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The reduced expression of endogenous duplications (REED) in the maize *R* gene family is mediated by DNA methylation

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The duplicated *R* and *Sn* genes regulate the maize anthocyanin biosynthetic pathway and encode tissue-specific products that are homologous to helix–loop–helix transcriptional activators. As a consequence of their coupling in the genome, *Sn* is partially silenced. Genomic restriction analysis failed to reveal gross structural DNA alterations between the strong original phenotype and the weak derivatives. However, the differences in pigmentation were inversely correlated with differences in the methylation of the *Sn* promoter. Accordingly, treatment with 5-azacytidine (AZA), a demethylating agent, restored a strong pigmentation pattern that was transmitted to the progeny and that was correlated with differential expression of the *Sn* transcript. Genomic sequencing confirmed that methylation of the *Sn* promoter was more apparent in the less pigmented seedlings and was greatly reduced in the AZA revertants. In addition, some methylcytosines were located in non-symmetrical C sequences. These findings provide an insight into *Sn* and *R* interaction, a process that we have termed Reduced Expression of Endogenous Duplications (REED). We propose that increasing the copy number of regulatory genes by endogenous duplication leads to such epigenetic mechanisms of silencing. Further understanding of the REED process may have broader implications for gene regulation and may identify new levels of regulation within eukaryotic genomes.

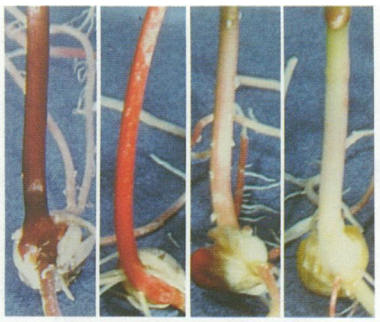
Keywords: azacytidine-induced phenotypic reversion/ DNA methylation/duplicated gene silencing/genomic sequencing/maize *R* genes

Introduction

During the last decade, epigenetic events that lead to modification in gene expression without alterations in the DNA sequence have been described in a variety of organisms. In some transgenic systems, both in mammals and in plants, an unexpected segregation of silenced genes has also been shown to occur (McGowan *et al.*, 1989; Matzke and Matzke, 1993; Meyer *et al.*, 1993). In addition, striking phenomena of endogenous gene suppression involving an interaction between alleles have been observed, with the most intriguing being paramutation (Brink, 1973; Coen and Carpenter, 1988; Patterson *et al.*, 1993). The mechanisms underlying this gene inactivation are still unclear, although ectopic pairing between homologous genes (Jorgensen, 1990) and interactions between sense and anti-sense RNA molecules (Grierson *et al.*, 1991) have been suggested.

Irrespective of the precise mechanism, the fact that repression of gene expression in transgenic plants is correlated with an increased number of integrated copies indicates that a cellular system exists that is somehow able to count the copy number of duplicated sequences in the genome. It may be relevant that the status of promoter methylation has been found to be influenced by copy number and by the chromosomal position of the transferred gene (Linn *et al.*, 1990), particularly because the presence of intense DNA methylation has been correlated with gene inactivation (Assaad *et al.*, 1993). Methylation is also involved in epigenetic phenomena, such as imprinting in mammals (Holliday, 1993), parental chromosome-dependent gene expression in transgenic mice (Brandeis *et al.*, 1993) and inactivation cycles of transposable elements (Chomet *et al.*, 1987; Banks and Fedoroff, 1988; Martienssen *et al.*, 1990; Brutnell and Dellaporta, 1994).

In this study, we have examined the interactions between the duplicated *R* and *Sn* genes, which regulate the maize anthocyanin biosynthetic pathway. In the presence of certain alleles of the *R* gene, *Sn* is partially silenced (Gavazzi *et al.*, 1990). To our knowledge, this is the first reported case in which the expression of an endogenous plant gene is reduced by the presence of a duplicated, non-allelic copy. The maize *R* gene family includes several alleles at the *R* locus on chromosome 10, as well as the *Sn* and *Lc* genes lying ~2 cM distal from them. The *B* gene, another member of the *R* family, is located on chromosome 2. All members are functionally duplicated genes encoding basic helix–loop–helix transcription factors that regulate the spatial and temporal accumulation of red anthocyanin pigments (Tonelli *et al.*, 1991; Consonni *et al.*, 1993). This process is generally light dependent. Each member of the *R* gene family controls a particular pattern of pigmentation; for example, the territories of *Sn* expression are the scutellar node and mesocotyl of the seedling, the leaf base and midrib of the growing plant and the cob glumes and pericarp of the adult plant. Several *Sn* alleles have been isolated. One of them, *Sn:bol3*, differs from the others in that it confers a light-dependent increase in pigmentation to the mesocotyl and to the seed integuments, and weak colour development in the dark. As a result of coupling with *R*, *Sn:bol3* is unstable, giving rise to weakly pigmented seedlings (Gavazzi *et al.* 1990). This phenomenon is similar to that of *R* paramutation, in which the paramutagenic effect of *R-st* over the paramutable and unstable *R-r* allele leads to a heritable reduction of *R-r* expression (Brink, 1973). The aim of the present study was to understand the molecular basis of *Sn* instability and to investigate how the expression of *Sn:bol3* is



	S	I	W	Tr
r Sn: bol 3	63	-	45	3
R-sc Sn: bol 3	-	66	8	10
(A_{530})	(68.7)	(12.1)	(4.4)	(2.6)

Fig. 1. Pigment accumulation in homozygous *r Sn:bol3* and *R-sc Sn:bol3* mesocotyls. The progeny of *r Sn:bol3* and *R-sc Sn:bol3* homozygous plants were scored for the mesocotyl anthocyanin content after 48 h of continuous illumination and visually classified as strong (S), intermediate (I), weak (W) and trace (Tr) according to the amount of pigment accumulated. Estimation of pigment content was determined spectrophotometrically after extraction of anthocyanins from individual mesocotyls. Values in brackets indicate the mean absorbance at 530 nm per g fresh weight. Each value is the mean of 10 independent determinations.

influenced by *R*. In order to distinguish this phenomenon from classical paramutation, we propose the term REED (Reduced Expression of Endogenous Duplications). We demonstrate that REED events occur through a methylation process involving the promoter sequence of the silenced gene. Interestingly, REED also involves the presence of a non-symmetrical type of C residue methylation, occurring outside the canonical CpG and CpNpG sites (Holliday and Pugh, 1975; Gruenbaum *et al.*, 1982). This pattern of methylation has only been reported previously in transgenic filamentous fungi (Selker, 1990a; Rhounim *et al.*, 1992) and in transgenic plants (Meyer *et al.*, 1994), and has never been found to occur in an endogenous gene.

Results

Sn:bol3 (from here on referred to as *Sn*) is distinguished from other *R* alleles because it confers a deep red mesocotyl pigmentation after light exposure and a weak pigment accumulation in the dark (Gavazzi *et al.*, 1990). Progeny of homozygous *r Sn* seedlings, selected for the presence of strong red mesocotyl pigmentation, consist of individuals with the parental phenotype, referred to as Sn-S, as well as seedlings with significantly lower pigment levels ranging from almost colourless to weakly coloured, referred to as Sn-tr and Sn-W respectively (Figure 1). These seedlings are totally light dependent. The change of Sn-S to Sn-W or Sn-tr is unidirectional, as no spontaneous revertants are ever observed among the weak and almost colourless progeny. Furthermore, continuous selfing of homozygous Sn-S results in a constant pattern of instability, with a frequency of 'suppressed', light-dependent derivatives varying from ~13 to 40%, according to the *R* allele present in the genome. Moreover, the association of *Sn* with *R-sc*, an allele that causes homogeneous pigmentation of the seed aleurone layer, leads to a significant reduction in the pigmentation of mesocotyls and scutellar nodes (Figure 1), with the Sn-S individuals being replaced by seedlings classified as having 'intermediate' (Sn-I) pigmentation. Similar reductions have been reported previously to occur in the pericarp, the seed tissue that is under *Sn* control (Gavazzi *et al.*, 1990; Mereghetti *et al.*, 1991).

To understand the molecular basis of *Sn* suppression, we focused our attention on *R-sc Sn*-I and *R-sc Sn*-tr derivatives. Because *R-sc* and *Sn* show a DNA polymorphism (Figure 2C), we performed Southern blot analyses of *Sn* derivatives to examine whether gross structural DNA alterations could be involved in the reduction of *Sn* expression. DNA extracted from Sn-I and Sn-tr seedlings (the highest and the lowest pigmented classes of *R-sc Sn* derivatives, respectively), was digested with *Hin*dIII and compared with that from the original strong pigmented seedlings (*r Sn*). Analysis was done using two probes: one of 550 bp from the promoter region that should detect a 4 kb *Sn* band, a 4 kb *r* band and a 4.6 kb *R-sc* band, and a second probe of 1.4 kb derived from the 3′ end of *Sn* cDNA, which should detect an *Sn* fragment of 7 kb, an *r* fragment of 6.2 kb and an *R-sc* fragment of 12 kb (Figure 2C). The results show a similar digestion profile in all the samples analysed (a pool of 10 seedlings for each sample), thus suggesting that no gross DNA alterations had occurred in the derivatives (Figure 2A).

As an alternative mechanism, we considered whether the different levels of *Sn* expression could be correlated with a change in the density of methyl-C residues in *Sn* DNA. DNA from mesocotyls and leaves of Sn-I and Sn-tr seedlings was extracted and cleaved with the methylation-sensitive restriction endonucleases *Hpa*II or *Bgl*II, together with *Hin*dIII. In order to discriminate between the *R-sc* and *Sn* bands in the Southern blot analysis, DNA from *R-sc* seedlings was loaded as a control. We did not include in the analysis DNA extracted from *r Sn*-S seedlings because, by sequence data, *r* and *Sn* have identical promoter regions. Figure 2C shows the restriction sites, relevant for this analysis, within the 4.0 kb *Sn Hin*dIII genomic fragment, which contains the transcription start site and the promoter region (Tonelli *et al.*, 1991). Three *Hpa*II sites are present, one in the promoter, at position –689 and two others at +40 and +140. Using the Sn-550 probe with *Hin*dIII–*Hpa*II-digested DNA, we therefore would expect to detect either: (i) a band of 4 kb if all three sites were methylated; (ii) a 3.1 kb band if methylation occurred at the +40 or +140 sites but not at –689; (iii) a 1.5 kb fragment if cleavage occurred at either one of the +40 or +140 sites; or (iv) a band of 700 bp if all sites were unmethylated.

The results (Figure 2B) show that the 1.5 kb and 700 bp fragments were present in both Sn-I and Sn-tr seedlings, while the 4.0 kb band, which indicates methylation at all three *Hpa*II sites, was detected only in the Sn-tr DNA from both mesocotyls and leaves (lanes 2 and 4). The absence of this band in the DNA of Sn-I seedlings (lanes 1 and 3) therefore reveals a correlation between the almost completely suppressed pigment production of Sn-tr seedlings and the methylated profile of the three *Hpa*II sites. Based on these results, we therefore concluded that

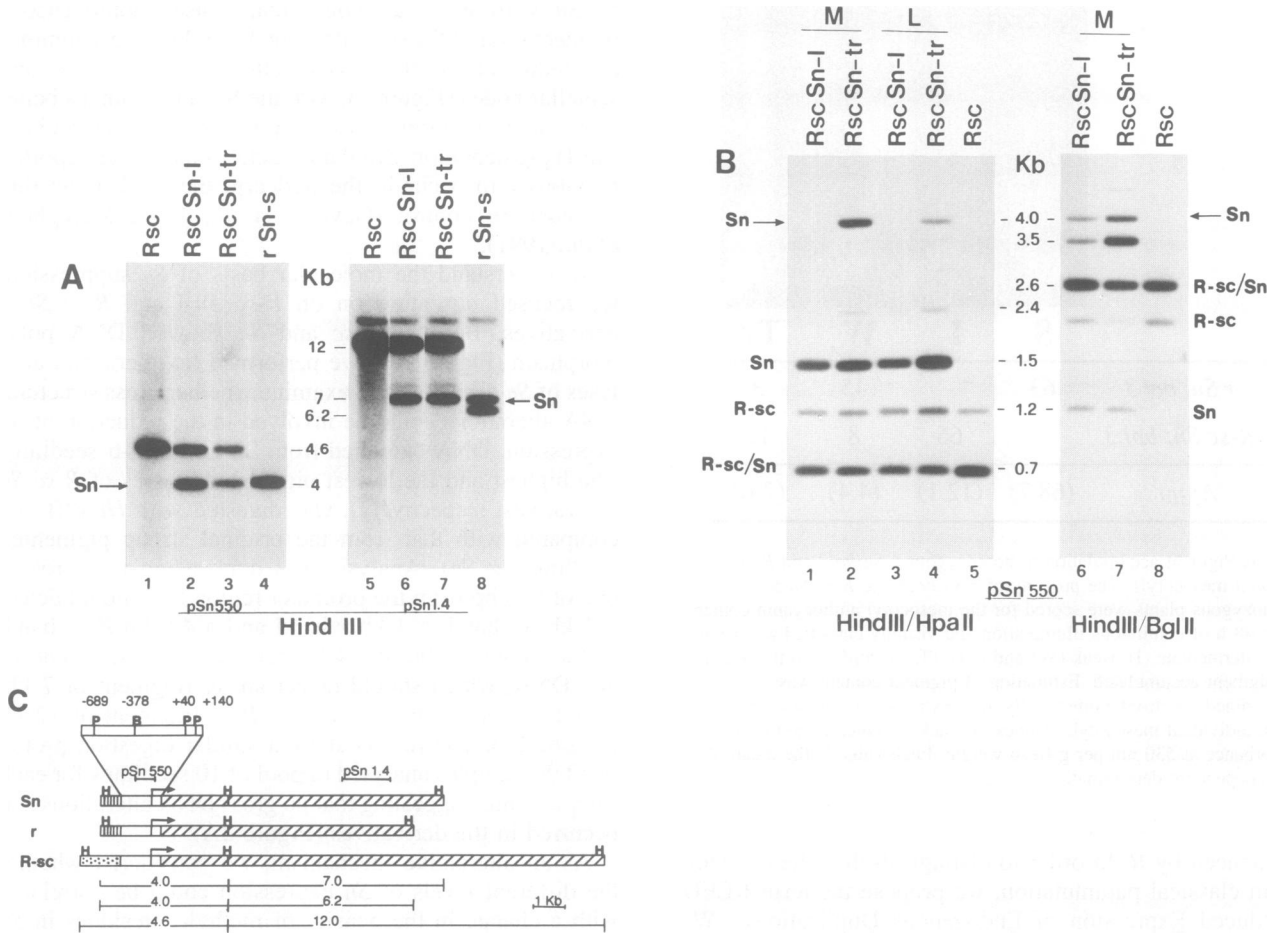


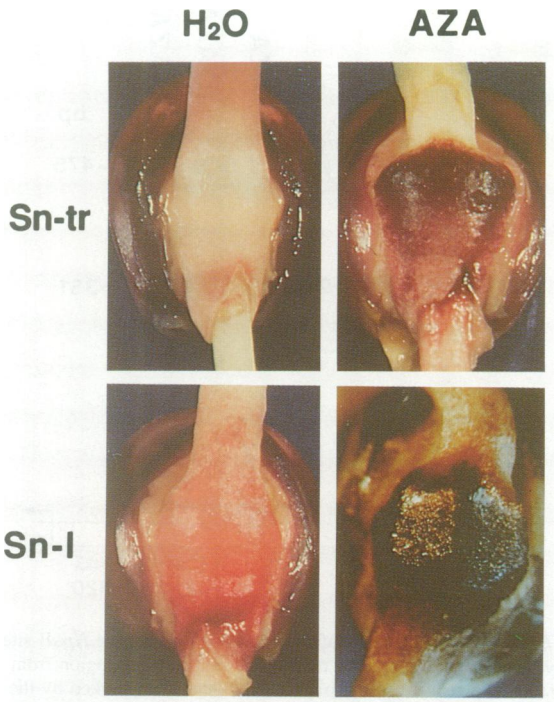
Fig. 2. Southern analysis of *Sn* derivatives. (**A**) Genomic DNA extracted from *R-sc Sn* seedlings showing intermediate (Sn-I) and traces (Sn-tr) of pigmentation was restricted with *Hin*dIII and compared with that from *r Sn*, exhibiting the strong (Sn-S) phenotype. DNA from *R-sc* seedlings was loaded as a control. Each DNA sample consisted of a pool of 10 seedlings. Filters were probed with promoter (pSn550) and cDNA (pSn1.4) fragments. The migration of the *Sn* gene (4 and 7 kb *Hin*dIII) is indicated by arrows. (**B**) DNA extracted separately from mesocotyls (M) and leaves (L) of *R-sc Sn* seedlings showing Sn-I and Sn-tr phenotype was cut with a combination of enzymes and hybridized with a promoter probe (pSn550) in order to assess the methylation pattern of *Sn*. The differences in the methylation profile of Sn-tr (lanes 2, 4 and 7) versus Sn-I (lanes 1, 3 and 6) in the *Hin*dIII–*Hpa*II and in the *Hin*dIII–*Bgl*II restriction digests are marked by arrows. (**C**) Schematic representation of the *Sn*, *r* and *R-sc* genes. The arrows indicate transcription start sites and direction of transcription. The region from –700 to +140 bp, showing 100% homology between *Sn*, *r* and *R-sc* genes is marked by an open box, while the 5′ end, which is 100% homologous between *Sn* and *r* (striped box), is highly polymorphic for *R-sc* (dotted box). From +140 to the *Hin*dIII site at the 3′ end the genomic fragments are schematically marked. The hybridizing regions of the pSn550 and pSn 1.4 are indicated above the genomic fragments. Restriction sites relevant to the analysis include: *Hin*dIII (H), *Hpa*II (P) and *Bgl*II (B).

the complete demethylation in Sn-I plants involves one of the two *Hpa*II sites located at position +40 and +140. In these individuals, the incomplete cleavage of the *Hpa*II site at –689 might indicate that Sn-I and Sn-tr, to some extent, share a common methylation status over the entire 5′ region of the gene. This hypothesis agrees with the observation that the *R-sc Sn*-I plants never reach the high levels of mesocotyl pigmentation observed in *r Sn*-S seedlings.

Although the profile of bands obtained after digestion with *Bgl*II and *Hin*dIII is the same in the two derivatives, the intensity of the bands is different (Figure 2B). Compared with Sn-tr (lane 7), Sn-I DNA (lane 6) showed a reduced intensity of the 4.0 and 3.5 kb bands (this smaller fragment is due to an additional *Bgl*II site in the intron), indicating a higher level of methylation in Sn-tr seedlings. An additional observation was the lack of the *R-sc* 2 kb band in Sn-tr samples, which suggests that *R-sc* sequences also have different methylation profiles in plants expressing the Sn-I and Sn-tr phenotypes. The same blots were hybridized with other probes covering the central and 3′ end of the *Sn* gene, and in no case did the resulting pattern show any differences between the different phenotypes (data not shown).

A demethylating agent restores the strong red Sn phenotype

If the transition from Sn-S plants to Sn-I and Sn-tr was caused by *de novo* methylation of certain sites of the *Sn:bol3* allele, it might be possible to restore the strong pigmented phenotype by treatment with 5-aza-2′-deoxycytidine (AZA). This cytidine analogue has been used to inhibit DNA maintenance methylase (Gartler and Goldman, 1994). Homozygous Sn-I and Sn-tr seeds were germinated in the dark in the presence of AZA. Five days after germination, the seedlings were exposed to light for 48 h and the anthocyanin content of the scutellar node was determined. The results revealed striking differences



Phenotype	Treatment	A_{530}/gfw	A_{530}/scutellar node
Sn-I	H_2O	8.15	0.407
	AZA	33.10	1.930
Sn-tr	H_2O	0.35	0.008
	AZA	22.70	0.665

Fig. 3. Effects of AZA on anthocyanin accumulation in Sn-I and Sn-tr derivatives. *R-sc Sn*-tr and *R-sc Sn*-I seeds were treated with AZA during germination and then exposed to light for 48 h. Pigment accumulation in scutellar nodes from treated (AZA) and untreated (H_2O) seedlings was compared and determined spectrophotometrically after extraction of anthocyanins from individual scutellar nodes. The mean of 10 independent determinations is expressed as absorbance values at 530 nm per g fresh weight (gfw) or per scutellar node.

in the level of pigmentation between treated and untreated plants (Figure 3). Compared with untreated controls, AZA-treated seedlings showed a 65- to 83-fold increase in pigment content. The effect of the analogue was also evident in homozygous Sn-I seedlings where a 4- to 5-fold increase in pigment content was observed. Furthermore, AZA-treated Sn-tr seedlings showed a higher anthocyanin content compared with that of untreated Sn-I seedlings. It was also interesting to observe that the exposure of *R-sc Sn* seedlings to AZA restored the characteristic light-independent expression of *Sn* in *r Sn*-S plants. In fact, in darkness, AZA-treated seedlings showed an anthocyanin content 500-fold higher than that of untreated ones (data not shown). Although the seedlings suffer from the AZA treatment (they show a reduced growth rate), the enhancement of pigmentation was strictly confined to the tissues under *Sn* control. To find out whether the epigenetic change was germinally transmissible, Sn-tr seeds were treated with AZA, seedlings were grown and the mature plants selfed. AZA treatment was performed for only a

Table I. The effect of 5-aza-2′-deoxycytidine on the progeny of treated plants

Parental plant	Progeny code	Phenotypic classification		
		I	W	tr
Sn-tr-AZA	G 65	38		
	G 66	40		
	G 68	36		
Sn-tr-H_2O	G 70			48
	G 71			32
Sn-I-H_2O	G 53	20	20	2
	G 54	10	34	
	G 55	4	2	

R-sc Sn-tr plants, treated with AZA (Sn-tr-AZA), were selfed and the progeny was compared with that of *R-sc Sn*-tr and *R-sc Sn*-I untreated plants (Sn-tr-H_2O and Sn-I-H_2O). Progeny seeds were germinated in the dark and then exposed to light for 72 h. Seedlings were scored for their mesocotyl pigment accumulation. I, W and tr stand for intermediate, weak and trace mesocotyl pigmentation.

brief period (24 h) in order not to inhibit growth and development significantly. Sibling plants from untreated seedlings showing Sn-I and Sn-tr phenotypes were used as controls. The progeny from these experiments was germinated and the pigment content of the seedlings was analysed (Table I). Progeny of AZA-treated seedlings not only showed a higher level of pigmentation compared with that of the Sn-I and Sn-tr control plants, but all individuals exhibited an intermediate phenotype, i.e. there were no weakly pigmented individuals. Therefore, the phenotypic reversion obtained with AZA was transmitted to the progeny, suggesting that the pattern of induced demethylation could pass through meiosis and subsequently be shared by all cells, since instability was no longer observed.

In order to correlate the accumulation of anthocyanins in AZA-treated and untreated seedlings with changes in the levels of *Sn* steady-state mRNA, a time course of mRNA induction was monitored by Northern blot analysis, using total RNA extracted at different time intervals during growth in the dark and light (Figure 4). Previous results had shown that *Sn:bol3* mRNA levels increased following light irradiation, reaching a peak after 24–48 h of illumination, while longer exposure (72–96 h) led to a subsequent decline. In contrast, the steady-state levels of the structural *C2* and *A1* genes are coordinately induced and maintained at high levels even after 96 h of light exposure (Tonelli *et al.*, 1991). Our results, while confirming these previously reported for untreated seedlings (Figure 4A), highlight striking differences for seedlings treated with AZA (Figure 4B). In complete accordance with the appearance of strong red pigmentation 2 days after germination, *Sn* transcript levels in AZA-treated seedlings displayed a dramatic transient increase, reaching the highest levels before light irradiation (Figure 4B). Since *Sn* expression is normally transient, the decline in the mRNA levels observed following this peak is not surprising. In a coordinated way, *C2* mRNA levels in AZA-treated scutellar nodes were very high values at the beginning of the time course and, as expected, this high level expression persisted longer than that of *Sn*, even after 48 h illumination. A similar profile of *A1* mRNA following AZA treatment was also detected (data not shown). These findings, therefore, suggest that

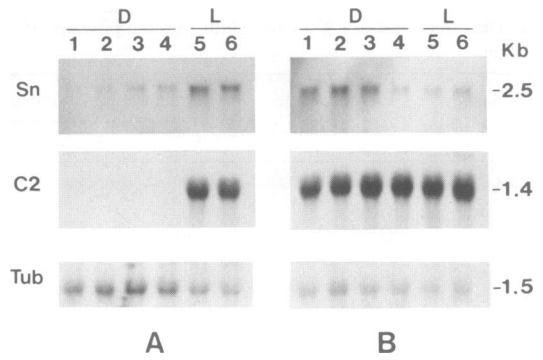


Fig. 4. Gene expression in *Sn* seedlings treated with AZA. Total RNA was extracted from scutellar nodes of *R-sc Sn:bol:3* seedlings, treated with AZA (**B**) or untreated (**A**). Seeds were germinated in the dark (D) and samples were collected after 4, 5, 6 and 7 days (lanes 1, 2, 3 and 4 respectively), then were exposed to light (L) and samples were collected after 24 and 48 h (lanes 5 and 6). Northern analysis was performed as described in Materials and methods. The probes used are: a 1.4 kb fragment (Sn) from *Sn* cDNA, a chalcone synthase (C2) cDNA fragment and an α-tubulin cDNA (Tub) as a control for loading. The sizes (kb) of transcripts are indicated on the right side of the panel.

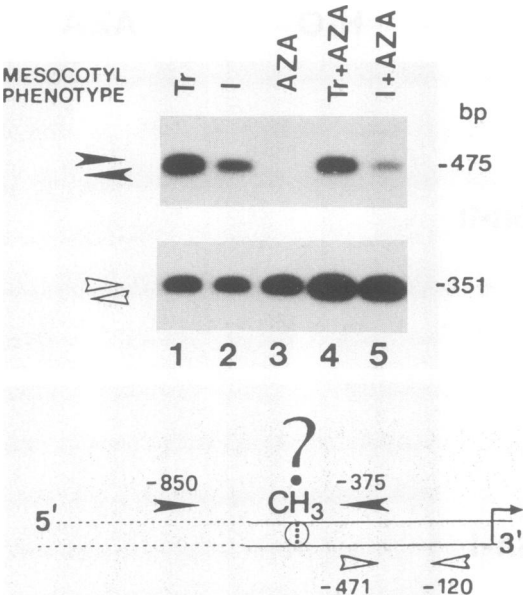


Fig. 5. PCR-mediated methylation analysis of the unique *Hpa*II site of the *Sn* promoter. A schematic map of the *Sn* promoter region from –1492 to 0 bp relative to the transcription start site (marked by the thin arrow) is shown in the lower panel. The continuous line indicates the region of 100% homology shared by the *Sn bol:3* and the *R-sc* promoter sequence, the *Hpa*II site analysed is located at –689 bp relative to the transcription start site. The dotted line marks the 5′ end region of divergence of the *Sn* promoter from *R-sc*. Arrowheads indicate the sets of primers used. In the first set (AR8 and OH1, black arrowheads), one primer (AR8 at –850) is highly specific for *Sn*. These primers amplify a fragment of 475 bp only when the DNA is not cleaved by *Hpa*II. The second couple of primers (AR18 and CT12, empty arrowheads) were used to amplify a smaller reference fragment of 351 bp from the region flanking the *Hpa*II site. In the top part is shown a PCR analysis of genomic DNA extracted from mesocotyls and cleaved with *Hpa*II. Lanes 1–3: amplification of 100 ng DNA from Sn-tr, Sn-I and Sn-AZA, respectively. Lane 4: amplification of 100 ng Sn-tr DNA mixed with 100 ng of Sn-AZA DNA. Lane 5: amplification of 100 ng Sn-I DNA mixed with 100 ng Sn-AZA DNA. The sizes (bp) of the amplified bands are indicated on the right of the figure, while on the left the black or empty arrowheads indicate the sets of primers used.

the enhanced pattern of pigmentation was correlated with a strong AZA-induced transcriptional activation of *Sn* during early seedling development.

PCR-mediated fine analysis of the HpaII site within the Sn promoter

In order to correlate the degree of anthocyanin accumulation with the methylation status of the *Sn* promoter, we performed a fine analysis of the 5′-CCGG-3′ *Hpa*II site at –689. However, the high level of sequence similarity shared between *R-sc* and *Sn* made this task difficult. To bypass this problem, we took advantage of the polymorphism at the 5′ end of the sequences by using a PCR approach. The *Hpa*II site is in fact very close to the breakpoint between *R-sc* and *Sn* homology (Figure 2C). Two primers flanking the site were used, with the most upstream one being specific for *Sn*. Genomic DNA, extracted from mesocotyls and leaves of Sn-I, Sn-tr and Sn-AZA-treated plants, was digested with *Hpa*II and analysed by PCR. The resulting PCR product, a 475 bp fragment, was expected only if the *Hpa*II site was methylated, while in the absence of methylation the site would not be protected and would be cut by *Hpa*II, hence inhibiting the PCR extension reaction. It would be expected that the intensity of the PCR fragment obtained should be directly correlated to the overall methylation state of the *Hpa*II site analysed. In order to measure slight differences in band intensities, PCRs were performed for 18 cycles and then blotted from agarose gels onto Pall membranes, to be probed with the *Sn* 550 bp promoter fragment. The results (Figure 5) show that in Sn-tr seedlings the intensity of the PCR band (lane 1) is stronger than that of Sn-I (lane 2), indicating a higher methylation state of the *Hpa*II site. The presence of a faint band in Sn-I suggests, however, that the *Hpa*II site in these plants might not be fully unmethylated but rather methylated in a few cases due to cell heterogeneity. The PCR product was absent in DNA from AZA-treated plants (lane 3), revealing that in these individuals the *Hpa*II site (–689) was digested to completion, as was expected in the case of an unmodified 5′-CCGG-3′ site. In order to prove that the system was sensitive to changes in DNA methylation, a mixing experiment was performed. After digestion with *Hpa*II, the DNAs from Sn-tr and Sn-AZA were mixed and then amplified (lane 4). The same was done with *Hpa*II-digested DNA derived from Sn-I and Sn-AZA (lane 5). The quantities of the PCR products were similar to that previously observed (compare lane 1 versus lane 4 and lane 2 versus lane 5). Taken together, these results support the hypothesis that the AZA-treated plants undergo a massive demethylation of specific *Sn* promoter sites.

Methylation status of the Sn promoter is correlated with pigment phenotypes

To determine more precisely the specific sites of cytosine methylation, we took advantage of a genomic sequencing protocol, based on bisulfite-induced modification of genomic DNA, which converts all unmethylated cytosines into U residues, leaving unaltered only the 5-methylcytosines (Frommer *et al.*, 1992). The treatment is followed by strand-specific PCR amplification and sequencing that

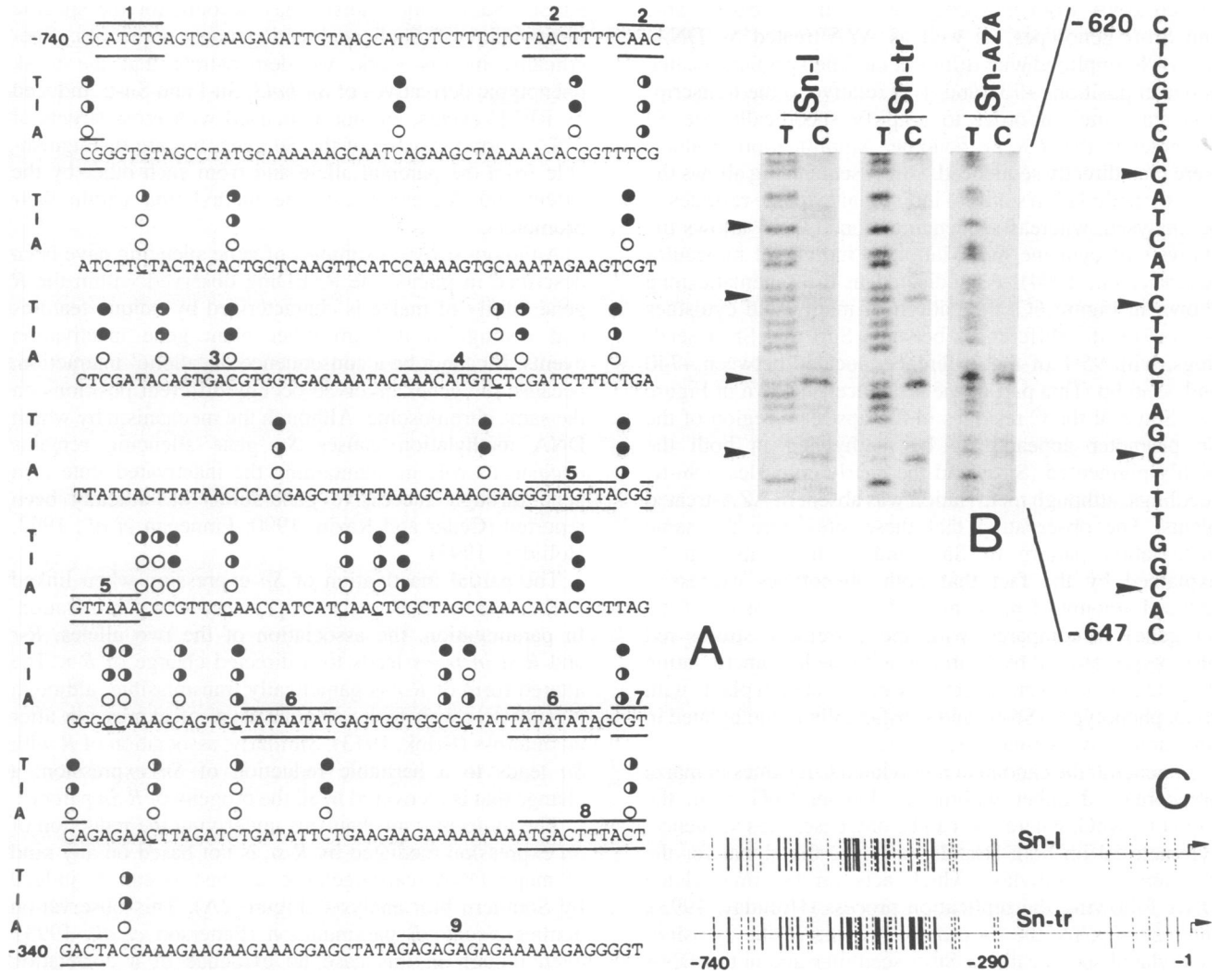


Fig. 6. Differences in the distribution of methylated cytosines in the promoter of Sn-I, Sn-tr and Sn-AZA-treated seedlings. (**A**) Distribution of the methylated cytosines in the region spanning from –740 to –340 bp relative to the transcription start site of the *Sn* promoter. The comparison has been made among mesocotyl DNA of Sn-tr, Sn-I and Sn-AZA-treated seedlings, respectively indicated as T, I and A samples. The data represent the average methylation state of the cells in the tissue analysed: most C residues methylated at the site are marked by a filled circle, an intermediate level of methylation is indicated by a half-filled circle, while an empty circle indicates mostly unmethylated cytosines at the site. C residues that are not located within the canonical CpG or CpNpG sequence are underlined if they show differences in their methylation pattern among the samples analysed. Putative transcription factor binding sites numbered on the top strand of the *Sn* promoter sequence include: (1) a putative binding site for *myc*-like proteins (Prendergast and Ziff, 1992); (2) two plant and viral *myb* factor consensus recognition sites (Paz-Ares *et al.*, 1987; Biedenkapp *et al.*, 1988); (3) a c-AMP regulated ATF protein site (Lin and Green, 1988), as well as a consensus site for plant leucine zipper proteins such as TGA1 tobacco factor (Katagiri *et al.*, 1989) and CPRF1 (Feldbrühgge *et al.*, 1994); (4) p53 consensus site (El-Deiry *et al.*, 1992); (5) two light-regulated GT factor binding sites (Gilmartin *et al.*, 1990); (6) two AT-rich regions with a putative role in tissue-specific regulation (Jacobsen *et al.*, 1990) or in local conformation of nucleosomes (Chen *et al.*, 1987); (7) a CRE, a mammalian cAMP-responsive element (Fink *et al.*, 1988); (8) two consensus sites for binding of leucine zipper proteins, such as c-Jun, GCN4 and CREB (Katagiri *et al.*, 1989), as well as an AP1 site recognized by a c-Jun/c-Fos heterodimer (Schutte *et al.*, 1989); and (9) a GAGA transcription factor binding site, with a role in the spatial and temporal development (Biggin and Tjian, 1989). (**B**) Example of a sequence of the upper strand of the *Sn* promoter region between positions –647 and –620 relative to the transcription start site. The lanes of thymines (T) and cytosines (C) of bisulfite-treated DNA extracted from Sn-I, Sn-tr and Sn-AZA-treated mesocotyls are reported from the sequencing gel. The black arrowheads indicate the C residues that underwent different degrees of bisulfite deamination depending on their methylation state. Complete demethylation is indicated by a cytosine that has been totally converted to thymine. The first arrowhead from the top marks a completely non-methylated C residue, while the second arrowhead indicates an intermediate level of methylation for Sn-tr DNA only. This cytosine does not belong to a canonical CpG or CpNpG site. The third arrow from the top shows a cytosine bearing a partial methylation state in all the samples, and the fourth one indicates a fully methylated C residue in both Sn-I and Sn-tr, which differs from that in the AZA-treated plants. (**C**) Differences in the methylation status of the Sn-I and Sn-tr promoter region between positions –740 and 0 relative to the transcription start (indicated by arrows) by plotting the bisulfite-analysed C residues. Each vertical thick line indicates the position of a cytosine methylated in most sites. The thin line indicates an unmethylated cytosine present at all sites, while the dashed line indicates the methyl residue in an intermediate number of sites. The promoter sequence is reported in Consonni *et al.* (1993).

allows all the unmethylated cytosines to be read as T residues. Because the localization of each 5-methylcytosine is indicated by a band on the sequencing gel, this result is far more reliable than that obtained with other methods which rely on the lack of bands in a corresponding lane. To identify modified C, an example of the methylation-dependent presence of T–C residues in a sequencing gel is given in Figure 6B. The upper strand of bisulfite-

treated DNA from mesocotyl of seedlings bearing Sn-I and Sn-tr genotypes, as well as AZA-treated *Sn* DNA, was PCR amplified with different modified primers located between positions –738 and +79 relative to the transcription start site, in order to amplify specifically the *Sn* promoter in the *R-sc Sn* genome. Amplification products were then directly sequenced. Direct sequencing allows the overall methylation status of individual cytosine residues to be analysed, whereas sequencing cloned DNA allows the analysis of cytosine methylation in individual molecules (Clark *et al.*, 1994). As indicated in the schematic map shown in Figure 6C, the pattern of methylated cytosines shows specific differences between Sn-I and Sn-tr seedlings, with 95% of the differences located between –740 and –336 bp. This part of the sequence is shown in Figure 6A. Some of the C residues in the upstream region of the *Sn* promoter appeared to be methylated in both the highly pigmented (Sn-I) and the nearly colourless (Sn-tr) seedlings, although methylation was absent in AZA-treated plants. The observation that these sites bore the same methylation pattern in Sn-I and Sn-tr plants can be explained by the fact that both phenotypes express a reduced amount of pigment (and thus a repression of the *Sn* gene) if compared with the extremely strong red phenotype restored by treatment with the hypomethylating drug. Other sites were selectively methylated in plants with weak phenotypes (Sn-tr) and substantially unmethylated in Sn-I and in AZA-treated samples.

In general, the canonical methylated C residues in maize are contained either within the doublet CpG or in the triplet CpNpG, where 'N' can be any base. This sequence, symmetrical on both strands, provides the signal for the maintenance methylase which acts on hemimethylated DNA following the replication process (Holliday, 1993). Interestingly, for the *Sn* promoter, some of the cytosines methylated specifically in Sn-tr seedlings and not in DNA from Sn-I or AZA-treated seedlings were not located within such a canonical context but were present in non-symmetrical sequences. The presence of methyl groups on C residues in unexpected sequence positions in plant genes has been shown recently in transgenic *Petunia hybrida* (Meyer *et al.*, 1994). In agreement with these studies, we found that the non-symmetrical methylation pattern within the *Sn* promoter was also not characterized by a strict sequence specificity.

How differences in methylation between Sn-I and Sn-tr seedlings over 740 bp of the *Sn* promoter sequence could influence *Sn* expression is not yet known. However, it may be significant that some putative binding sites for transcription factors are present in regions of the *Sn* promoter in which we have observed differential methylation (for details see Figure 6A).

Discussion

This work highlights a novel example of interaction between genes that share a high degree of sequence homology (>90% homology at the cDNA level, Perrot and Cone, 1989; Consonni *et al.*, 1993). This interaction results in the partial silencing of one of the genes involved. Because it most likely represents a control mechanism operating among different members of a gene family, we propose to refer to this event with the general term REED. Such a mechanism may account for the specific inactivation of duplicated endogenous sequences. Specifically, in this work, we demonstrate that the weak phenotypic derivatives of *Sn:bol3*, Sn-I and Sn-tr, induced by REED events, are not associated with gross structural DNA rearrangements of the *Sn* gene, but are distinguishable from the parental allele and from each other by the extent and degree of cytosine methylation within their promoters.

Although several examples of gene silencing have been described in plants, the REEDing observed within the *R* gene family of maize is characterized by unique features that distinguish it from other plant gene inactivation events. It cannot be a consequence of 'allelic' interaction, because the genes involved occupy different positions on the same chromosome. Although the mechanism by which DNA methylation causes *Sn* gene silencing remains unclear, its role in maintaining the inactivated state of a gene through successive generations has already been reported (Cedar and Razin, 1990; Finnegan *et al.*, 1993; Holliday, 1993).

The partial inactivation of *Sn* expression when linked to *R-sc* can be envisaged as a special case of paramutation. In paramutation, the association of the two alleles, *R-r* and *R-st in trans* leads to a directed change of *R-r*. The altered form of *R-r* is gametically transmissible, although it tends to revert to its original level of expression after an outcross (Brink, 1973). Similarly, association of *R* with *Sn* leads to a heritable reduction of *Sn* expression, a change that is recovered in all the progeny of *R Sn* parents.

The molecular mechanisms underlying the reduction of *Sn* expression mediated by *R-sc* is not based on any kind of major DNA rearrangement, at least as can be judged by Southern blot analysis (Figure 2A). This observation applies also to *B* paramutation (Patterson *et al.*, 1993), even though in this case no evidence of a correlation between hypermethylation and *B* inactivation was observed. Our data, however, may not be directly comparable with the results with *B*, because different assays were used in the two systems. In the case of *B*, because no genomic sequencing analysis was performed, it remains possible that the same mechanism may be involved. Evidence for the role of DNA methylation in modulating *Sn* expression was first obtained through the use of methylation-sensitive enzymes (Figure 2B). Their use allowed the following conclusions: (i) plants with the most suppressed *Sn* phenotype (Sn-tr) also contain the most methylated cytosines within the Sn promoter; and (ii) a partial methylation profile was found for the Sn-I derivatives, data that correlate with the phenotypic reduction of mesocotyl expression in *R-sc Sn*-I seedlings, which never spontaneously revert to produce the amount of pigment observed in plants with the *r Sn*-S original genotype (Gavazzi *et al.*, 1990).

The change from strong to weak pigmentation determined by the *Sn* derivatives is not confined to mesocotyl but is observed in all pigmented tissues under *Sn* control (e.g. scutellar nodes and midrib). On the other hand, the different degrees of methylation are not tissue-specific: we observed, in fact, the same pattern of methylation in the mesocotyls and in the leaves, even though *Sn* transcripts are not detectable in leaves (Tonelli *et al.*, 1991). These data support the idea that epigenetic events occur regard-

less of whether *Sn* is expressed in the tissue. Similar results have been reported for the *P* gene (Das and Messing, 1994). On the other hand, genes encoding storage proteins are specifically unmethylated only in the maize endosperm, pointing to a role for methylation in tissue-specific regulation (Bianchi and Viotti, 1988). Also, in our system, methylation appears to modulate *Sn* expression in a given tissue. The results with AZA are in agreement with this interpretation. The response of *Sn* to the demethylating agent is confined to the mesocotyl and the scutellar node; in fact, even if the *Sn* promoter becomes unmethylated following AZA treatment, ectopic *Sn* expression is never observed in the seedlings. This suggests that cell-specific factors must be involved in the regulation of *Sn* activation through their interaction with methylated/unmethylated tissue-specific *cis*-elements. In addition, it has been reported by Gartler and Goldman (1994) that reactivation induced by AZA may not always be tied to advance in replication timing of a whole chromosomal region, and it is possible that, in some instances, reactivation could be restricted to a single locus.

Genomic sequencing using the bisulfite method allowed us to correlate the level of *Sn* expression in Sn-I, Sn-tr and Sn-AZA plants with the presence of methyl-C residues within the promoter. The degree of cytosine methylation has been shown to be very low in the AZA-treated *Sn* promoter, compared with Sn-I and Sn-tr DNA, while the latter showed the highest level of methyl-C residues. Thus, the level of pigmentation was inversely correlated to the level of *Sn* promoter methylation. The presence of methyl-C residues inside or close to putative transcription factor binding sites might be responsible for the spatial, developmental and light-induced expression of Sn. The activation of *Sn* and anthocyanin accumulation has been reported to be regulated by light, in particular by a blue photoreceptor (Mereghetti *et al.*, 1991). The observed differences in methylation of a C residue near a putative GT binding site, present within several light-regulated genes (Gilmartin *et al.* 1990), could account for the different light responses of the *Sn* derivatives and in the AZA-treated seedlings. It is likely that activation of *Sn* results from the synergistic effect of light together with unidentified developmental factors that bind to the *Sn* promoter. Indeed, developmental and light regulation of *Sn*, in terms of pigment accumulation, has been observed in the immature pericarp (Mereghetti *et al.*, 1991). Specifically, our results have shown that: (i) AZA-treated *Sn* seedlings, which exhibit a very strong pigmentation in the dark, display a very early light-independent activation of *Sn* transcript (Figures 3 and 4B); (ii) the original *r Sn*-S, which shows weak pigmentation in the dark but strong pigment accumulation following light exposure, displays a partial light-independent *Sn* regulation (Tonelli *et al.*, 1991); and (iii) weak derivatives, which have a totally light-dependent pigmentation, show only a late *Sn* activation (Figure 4A). The hypothesis that the developmental regulation of the *Sn* promoter is controlled by the methylation status of the promoter itself is supported by the results of the azacytidine demethylating treatment. In fact, using Northern analysis, we have demonstrated that in AZA-treated seedlings an *Sn* peak of activation occurs very early in development, whereas the influence of light is less pronounced. The partial demethylation of the sequences involved in this developmental control could account for the light-independent expression of Sn-S versus Sn-I and Sn-tr. To understand this process, it will be interesting to analyse the putative binding sites that show a pattern of cytosine methylation that is identical in Sn-I and Sn-tr but different in AZA-treated plants (Figure 5B).

The genomic sequencing of the *Sn* promoter has emphasized the existence of methylation of C residues that are not confined to symmetrical sequences such as CpG and CpNpG, which should be involved in the faithful maintenance of the methylation pattern (Holliday and Pugh, 1975). To our knowledge, this is the first example of an endogenous plant gene that contains such a pattern of methylation. In fungi, such as *Ascobolus immersus* (Goyon and Faugeron, 1989) and *Neurospora crassa* (Selker, 1990b), non-symmetrical DNA methylation has been observed, while in plants it has been observed by sequencing only in transgenic petunia systems (Meyer *et al.*, 1994). The maintenance of such methylation might involve a mechanism of recognition of hemimethylated regions of DNA, based on changes in the chromatin structure, as proposed by Selker (1990b). It is still unclear, however, whether the presence of methyl groups at some C residues in non-canonical positions has a specific function in gene silencing. Because some differences in the methylation profile of Sn-tr versus Sn-I are characterized by such residues, methylation at both canonical and non-canonical sites could be correlated with the inheritable absence of *Sn* activation and pigment accumulation in Sn-tr seedlings.

Similarities of REED to other genetic phenomena

Even if methylation accounts for the *Sn* REED phenomenon, the question of whether methylation is a consequence or a cause of altered gene expression has not yet been established. Other factors could exist which coordinate both the expression level and the methylation state of a gene sequence. Conformational changes of the chromatin structure could be the cause of gene silencing and of the establishment of a determined epigenetic pathway (Pfeifer and Riggs, 1991; Peterson and Sapienza, 1993). Well known, for example, are phenomena in which heterochromatin formation is involved in gene inactivation, such as position-effect variegation (PEV) described in *Drosophila* (Henikoff, 1992), X-chromosome inactivation and cell memory (reviewed by Riggs and Pfeifer, 1992) and telomeric position effects in yeast (Aparicio *et al.*, 1991). It could be hypothesized that a spread of methylation caused by the presence of a heterochromatic region neighbouring a euchromatic region of the chromosome is able to create a state of inactivation that could be inherited and be both stable and clonal. Our Southern analyses have demonstrated the presence of bands due to methylation of some sites belonging to *R-sc*-specific sequences in DNA extracted from Sn-tr seedlings. Therefore, the extensive methylation of *Sn* sequences is not confined to the gene sequence but may result from a spreading of methylation into flanking DNA regions. A similar case has been observed in maize for the *P* gene hosting an inactive *Ac* element (Brutnell and Dellaporta, 1994). This possibility should be taken into account to explain changes in the epigenetic status of a whole chromosomal region, which spans from one region of homology (*R*) to the other (*Sn*).

Potential similarities between the *Sn* REED events and other phenomena observed in fungi can be proposed. These processes are the 'methylation-induced premeiotically' response (MIP) in *A.immersus* (Rhounim *et al.*, 1992) and the 'repeat-induced point mutation' (RIP) in *N.crassa* (Selker, 1990a). In the former, artificial tandem DNA repeats are inactivated through methylation and faithfully transmitted to the progeny. In the latter, base modification processes are accompanied by mutation of the sequence. These kinds of mechanism are proposed to target natural DNA repeats for inactivation in order to contribute to genome stability by preventing chromosome rearrangement (Faugeron *et al.*, 1990).

Kricker *et al.* (1992) proposed that uninterrupted homologous sequences longer than 0.3 kb are targeted for methylation as a defensive strategy of the genome. In our case, the simultaneous presence in the genome of both *R-sc* and *Sn* homologous sequences could have activated similar mechanisms. This model also applies to the repeat-induced gene silencing (RIGS), described in transgenic *Arabidopsis*, in which single gene inserts are fully expressed, while multi-gene inserts, which represent duplicated sequences, segregate silenced progeny (Assaad *et al.*, 1993). *Sn* and *R-sc*, besides representing duplicated sequences, are also transcriptional activators exerting a critical role in gene regulation. Therefore, their copy number must be retained in the cell under a strict dosage control and must be properly titrated in the genome by as yet unidentified mechanisms.

Materials and methods

Genetic stocks

The *R locus*: detailed descriptions of the origin, phenotype and structural characteristics of the *R* alleles used in this study can be found in Dooner and Kermicle (1974). *R-sc*: coloured aleurone, green plant, a germinal derivative of the paramutagenic allele *R-st*, composed of (*Sc*) (*I-R*)(*Nc*), obtained by loss of the (*I-R*) component (Kermicle, 1984).

The *Sn locus*: a factor lying two map units distal to *R*, conferring specific tissue pigmentation (following light exposure) to the scutellar node, mesocotyl, leaf base and midrib and to the seed integuments (glumes and pericarps). Of the four independent accessions identified, *Sn:bol3* differs from the others in that it confers (following light exposure) a higher pigmentation level and a weak colour development even in the dark. *Sn:bol3* is located on an *r* marked chromosome which confers pigmentation to the anthers (Gavazzi *et al.*, 1990; Tonelli *et al.*, 1991).

R-sc Sn:bol3: this line results from recombination of a heterozygous *R-sc sn/r Sn:bol3* (Gavazzi *et al.*, 1990).

Germination and anthocyanin extraction

Seeds were allowed to germinate in darkness for 5 days at 24°C until a mesocotyl ~3 cm long had developed. Seedlings were then exposed to continuous light or kept in darkness at 21°C. Anthocyanins from individual excised mesocotyls were extracted with a fixed volume of 1% HCl in ethanol. The extracts were centrifuged twice and absorption determined spectrophotometrically at 530 nm. Anthocyanin concentration is expressed as absorbance value at 530 nm per scutellar node or per g of fresh weight. Mean values represent 10 independent replicates. Standard errors of the means are <5%.

Treatment with azacytidine

Seeds were incubated in a 30 μM solution of 5-aza-2′-deoxycytidine (Sigma) for 48 h in rotating flasks, under dark germination conditions. They were then plated in Plexiglass boxes on wet filter paper and kept in the dark for 48 h. Seedlings were then watered with a 30 μM AZA solution for 72 h; finally they were transferred into clean boxes and exposed to light. The procedure used in order to obtain plant maturation and flowering was modified by incubating seeds in azacytidine solution for only 24 h.

DNA and RNA analysis

Genomic DNA isolation and Southern analysis were performed as previously described (Tonelli *et al.*, 1991). To perform a time course analysis of RNA accumulation, scutellar nodes and mesocotyls were excised at different stages of seedling development, both in the dark and after exposure to white light for 24 and 48 h. Power Stars-HQ1-T400 W/DV Osram lamps were used as the light source with fluence rates of 68 W/m^2. RNA was extracted by grinding in liquid nitrogen using a pestle and mortar, and purified according to the method described in Van Tunen *et al.* (1988). For Northern blot analysis, 10 μg of total mRNA were loaded on formaldehyde gels (Maniatis *et al.*, 1982) and, after electrophoresis, blotted on Biodyne nylon membrane (Pall). Hybridization and filter washing were performed as described for Southern blotting. RNA molecular weight markers were from Bethesda Research Laboratory. Probes used for Southern and Northern hybridization were: (i) Sn 550, a fragment from –471 to +59 bp (relative to the transcription start site), at the 5′ promoter region of the *Sn* gene (Tonelli *et al.*, 1991); (ii) 1.4 kb, a *Pst*I–*Eco*RI fragment derived from the *Sn* cDNA (Tonelli *et al.*, 1991); (iii) C2, a 1.5 kb cDNA of the *C2* gene (Wienand *et al.*, 1987); (iv) Tub, a 1 kb *Eco*RI fragment from an α-tubulin cDNA from maize (Dolfini *et al.*, 1993).

PCR-mediated methylation analysis

For the analysis of DNA methylation at the *Hpa*II site located at position –689 bp relative to the transcription start in the *Sn* promoter, the following primers were used: primer AR8 (5′ CTACCCCAAACAACGGTCACTCATCA), located at 161 bp upstream of the *Hpa*II site and the primer OH1 (5′ ATCTAAGTTCTCTGACG) located 314 bp downstream.

As a reference for amplification, two primers were used that are both located in the downstream flanking region of the *Hpa*II site, primer AR18 (5′ TTCCAACCATCATCAACTCGCT) at –471 bp relative to the transcription start site and primer CT12 (5′ GATATGCCACGAACCTTGG) located at position –120 bp. For each amplification reaction, 100 or 200 ng of genomic DNA digested with the restriction enzyme *Hpa*II were used. The 50 μl reaction mix contained dATP, dTTP, dCTP and dGTP (200 μM each), two primers (25 pmol each), PCR buffer (2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris–HCl pH 9.0 at 25°C, 0.1% Triton X-100), and 2.5 units of *Taq* polymerase (Promega). Following the first denaturation step (2.5 min at 94°C), the reaction mix underwent 18 amplification cycles consisting of 45 s at 94°C, 1 min at 50°C (with primers AR8 and OH1) or 60°C (with primers AR18 and CT12) and 1 min at 72°C. An aliquot of the reaction was then loaded on a 1% agarose gel in TBE buffer and transferred onto a nylon membrane (Pall Bydine) after electrophoresis. The filter was hybridized with the *Sn* promoter fragment of 550 bp.

Bisulfite treatment, PCR and sequencing of genomic DNA

Bisulfite treatment was essentially performed as described by Frommer *et al.* (1992), with some modifications. The maize mesocotyl DNA (20 μg) was first cleaved with *Hin*dIII and then was subjected to the preparation steps for the bisulfite treatment according to the protocol, with a final additional denaturation step at 95°C for 3 min. DNA was then incubated with a freshly made 3.1 M sodium bisulfite solution, 0.5 mM hydroquinone, pH 5.0 in a final volume of 1.2 ml, overlayed with 200 μl of mineral oil and kept at 50°C in the dark for 18 h. After dialysis, the samples were dried under vacuum, resuspended in 150 μl of sterile water and stored in aliquots at –20°C.

PCR was performed in a 50 μl reaction mix containing 100 ng of bisulfite-treated DNA, 1 μl of primers (25 pmol each), 1 μl of dNTP (10 mM each), 5 μl of buffer (5 mM $MgCl_2$, 67 mM Tris–HCl pH 8.8 at 25°C, 16 mM ammonium sulfate, 0.01% Tween-20). Two modified primers were used to amplify the top strand. One 24mer, AR4 (5′ TTGAAAGGTATGTGAGTGTAAGAG), located at –738 bp relative to the transcription start, and a 23mer, AR5 (5′ AAACAATACAAAAACTTCCATAC), located at +79 bp. The reaction mixture was subjected to an initial denaturation step of 2.5 min at 94°C, followed by eight amplification cycles (45 s at 94°C, 1 min at 58°C, 2 min at 72°C), and 34 cycles (45 s at 94°C, 1 min at 58°C and 1 min at 72°C). The last cycle consisted of 45 s at 94°C, 1 min at 58°C and 5 min at 72°C. The resulting 817 bp amplified fragment was purified through a 'Micropure-0.22 Separator and Microcon 50 Microconcentrator' (Amicon) from a 1% agarose gel and stored until sequenced.

The sequencing reactions were performed directly on the purified

fragments using the 'fmol DNA Sequencing System Sample' (Promega). In addition to the modified AR4 and AR5 primers, two other modified primers were used, the 24mer AR19 (5′ GTTTTATTAAAGATAAAGAGGGTT) located at –226 bp, and the 21mer AR21 (5′ ACTCTAATTAAATTTAACTTC) at –224 bp.

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