al.*, 1999) suggest that, as depicted in Figure 2, PTGS comprises several phenomena. It is likely that different viruses have evolved to tackle PTGS depending on the nature of their co-evolution with their plant hosts.

Evolutionary implications of DNA modification

The association of DNA methylation with transposon silencing, inactivation of extraneous and duplicated sequences and hence with TGS and PTGS has led to the view that DNA methylation serves as a defense rampart in eukarya that evolved from the restriction-modification system of bacteria (Bestor and Tycko, 1996). An alternative proposal is that DNA methyla-

tion evolved as a global repressor of transcriptional noise in organisms concomitant with an increase in genome size and complexity, to prevent spurious expression of genes in differentiated cells (Bird, 1995). Although DNA methylation may affect DNA-protein interactions, it is now widely accepted that DNA methylation recruits chromatin modifying or remodeling components that change the chromatin environment of a DNA sequence, leading to repression (Bird and Wolffe, 1999). The crown group of eukarya includes animals, plants and fungi (Sogin and Silberman, 1998) and, while DNA methylation was probably ancestral to this group (Wolffe and Matzke, 1999), not all crown group lineages use DNA methylation extensively in defense and differential regulation of the genome. For example, *C. elegans* and *Schizosaccharomyces pombe* lack detectable DNA methylation although the latter has an inactive DNA methyltransferase (Wilkinson *et al.*, 1995; Yoder and Bestor, 1998). *Drosophila* shows very little detectable methylation despite encoding at least two methyltransferases (Hung *et al.*, 1999) while plants and vertebrates show extensive DNA methylation of their genomes. However, the proteins that cause chromatin remodeling (such as the SNF2 helicase) or chromatin modifications (such as histone acetylases or deacetylases that are the downstream effectors of DNA methylation) are present in all members of the crown group. Additionally, TGS, repeat-induced silencing phenomena and protein modules (such as the chromo domain) that are closely associated with methylation in plants, vertebrates and fungi are also found in organisms lacking methylation. Thus, it is possible that while DNA methylation is ancestral, the adaptation to genome-wide methylation and its associated effects evolved in these lineages concomitant with pressure from extragenomic and intragenomic selfish elements like viruses, transposons and retrotransposons, providing an additional defense reinforcement (Bestor, 1998) or possibly an evolutionary cost benefit (Bird, 1995).

DNA methylation in plants has been implicated in differential regulation of some genes, paramutation, parent-specific gene imprinting, nucleolar dominance, controlling expression of selfish elements, TGS and PTGS. DNA methylation (which is predominantly at the 5-C position of cytosine in eukaryotes) activity is classically differentiated into the post-replicative maintenance methylation, which methylates the unmethylated strand of a hemi-methylated DNA template (and thus requires symmetric C residues such as CG or CNG), and *de novo* methylation which arises as a result of various features of the target DNA and its environment. These include DNA repeats, allelic and non-allelic DNA pairing, unusual (e.g. cruciform) DNA structures and proximity of the target gene to heterochromatin (reviewed in Kumpatla *et al.*, 1998; Matzke and Matzke, 1998a). Alternatively, DNA methylation can also be triggered by homologous RNA-DNA interactions (Wassenegger and Pélissier, 1998; Wolffe and Matzke, 1999) that are probably caused by the formation of aberrant RNA of promoter or coding region sequences. Several DNA methyltransferases have been described in *Arabidopsis* (Genger *et al.*, 1999) that may have roles similar to those seen in *Ascobolus*, which has a methyltransferase (MASC-1) responsible for *de novo* methylation of repeats (MIP), and at least two more methyltransferases, one of which is a maintenance methylase (Selker, 1999). Recently, an *Arabidopsis* methyltransferase fused to a chromo domain that is involved in protein-protein interactions of chromatin-associated proteins was described (Henikoff and Comai, 1998) that suggests a chromatin recruiting function for this protein. Interestingly, several natural strains of *Arabidopsis* have inactive copies of this protein. It is unclear if different plant lineages will have diverse DNA methyltransferase families, or if all the different DNA methyltransferases were already present in ancestors of flowering plants.

Putative insight into the stochastic nature of silencing

A perplexing feature in transgenic plants showing TGS is the unpredictable nature of the induction of silencing in progeny, often correlated with increased methylation of promoter sequences (Kilby *et al.*, 1992; Assaad *et al.*, 1993; Kumpatla and Hall, 1998b). Typically, plants that are silenced maintain their epigenetic state in their progeny. Several lines of evidence give insight to the processes that operate during TGS. Studies in *Arabidopsis* showed that establishment of nucleolar dominance, where rDNA from one haplotype is methylated and silenced, is a progressive process that takes one or two generations of self-pollination and propagation. In another series of elegant experiments, crosses between a mutant defective in methylation (*ddm*1, now recognized to be a helicase of the SWI/SNF2 family) and a transcriptionally silenced gene resulted in progressive reversion of transgene silencing that took up to two generations for complete activation and coincided with a progressive decrease in methylation (Jeddeloh *et al.*, 1998). As both sym-

metric and asymmetric methylation is observed during TGS, maintenance or *de novo* methylation (that are post-replicative processes) seem to be cumulative, with methylation density increasing in each round of replication. Increased methylation would cause a greater accrual of methylated DNA-binding proteins that cause repressive chromatin structures. Since the number of replication rounds a somatic cell undergoes before it becomes a gametic cell is unpredictable, progeny will have different levels of accumulation of methylation; this may contribute to the stochastic induction of silencing in progeny of expressing plants. The progressive nature of the methyltransferase, however, is not understood and may be tied to the efficiencies and nature of how the maintenance and *de novo* methyltranferases interact with chromatin to propagate a repressed state.

In regard to PTGS, if the methylation density or the amount of aberrant RNA is increased in a cell (perhaps reflecting different ploidy status of individual cells: Matzke *et al.*, 1999), these events may be self-perpetuating, further augmenting coding region methylation levels in that cell. If systemic spread to surrounding cells ensues, this would generate a tissue source for increased transcription of aberrant RNA, determining the extent of systemic silencing.

A prediction from the above concepts is that seeds derived from gametes that were formed late in development (i.e. after many divisions) should show more silencing than those derived from gametes that were formed early. This is consistent with the increase in methylation observed in plant development (Richards, 1997).

Avoidance of silencing

From a pragmatic point of view, the development of strategies to avoid transgene silencing is an urgent goal. Some of the following approaches towards this goal are considered in greater detail in Kumpatla *et al.* (1998). Gene constructs should contain base substitutions so that they contain as little sequence similarity as possible to putative endogenous sequences, or to similar sequences in the same construct. As reflected in many of the articles cited here, although sequence similarity is closely associated with methylation-based silencing, many endogenous genes are present in multiple copies. The small sequence differences present in such genes may be very important in avoiding detection by genome surveillance processes. Additionally, introns may be important in providing sequence diversity. The relative GC or AT richness of DNA may signal a region as being ‘gene space’ compatible with a coding region and one approach to establish a GC-rich ‘isochore’-like environment would be to flank each end of the gene construct with CpG sequences of ca. 300 bp and not less than 50 bp that contain at least 20% of A and T residues dispersed along their length. Since genes are typically organized in chromatin loops of various sizes that are attached to the proteinaceous nuclear matrix at locations known as matrix attachment regions (MARs), flanking transgene inserts with MARs may help to reduce variance in expression levels, make expression proportionate to gene copy number and reduce position effect in transgenic organisms (Vain *et al.*, 1999). A detailed consideration of MARs in reducing silencing is given elsewhere in this volume (Allen *et al.*, 2000). The addition of transcriptional terminators on either side of the transgene construct is probably advisable to prevent transcriptional read-through from promoters present in flanking genomic regions, thereby preventing collision of transcription complexes. Another important precaution is to ensure that transgene constructs contain little, preferably no, plasmid or phage vector sequences since these may be recognized as alien to the recipient genome and serve as targeting elements for surveillance systems. An elegant approach to eliminate multiple identical copies was recently demonstrated in wheat: the transgene was flanked by *lox* recombination sites in an inverted orientation; by crossing lines transgenic for this construct with a line expressing the CRE recombinase, progeny carrying a single-copy insert were resolved from the 4-copy parental line (Srivastava *et al.*, 1999). As more insight is gained into the establishment of methylation patterns, duplicated sequence recognition, the role of chromatin structure and the role of RdRP in TGS and PTGS systems, it is likely that additional avoidance strategies can be developed.

Conclusions

Studies on gene silencing are revealing many novel aspects of gene expression and are providing unifying concepts of epigenetic regulation and the involvement of chromatin environment as well as implicating newly identified host factors as major regulators of genetic behavior. This insight, combined with new findings from large-scale genomic analyses currently underway, will undoubtedly enable the development

of transgenic plants that reliably express the desired novel traits. Stability of expression is vital for future increases in performance of major monocot crops, where exciting potentials exist for many improvements, including disease resistance and nutritional and processing qualities as well as abiotic stress tolerance and overall yield enhancement.

Few, if any, of the silencing processes reviewed here will prove to be unique to monocots; indeed, part of the recent excitement in this field is the recognition that silencing events are essentially ubiquitous among eukaryotes and that they are present in many prokaryotes. However, the occurrence of silencing in monocots has been especially evident because of the widespread use of direct DNA approaches for transformation. While important instances of single- or low-copy-number inserts have been attained with this procedure, the data reviewed here make it clear that the vast majority of inserts are multicopy and include many sequence rearrangements. The novel strategy described by Srivastava *et al.* (1999) for resolving multicopy inserts may alleviate this major drawback to direct transfer techniques.

Even where single-copy inserts are present, there are worrisome instances of gene silencing. Presently, we have little insight into how or why such sequences are targeted, although it is clear that in many instances excessive expression levels lead to PTGS. We also have little insight into the stochastic processes that lead to silencing in some lines while sibling progeny with an apparently identical genomic complement and organization express transgenic information reliably and at high levels.

While it is possible that a specific attribute of a transgene is the key feature that triggers an initial silencing response, there is no reason to exclude the possibility that additional characteristics stimulate further responses. Hopefully, the diagrams in Figures 1 and 2 convey the message that there are multiple safeguards and regulators of gene expression that can respond to intrusive DNA or RNA derived therefrom. With this in mind, it is very possible that some of the more enigmatic aspects of transgene silencing reflect the response of more than one genomic surveillance process. It is unclear at present whether certain genomes are more susceptible to silencing and, if so, whether this is correlated with genome size or content (e.g. the amount of repetitive sequences present) or the presence of specific surveillance arrays. Do specific nucleotide sequences or structures of transgenes mark them as being alien to the recipient genome? Are particular promoters, coding regions and terminators preferential targets for silencing and, if so, why? The challenge to unravel these puzzles should stimulate important and undoubtedly fruitful avenues for future biological research.

Acknowledgements

We thank Jinjiang Dong, Pushpa Kharb, Guofu Li, Magda Cervera, Tao Wang, Prapapan Teerawanichpan and Xin Zhou for discussions, L. Aravind, Peter Shewry and David Somers for unpublished information, and Marge Matzke, Erik Mirkov, Ivan Ingelbrecht and Jeffrey Chen for critically reading the manuscript. Aspects of this work were funded by grants from NSF (MCB-9974706), the Rockefeller Foundation and the Texas Advanced Technology Program (92-1999).

References

Allen, G., Spiker, S. and Thompson, W. 2000. The use of matrix attachment regions MARs to minimize transgene silencing. Plant Mol. Biol. 43: 361–376 (this issue).

Alvarez, M.L., Guelman, N.G., Halford, N.G., Lustig, S., Reggiardo, M.I., Ryabushkina, N., Shewry, P., Stein, J. and Vallejos, R.H. 2000. Silencing of HMW glutenins in transgenic wheat expressing extra HMW subunits. Theor. Appl. Genet. 100: 319–327.

Anandalakshmi, R., Pruss, G.J., Ge, X., Marathe, R., Mallory, A.C., Smith, T.H. and Vance, V.B. 1998. A viral suppressor of gene silencing in plants. Proc. Natl. Acad. Sci. USA 95: 13079–13084.

Assaad, F.F., Tucker, K.L. and Signer, E.R. 1993. Epigenetic repeat-induced gene silencing (RIGS) in *Arabidopsis*. Plant Mol. Biol. 22: 1067–1085.

Bahramian, M.B. and Zarbl, H. 1999. Transcriptional and posttranscriptional silencing of rodent alpha1(I) collagen by a homologous transcriptionally self-silenced transgene. Mol Cell. Biol. 19: 274–283.

Barcelo, P., Hagel, C., Becker, D., Martin, A. and Lorz, H. 1994. Transgenic cereal (tritordeum) plants obtained at high efficiency by microprojectile bombardment of inflorescence tissue. Plant J. 5: 583–592.

Battraw, M.J. and Hall, T.C. 1990. Histochemical analysis of *CaMV 35S* promoter-β-glucuronidase gene expression in transgenic rice plants. Plant Mol. Biol. 15: 527–538.

Battraw, M.J. and Hall, T.C. 1991. Stable transformation of *Sorghum bicolor* protoplasts with chimeric neomycin phosphotransferase II and β-glucuronidase genes. Theor. Appl. Genet. 82: 161–168.

Baulcombe, D.C. 1996. RNA as a target and an initiator of post-transcriptional gene silencing in transgenic plants. Plant Mol. Biol. 32: 79–88.

Baulcombe, D. 1999. Viruses and gene silencing in plants. Arch. Virol. Suppl. 15: 189–201.

Baulcombe, D.C. and English, J.J. 1996. Ectopic pairing of homologous DNA and post-transcriptional gene silencing in transgenic plants. Curr. Opin. Biotechnol. 7: 173–180.

Beclin, C., Berthome, R., Palauqui, J.C., Tepfer, M. and Vaucheret, H. 1998. Infection of tobacco or *Arabidopsis* plants by CMV counteracts systemic post-transcriptional silencing of nonviral (trans)genes. Virology 252: 313–317.

Bernardi, G. 1995. The human genome: organization and evolutionary history. Annu. Rev. Genet. 29: 445–476.

Bestor, T.H. 1998. The host defence function of genomic methylation patterns. Novartis Found. Symp. 214: 187–195.

Bestor, T.H. and Tycko, B. 1996. Creation of genomic methylation patterns. Nature Genet. 12: 363–367.

Bhalla, P.L., Swoboda, I. and Singh, M.B. 1999. Antisense-mediated silencing of a gene encoding a major ryegrass pollen allergen. Proc Natl. Acad. Sci. USA 96: 11676–11680.

Bi, X. and Broach, J.R. 1997. DNA in transcriptionally silent chromatin assumes a distinct topology that is sensitive to cell cycle progression. Mol. Cell. Biol. 17: 7077–7087.

Bird, A.P. 1995. Gene number, noise reduction and biological complexity. Trends Genet. 11: 94–100.

Bird, A.P. and Wolffe, A.P. 1999. Methylation-induced repression: belts, braces, and chromatin. Cell 99: 451–454.

Birney, E., Kumar, S. and Krainer, A.R. 1993. Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. Nucl. Acids Res. 21: 5803–5816.

Blechl, A.E. and Anderson, O.D. 1996. Expression of a novel high-molecular-weight glutenin subunit gene in transgenic wheat. Nature Biotechnol. 14: 875–879.

Brigneti, G., Voinnet, O., Li, W.X., Ji, L.H., Ding, S.W. and Baulcombe, D.C. 1998. Viral pathogenicity determinants are suppressors of transgene silencing in *Nicotiana benthamiana*. EMBO J. 17: 6739–6746.

Bundock, P., den Dulk-Ras, A., Beijersbergen, A. and Hooykaas, P.J.J. 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to *Saccharomyces cerevisiae*. EMBO J. 14: 3206–3214.

Cao, J., Duan, X.L., McElroy, D. and Wu, R. 1992. Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells. Plant Cell Rep. 11: 586–591.

Carels, N., Barakat, A. and Bernardi, G. 1995. The gene distribution of the maize genome. Proc. Natl. Acad. Sci. USA 92: 11057–11060.

Casas, A.M., Kononowicz, A.K., Zehr, U.B., Tomes, D.T., Axtell, J.D., Butler, L.G., Bressan, R.A. and Hasegawa, P.M. 1993. Transgenic sorghum plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA 90: 11212–11216.

Chareonpornwattana, S., Thara, K.V., Wang, L., Datta, S.K., Panbangred, W. and Muthukrishnan, S. 1999. Inheritance, expression, and silencing of a chitinase transgene in rice. Theor. Appl. Genet. 98: 371–378.

Chen, F.-C., Kuehnle, A.R. and Sugii, N. 1997. Anthurium roots for micropropogation and *Agrobacterium tumefaciens* mediated gene transfer. Plant Cell Tiss. Org. Cult. 49: 71–74.

Chen, W.P., Gu, X., Liang, G.H., Muthukrishnan, S., Chen, P.D., Liu, D.J. and Gill, B.S. 1998. Introduction and constitutive expression of a rice chitinase gene in bread wheat using biolistic bombardment and the *bar* gene as a selectable marker. Theor. Appl Genet. 97: 1296–1306.

Cheng, X., Sardana, R., Kaplan, H. and Altosaar, I. 1998. *Agrobacterium*-transformed rice plants expressing synthetic cryIA(b) and cryIA(c) genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA 95: 2767–2772.

Cho, M.-J., Jiang, W. and Lemaux, P.G. 1999. High-frequency transformation of oat via microprojectile bombardment of seed-derived highly regenerative cultures. Plant Sci. 148: 9–17.

Cogoni, C. and Macino, G. 1999a. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature 399: 166–169.

Cogoni, C. and Macino, G. 1999b. Posttranscriptional gene silencing in *Neurospora* by a RecQ DNA helicase. Science 286: 2342–2344.

Cooley, J., Ford, T. and Christou, P. 1995. Molecular and genetic characterization of elite transgenic rice plants produced by electric-discharge particle acceleration. Theor. Appl. Genet. 90: 97–104.

De Buck, S., Jacobs, A., Van Montagu, M. and Depicker, A. 1999. The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. Plant J. 20: 295–304.

Dellaire, G. and Chartrand, P. 1998. Direct evidence that transgene integration is random in murine cells, implying that naturally occurring double-strand breaks may be distributed similarly within the genome. Radiat. Res. 149: 325–329.

Depicker, A. and Van Montagu, M. 1997. Post-transcriptional gene silencing in plants. Curr. Opin. Cell Biol. 9: 373–382.

D'Halluin, K., Bonne, E., Bossut, M., De Beuckeleer, M. and Leemans, J. 1992. Transgenic maize plants by tissue electroporation. Plant Cell 4: 1495– 1505.

Dong, J.J., Teng, W.M., Buchholz, W.G. and Hall, T.C. 1996. *Agrobacterium*-mediated transformation of javanica rice. Mol. Breed. 2: 267–276.

Dorer, D.R. and Henikoff, S. 1997. Transgene repeat arrays interact with distant heterochromatin and cause silencing *in cis* and *trans*. Genetics 147: 1181–1190.

Elmayan, T., Balzergue, S., Beon, F., Bourdon, V., Daubremet, J., Guenet, Y., Mourrain, P., Palauqui, J.C., Vernhettes, S., Vialle, T., Wostrikoff, K. and Vaucheret, H. 1998. *Arabidopsis* mutants impaired in cosuppression. Plant Cell 10: 1747–1758.

English, J.J., Davenport, G.F., Elmayan, T., Vaucheret, H. and Baulcombe, D.C. 1997. Requirement of sense transcription for homology-dependent virus resistance and trans-inactivation. Plant J. 12: 597–603.

English, J.J., Mueller, E. and Baulcombe, D.C. 1996. Suppression of virus accumulation in transgenic plants exhibiting silencing of nuclear genes. Plant Cell 8: 179–188.

Fanti, L., Dorer, D.R., Berloco, M., Henikoff, S. and Pimpinelli, S. 1998. Heterochromatin protein 1 binds transgene arrays. Chromosoma 107: 286–292.

Festenstein, R., Tolaini, M., Corbella, P., Mamalaki, C., Parrington, J., Fox, M., Miliou, A., Jones, M. and Kioussis, D. 1996. Locus control region function and heterochromatin-induced position effect variegation. Science 271: 1123–1125.

Festenstein, R., Sharghi-Namini, S., Fox, M., Roderick, K., Tolaini, M., Norton, T., Saveliev, A., Kioussis, D. and Singh, P. 1999. Heterochromatin protein 1 modifies mammalian PEV in a dose- and chromosomal-context-dependent manner. Nature Genet. 23: 457–461.

Fire, A. 1999. RNA-triggered gene silencing. Trends Genet. 15: 358–363.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806–811.

Flavell, R.B. 1994. Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA 91: 3490–3496.

Francastel, C., Walters, M.C., Groudine, M. and Martin, D.I. 1999. A functional enhancer suppresses silencing of a transgene and prevents its localization close to centrometric heterochromatin. Cell 99: 259–269.

Fromm, M.E., Morrish, F., Armstrong, C., Williams, R., Thomas, J. and Klein, T.M. 1990. Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/technology 8: 833–839.

Garrick, D., Fiering, S., Martin, D.I. and Whitelaw, E. 1998. Repeat-induced gene silencing in mammals. Nature Genet. 18: 56–59.

Genger, R.K., Kovac, K.A., Dennis, E.S., Peacock, W.J. and Finnegan, E.J. 1999. Multiple DNA methyltransferase genes in *Arabidopsis thaliana*. Plant Mol. Biol. 41: 269–278.

Goodwin, J., Chapman, K., Swaney, S., Parks, T.D., Wernsman, E.A. and Dougherty, W.G. 1996. Genetic and biochemical dissection of transgenic RNA-mediated virus resistance. Plant Cell 8: 95–105.

Goossens, A., Dillen, W., De Clercq, J., Van Montagu, M. and Angenon, G. 1999. The arcelin-5 gene of *Phaseolus vulgaris* directs high seed-specific expression in transgenic *Phaseolus acutifolius* and *Arabidopsis* plants. Plant Physiol. 120: 1095–1104.

Guo, H.S., Lopez-Moya, J.J. and Garcia, J.A. 1999. Mitotic stability of infection-induced resistance to plum pox potyvirus associated with transgene silencing and DNA methylation. Mol. Plant-Microbe Interact. 12: 103–111.

Hamilton, A.J. and Baulcombe, D.C. 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286: 950–952.

Henikoff, S. and Comai, L. 1998. A DNA methyltransferase homolog with a chromodomain exists in multiple polymorphic forms in *Arabidopsis*. Genetics 149: 307–318.

Hernalsteens, J.-P., Thia-Toong, L., Schell, J. and Van Montagu, M. 1984. An *Agrobacterium*-transformed cell culture from the monocot *Asparagus officinalis*. EMBO J. 3: 3039–3041.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6: 271–282.

Hohn, T., Corsten, S., Rieke, S., Muller, M. and Rothnie, H. 1996. Methylation of coding region alone inhibits gene expression in plant protoplasts. Proc Natl. Acad. Sci. USA 93: 8334–8339.

Hollick, J.B., Dorweiler, J.E. and Chandler, V.L. 1997. Paramutation and related allelic interactions. Trends Genet. 13: 302–308.

Hung, M.S., Karthikeyan, N., Huang, B., Koo, H.C., Kiger, J. and Shen, C.J. 1999. *Drosophila* proteins related to vertebrate DNA (5-cytosine) methyltransferases. Proc. Natl. Acad. Sci. USA 96: 11940–11945.

Iglesias, V.A., Moscone, E.A., Papp, I., Neuhuber, F., Michalowski, S., Phelan, T., Spiker, S., Matzke, M. and Matzke, A.J.M. 1997. Molecular and cytogenetic analysis of stably and unstably expressed transgene loci in tobacco. Plant Cell 9: 1251–1264.

Ingelbrecht, I., Van Houdt, H., Van Montagu, M. and Depicker, A. 1994. Posttranscriptional silencing of reporter transgenes in tobacco correlates with DNA methylation. Proc. Natl. Acad. Sci. USA 91: 10502–10506.

Ingelbrecht, I.L., Irvine, J.E. and Mirkov, T.E. 1999. Posttranscriptional gene silencing in transgenic sugarcane. Dissection of homology-dependent virus resistance in a monocot that has a complex polyploid genome. Plant Physiol. 119: 1187–1198.

Ishida, Y., Saito, H., Ohta, S., Hiei, Y., Komari, T. and Kumashiro, T. 1996. High efficiency transformation of maize (*Zea mays* L.) mediated by *Agrobacterium tumefaciens*. Nature Biotechnol. 14: 745–750.

Itoh, K., Nakajima, M. and Shimamoto, K. 1997. Silencing of waxy genes in rice containing Wx transgenes. Mol. Gen. Genet. 255: 351–358.

Jakowitsch, J., Papp, I., Moscone, E.A., van der Winden, J., Matzke, M. and Matzke, A.J. 1999. Molecular and cytogenetic characterization of a transgene locus that induces silencing and methylation of homologous promoters in trans. Plant J. 17: 131–140.

Jeddeloh, J.A., Bender, J. and Richards, E.J. 1998. The DNA methylation locus DDM1 is required for maintenance of gene silencing in *Arabidopsis*. Genes Dev. 12: 1714–1725.

Jones, A.L., Thomas, C.L. and Maule, A.J. 1998. De novo methylation and co-suppression induced by a cytoplasmically replicating plant RNA virus. EMBO J. 17: 6385–6393.

Jones, L., Hamilton, A.J., Voinnet, O., Thomas, C.L., Maule, A.J. and Baulcombe, D.C. 1999. RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. Plant Cell 11: 2291–2302.

Jorgensen, R.A., Cluster, P.D., English, J., Que, Q. and Napoli, C.A. 1996. Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single-copy vs. complex T-DNA sequences. Plant Mol. Biol. 31: 957–973.

Kamo, K., Blowers, A., Smith, F., van Eck, J. and Lawson, R. 1995. Stable transformation of *Gladiolus* using suspension cells and callus. J. Am. Soc. Hort. Sci. 120: 347–352.

Kasschau, K.D. and Carrington, J.C. 1998. A counterdefensive strategy of plant viruses: suppression of posttranscriptional gene silencing. Cell 95: 461–470.

Kennerdell, J.R. and Carthew, R.W. 1998. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 95: 1017–1026.

Ketting, R.F., Haverkamp, T.H., van Luenen, H.G. and Plasterk, R.H. 1999. *mut-7* of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell 99: 133–141.

Kilby, N.J., Leyser, H.M.O. and Furner, I.J. 1992. Promoter methylation and progressive transgene inactivation in *Arabidopsis*. Plant Mol. Biol. 20: 103–112.

Klein, T.M., Kornstein, L. and Fromm, M.E., 1990. Genetic transformation of maize cells by particle bombardment and the influence of methylation on foreign-gene expression. In: J.P. Gustafson (Ed.) Gene Manipulation in Plant Improvement II: 19th Stadler Genetics Symposium, Plenum Press, New York, pp. 265–288.

Kohli, A., Gahakwa, D., Vain, P., Laurie, D.A. and Christou, P. 1999a. Transgene expression in rice engineered through particle bombardment: molecular factors controlling stable expression and transgene silencing. Planta 208: 88–97.

Kohli, A., Griffiths, S., Palacios, N., Twyman, R.M., Vain, P., Laurie, D.A. and Christou, P. 1999b. Molecular characterization of transforming plasmid rearrangements in transgenic rice reveals a recombination hotspot in the CaMV 35S promoter and confirms the predominance of microhomology mediated recombination. Plant J. 17: 591–601.

Kohli, A., Leech, M., Vain, P., Laurie, D.A. and Christou, P. 1998. Transgene organization in rice engineered through direct DNA transfer supports a two-phase integration mechanism mediated by the establishment of integration hot spots. Proc. Natl. Acad. Sci. USA 95: 7203– 7208.

Koonin, E.V., Zhou, S. and Lucchesi, J.C. 1995. The chromo superfamily: new members, duplication of the chromo domain and possible role in delivering transcription regulators to chromatin. Nucl. Acids Res. 23: 4229–4233.

Kumpatla, S.P. 1997. Transgene integrity, chimerism, silencing and stability in rice. Ph.D. thesis, Biology Dept., Texas A&M University, College Station, TX.

Kumpatla, S.P. and Hall, T.C. 1998a. Longevity of 5-azacytidine-mediated gene expression and re-establishment of silencing in transgenic rice. Plant Mol. Biol. 38: 1113–1122.

Kumpatla, S.P. and Hall, T.C. 1998b. Recurrent onset of epigenetic silencing in rice harboring a multi-copy transgene. Plant J. 14: 129–135.

Kumpatla, S.P. and Hall, T.C. 1999. Organizational complexity of a rice transgene locus susceptible to methylation-based silencing. IUBMB Life 48: 459–467.

Kumpatla, S.P., Teng, W., Buchholz, W.G. and Hall, T.C. 1997. Epigenetic transcriptional silencing and 5-azacytidine-mediated reactivation of a complex transgene in rice. Plant Physiol. 115: 361–373.

Kumpatla, S.P., Chandrasekharan, M.B., Iyer, L.M., Li, G. and Hall, T.C. 1998. Genome intruder scanning and modulation systems and transgene silencing. Trends Plant Sci. 3: 97–104.

Lambé, P., Dinant, M. and Matagne, R.F. 1995. Differential long-term expression and methylation of the hygromycin phosphotransferase (hph) and β-glucuronidase (GUS) genes in transgenic pearl millet (*Pennisetum glaucum)* callus. Plant Sci. 108: 51–62.

Lazerri, P.A., Brettschneider, R., Luhrs, R. and Lorz, H. 1991. Stable transformation of barley via PEG-induced direct DNA uptake into protoplasts. Theor. Appl. Genet. 81: 437–444.

Li, Z., Burow, M.D. and Murai, N. 1990. High frequency generation of fertile transgenic rice plants after PEG-mediated protoplast transformation. Plant Mol. Biol. Rep. 8: 276–291.

Lindbo, J.A., Silva Rosales, L., Proebsting, W.M. and Dougherty, W.G. 1993. Induction of a highly specific antiviral state in transgenic plants: implications for regulation of gene expression and virus resistance. Plant Cell 5: 1749–1759.

Lohmann, J.U., Endl, I. and Bosch, T.C. 1999. Silencing of developmental genes in *Hydra*. Dev. Biol. 214: 211–214.

Luff, B., Pawlowski, L. and Bender, J. 1999. An inverted repeat triggers cytosine methylation of identical sequences in *Arabidopsis*. Mol. Cell 3: 505–511.

Malagnac, F., Wendel, B., Goyon, C., Faugeron, G., Zickler, D., Rossignol, J.L., Noyer-Weidner, M., Vollmayr, P., Trautner, T.A. and Walter, J. 1997. A gene essential for de novo methylation and development in *Ascobolus* reveals a novel type of eukaryotic DNA methyltransferase structure. Cell 91: 281–290.

Maqbool, S.B. and Christou, P. 1999. Multiple traits of agronomic importance in transgenic indica rice plants: analysis of transgene integration patterns, expression levels and stability. Mol. Breed. 5: 471–480.

Marathe, R., Anandalakhsmi, R., Smith, T.H., Pruss, G.J. and Vance, V.B. 2000. RNA viruses as inducers, suppressors, and targets of post-transcriptional gene silencing. Plant Mol Biol., this issue.

Matzke, M.A. and Matzke, A.J.M. 1995. How and why do plants inactivate homologous (trans)genes. Plant Physiol. 107: 679–685.

Matzke, A.J. and Matzke, M.A. 1998a. Position effects and epigenetic silencing of plant transgenes. Curr. Opin. Plant Biol. 1: 142–148.

Matzke, M.A. and Matzke, A.J.M. 1998b. Epigenetic silencing of plant transgenes as a consequence of diverse cellular defence responses. Cell Mol. Life Sci. 54: 94–103.

Matzke, M.A., Primig, M., Trnovsky, J. and Matzke, A.J.M. 1989. Reversible methylation and inactivation of marker genes in sequentially transformed tobacco plants. EMBO J. 8: 643–649.

Matzke, M.A., Scheid, O.M. and Matzke, A.J. 1999. Rapid structural and epigenetic changes in polyploid and aneuploid genomes. Bioessays 21: 761–767.

Melquist, S., Luff, B. and Bender, J. 1999. Arabidopsis PAI gene arrangements, cytosine methylation and expression. Genetics 153: 401–413.

Mette, M.F., van der Winden, J., Matzke, M.A. and Matzke, A.J. 1999. Production of aberrant promoter transcripts contributes to methylation and silencing of unlinked homologous promoters in trans. EMBO J. 18: 241–248.

Metzlaff, M., O'Dell, M., Cluster, P.D. and Flavell, R.B. 1997. RNA-mediated RNA degradation and chalcone synthase A silencing in *Petunia*. Cell 88: 845–854.

Meyer, P. 1996. Repeat-induced gene silencing: common mechanisms in plants and fungi. Biol. Chem. Hoppe Seyler 377: 87–95.

Meyer, P. and Heidmann, I. 1994. Epigenetic variants of a transgenic petunia line show hypermethylation in transgene DNA: an indication for specific recognition of foreign DNA in transgenic plants. Mol. Gen. Genet. 243: 390–399.

Montgomery, M.K. and Fire, A. 1998. Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. Trends Genet. 14: 255–258.

Montgomery, M.K., Xu, S. and Fire, A. 1998. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA 95: 15502–15507.

Morel, J.-B. and Vaucheret, H. 2000. Post-transcriptional gene silencing mutants. Plant Mol. Biol. 43: 275–289 (this issue).

Morino, K., Olsen, O.A. and Shimamoto, K. 1999. Silencing of an aleurone-specific gene in transgenic rice is caused by a rearranged transgene. Plant J. 17: 275–285.

Müller, E., Lörz, H. and Lutticke, S. 1996. Variability of transgene expression in clonal cell lines of wheat. Plant Sci. 114: 71–82.

Mysore, K.S., Nam, J. and Gelvin, S.B. 2000. An *Arabidopsis* histone H2A mutant is deficient in *Agrobacterium* T-DNA integration. Proc. Natl. Acad. Sci. USA 97: 948–953.

Nan, X., Cross, S. and Bird, A. 1998. Gene silencing by methyl-CpG-binding proteins. Novartis Found. Symp. 214: 6–16.

Napoli, C., Lemieux, C. and Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into *Petunia* results in reversible co-suppression of homologous genes *in trans*. Plant Cell 2: 279–289.

Neuhuber, F., Park, Y.D., Matzke, A.J.M. and Matzke, M.A. 1994. Susceptibility of transgene loci to homology-dependent gene silencing. Mol. Gen.Genet. 224: 230–241.

Ng, H.H., Zhang, Y., Hendrich, B., Johnson, C.A., Turner, B.M., Erdjument-Bromage, H., Tempst, P., Reinberg, D. and Bird, A. 1999. MBD2 is a transcriptional repressor belonging to the MeCP1 histone deacetylase complex. Nature Genet. 23: 58–61.

Oard, J.H., Linscombe, S.D., Braverman, M.P., Jodari, F., Blouin, D.C., Leech, M., Kohli, A., Vain, P., Cooley, J.C. and Christou, P. 1996. Development, field evaluation, and agronomic performance of transgenic herbicide resistant rice. Mol. Breed. 2: 359–368.

Palauqui, J.C., Elmayan, T., Pollien, J.M. and Vaucheret, H. 1997. Systemic acquired silencing: transgene-specific post-transcriptional silencing is transmitted by grafting from silenced stocks to non-silenced scions. EMBO J. 16: 4738–4345.

Pal-Bhadra, M., Bhadra, U. and Birchler, J.A. 1997. Cosuppression in *Drosophila*: gene silencing of alcohol dehydrogenase by white-Adh transgenes is Polycomb dependent. Cell 90: 479–490.

Pal-Bhadra, M., Bhadra, U. and Birchler, J.A. 1999. Cosuppression of nonhomologous transgenes in *Drosophila* involves mutually related endogenous sequences. Cell 99: 35–46.

Park, S.H., Pinson, S.R. and Smith, R.H. 1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. Plant Mol. Biol. 32: 1135–1148.

Paro, R., Strutt, H. and Cavalli, G. 1998. Heritable chromatin states induced by the Polycomb and trithorax group genes. Novartis Found. Symp. 214: 51–61.

Pawlowski, W.P. and Somers, D.A. 1996. Transgene inheritance in plants genetically engineered by microprojectile bombardment. Mol. Biotechnol. 6: 17–30.

Pawlowski, W.P. and Somers, D.A. 1998. Transgenic DNA integrated into the oat genome is frequently interspersed by host DNA. Proc. Natl. Acad. Sci. USA 95: 12106–12110.

Pawlowski, W.P., Torbert, K.A., Rines, H.W. and Somers, D.A. 1998. Irregular patterns of transgene silencing in allohexaploid oat. Plant Mol. Biol. 38: 597–607.

Peng, J., Wen, F., Lister, R.L. and Hodges, T.K. 1995. Inheritance of *gusA* and *neo* genes in transgenic rice. Plant Mol. Biol. 27: 91–104.

Pinto, Y.M., Kok, R.A. and Baulcombe, D.C. 1999. Resistance to rice yellow mottle virus (RYMV) in cultivated African rice varieties containing RYMV transgenes. Nature Biotechnol. 17: 702–707.

Que, Q. and Jorgensen, R.A. 1998. Homology-based control of gene expression patterns in transgenic petunia flowers. Dev. Genet. 22: 100–109.

Qu, R., De Kochko, A., Zhang, L., Marmey, P., Li, L., Tian, W., Zhang, S., Fauquet, C.M. and Beachy, R.N. 1996. Analysis of a large number of independent transgenic plants produced by the biolistic method. In Vitro Cell Devel. Biol. Plant 32: 233–240.

Ratcliff, F., Harrison, B.D. and Baulcombe, D.C. 1997. A similarity between viral defense and gene silencing in plants. Nature 276: 1558–1560.

Ratcliff, F.G., MacFarlane, S.A. and Baulcombe, D.C. 1999. Gene silencing without DNA. RNA-mediated cross-protection between viruses. Plant Cell 11: 1207–1216.

Rathore, K.S., Chowdhury, V.K. and Hodges, T.K. 1993. Use of *bar* as a selectable marker gene and for the production of herbicide-resistant rice plants from protoplasts. Plant Mol. Biol. 21: 871–884.

Register, J.C., III, Peterson, D.J., Bell, P.J., Bullock, W.P., Evans, I.J., Frame, B., Greenland, A.J., Higgs, N.S., Jepson, I., Jiao, S. *et al.* 1994. Structure and function of selectable and non-selectable transgenes in maize after introduction by particle bombardment. Plant Mol. Biol. 25: 951–961.

Richards, E.J. 1997. DNA methylation and plant development. Trends Genet. 13: 319–323.

Rossi, L., Hohn, B. and Tinland, B. 1996. Integration of complete transferred DNA units is dependent on the activity of virulence E2 protein of *Agrobacterium tumefaciens*. Proc. Natl. Acad. Sci. USA 93: 126–130.

Rossignol, J.L. and Faugeron, G. 1994. Gene inactivation triggered by recognition between DNA repeats. Experientia 50: 307–317.

Sagi, L., Panis, B., Remy, S., Schoofs, H., De Smet, K., Swennen, R. and Cammue, B.P. 1995. Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. Bio/technology 13: 481–485.

Salomon, S. and Puchta, H. 1998. Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. EMBO J. 17: 6086–6095.

Schenk, P.M., Sagi, L., Remans, T., Dietzgen, R.G., Bernard, M.J., Graham, M.W. and Manners, J.M. 1999. A promoter from sugarcane bacilliform badnavirus drives transgene expression in banana and other monocot and dicot plants. Plant Mol. Biol. 39: 1221–1230.

Schiebel, W., Haas, B., Marinkovic, S., Klanner, A. and Sanger, H.L. 1993. RNA-directed RNA Polymerase from tomato leaves. I. Purification and physical properties. J. Biol. Chem. 263: 11851–11857.

Schiebel, W., Pélissier, T., Riedel, L., Thalmeir, S., Schiebel, R., Kempe, D., Lottspeich, F., Sanger, H.L. and Wassenegger, M. 1998. Isolation of an RNA-directed RNA polymerase-specific cDNA clone from tomato. Plant Cell 10: 2087–2101.

Schuh, W., Nelson, M.R., Bigelow, D.M., Orum, T.V., Orth, C.E., Lynch, P.T., Eyles, P.S., Blackhall, N.W., Jones, J., Cocking, E.C. and Davey, M.R. 1993. The phenotypic characterisation of R2 generation transgenic rice plants under field conditions. Plant Sci. 89: 69–79.

Selker, E.U. 1997. Epigenetic phenomena in filamentous fungi: useful paradigms or repeat-induced confusion? Trends Genet. 13: 296–301.

Selker, E.U. 1999. Gene silencing: repeats that count. Cell 97: 157–160.

Shimamoto, K. 1995. The molecular biology of rice. Science 270: 1772–1773.

Sijen, T., Wellink, J., Hiriart, J.-B. and van Kammen, A. 1996. RNA-mediated virus resistance: role of repeated transgenes and delineation of targeted regions. Plant Cell 8: 2277–2294.

Siomi, H. and Dreyfuss, G. 1997. RNA-binding proteins as regulators of gene expression. Curr. Opin. Genet. Dev. 7: 345–353.

Sogin, M.L. and Silberman, J.D. 1998. Evolution of the protists and protistan parasites from the perspective of molecular systematics. Int. J. Parasitol. 28: 11–20.

Somers, D.A., Rines, H.W., Gu, W., Kaeppler, H.F. and Bushnell, W.R. 1992. Fertile, transgenic oat plants. Bio/technology 10: 1589–1594.

Sonti, R.V., Chiurazzi, M., Wong, D., Davies, C.S., Harlow, G.R., Mount, D.W. and Signer, E.R. 1995. *Arabidopsis* mutants deficient in T-DNA integration. Proc. Natl. Acad. Sci. USA 92: 11786–11790.

Srivastava, V., Anderson, O.D. and Ow, D.W. 1999. Single-copy transgenic wheat generated through the resolution of complex integration patterns. Proc. Natl. Acad. Sci. USA 96: 11117–11121.

Stam, M., Viterbo, A., Mol, J.N. and Kooter, J.M. 1998. Position-dependent methylation and transcriptional silencing of transgenes in inverted T-DNA repeats: implications for posttranscriptional silencing of homologous host genes in plants. Mol. Cell Biol. 18: 6165–6177.

Sun, F.L. and Elgin, S.C. 1999. Putting boundaries on silence. Cell 99: 459–462.

Svitashev, S., Ananiev, E., Pawlowski, W.P. and Somers, D.A. 2000. Association of transgene integration sites with chromosome rearrangements in hexaploid oat. Theor. Appl. Genet. 100: 872–880.

Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A. and Mello, C.C. 1999. The *rde-1* gene, RNA interference, and transposon silencing in *C. elegans*. Cell 99: 123–132.

Takano, M., Egawa, H., Ikeda, J.E. and Wakasa, K. 1997. The structures of integration sites in transgenic rice. Plant J. 11: 353-361.

Vain, P., Buyser, J.D., Bui Trang, V. and Henry, Y. 1995. Foreign gene delivery into monocotyledonous species. Biotechnol. Adv. 13: 653–671.

Vain, P., Worland, B., Kohli, A., Snape, J.W., Christou, P., Allen, G.C. and Thompson, W.F. 1999. Matrix attachment regions increase transgene expression levels and stability in transgenic rice plants and their progeny. Plant J. 18: 233–242.

van Blokland, R., ten Lohuis, M. and Meyer, P. 1997. Condensation of chromatin in transcriptional regions of an inactivated plant transgene: evidence for an active role of transcription in gene silencing. Mol. Gen. Genet. 257: 1–13.

van Blokland, R., van der Geest, N., Mol, J.N.M. and Kooter, J.M. 1994. Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. Plant J. 6: 861–877.

van der Geest, A.H.M., Hall, G.E.J., Spiker, S. and Hall, T.C. 1994. The β-phaseolin gene is flanked by matrix attachment regions. Plant J. 6: 413–423.

van der Krol, A.R., Mur, L.A., Beld, M., Mol, J.N.M. and Stuitje, A.R. 1990. Flavonoid genes in *Petunia*: addition of a limited number of gene copies may lead to a suppression of gene expression. Plant Cell 2: 291–299.

van der Maas, H.M., de Jong, E.R., Rueb, S., Hensgens, L.A. and Krens, F.A. 1994. Stable transformation and long-term expression of the *gusA* reporter gene in callus lines of perennial ryegrass (*Lolium perenne* L.). Plant Mol. Biol. 24: 401–405.

van Eldik, G.J., Litiere, K., Jacobs, J.J., Van Montagu, M. and Cornelissen, M. 1998. Silencing of β-1,3-glucanase genes in tobacco correlates with an increased abundance of RNA degradation intermediates. Nucl. Acids Res. 26: 5176–5181.

Vaucheret, H. 1993. Identification of a general silencer for 19S and 35S promoters in a transgenic tobacco plant: 90 bp of homology in the promoter sequence are sufficient for trans-inactivation. C. R. Acad. Sci. Paris, Sci. Vie/Life Sci. 316: 1471–1483.

Voinnet, O., Vain, P., Angell, S. and Baulcombe, D.C. 1998. Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. Cell 95: 177–187.

Voinnet, O., Pinto, Y.M. and Baulcombe, D.C. 1999. Suppression of gene silencing: a general strategy used by diverse DNA and RNA viruses of plants. Proc. Natl. Acad. Sci. USA 96: 14147–14152.

Wade, P.A., Jones, P.L., Vermaak, D., Veenstra, G.J., Imhof, A., Sera, T., Tse, C., Ge, H., Shi, Y.B., Hansen, J.C. and Wolffe, A.P. 1998. Histone deacetylase directs the dominant silencing of transcription in chromatin: association with MeCP2 and the Mi-2 chromodomain SWI/SNF ATPase. Cold Spring Harb. Symp. Quant. Biol. 63: 435–445.

Wade, P.A., Gegonne, A., Jones, P.L., Ballestar, E., Aubry, F. and Wolffe, A.P. 1999. Mi-2 complex couples DNA methylation to chromatin remodelling and histone deacetylation. Nature Genet. 23: 62–66.

Wan, Y. and Lemaux, P.G. 1994. Generation of large numbers of independently transformed fertile barley plants. Plant Physiol. 104: 37–48.

Wassenegger, M. and Pélissier, T. 1998. A model for RNA-mediated gene silencing in higher plants. Plant Mol. Biol. 37: 349–362.

Watad, A.A., Yun, D.-J, Matsumoto, T., Niu, X., Wu, Y., Kononowicz, A.K., Bressan, R.A. and Hasegawa, P.M. 1998. Microprojectile bombardment-mediated transformation of *Lilium longiflorum*. Plant Cell Rep. 17: 262–267.

Waterhouse, P.M., Graham, M.W. and Wang, M.B. 1998. Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. Proc. Natl. Acad. Sci. USA 95: 13959–13964.

Wilkinson, C.R., Bartlett, R., Nurse, P. and Bird, A.P. 1995. The fission yeast gene pmt1+ encodes a DNA methyltransferase homologue. Nucl. Acids Res. 23: 203–210.

Wolffe, A.P. and Matzke, M.A. 1999. Epigenetics: regulation through repression. Science 286: 481–486.

Xu, Y., Buchholz, W.G., DeRose, R.T. and Hall, T.C. 1995. Characterization of a rice gene family encoding root-specific proteins. Plant Mol. Biol. 27: 237–248.

Ye, F. and Signer, E.R. 1996. RIGS (repeat-induced gene silencing) in *Arabidopsis* is transcriptional and alters chromatin configuration. Proc. Natl. Acad. Sci. USA 93: 10881–10886.

Yin, Y., Zhu, Q., Dai, S., Lamb, C. and Beachy, R.N. 1997. RF2a, a bZIP transcriptional activator of the phloem-specific rice tungro bacilliform virus promoter, functions in vascular development. EMBO J. 16: 5247–5259.

Yoder, J.A. and Bestor, T.H. 1998. A candidate mammalian DNA methyltransferase related to pmt1p of fission yeast. Hum. Mol. Genet. 7: 279–284.

Zhang, H.M., Yang, H., Rech, E.L., Golds, T.J., Davis, A.S., Mulligan, B.J., Cocking, E.C. and Davey, M.R. 1988. Transgenic rice plants produced by electroporation-mediated plasmid uptake into protoplasts. Plant Cell Rep. 7: 379–384.

Zhang, S., Warkentin, D., Sun, B., Zhong, H. and Sticklen, M. 1996. Variation in the inheritance of expression among subclones for unselected (*uidA*) and selected (*bar*) transgenes in maize (*Zea mays* L.). Theor. Appl Genet. 92: 752–761.