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Transgenerational changes in the genome stability and methylation in pathogen-infected plants

(Virus-induced plant genome instability)

Alexander Boyko[1], Palak Kathiria[1], Franz J. Zemp[1], Youli Yao[1], Igor Pogribny[2] and Igor Kovalchuk[1,*]

[1]Department of Biological Sciences, University of Lethbridge, Lethbridge, AB. T1K 3M4, Canada, and
[2]Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA

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ABSTRACT

Previously, we reported the generation of a virus-induced systemic signal that increased the somatic and meiotic recombination rates in tobacco mosaic virus (TMV)-infected tobacco plants. Here, we analyzed the progeny of plants that received the signal and found that these plants also have a higher frequency of rearrangements in the loci carrying the homology to LRR region of the gene of resistance to TMV (*N*-gene). Analysis of the stability of repetitive elements from *Nicotiana tabacum* loci and 5.8S ribosomal RNA loci did not show any changes. Further analysis of the changes in the progeny of infected plants revealed that they had substantially hypermethylated genomes. At the same time, loci-specific methylation analysis showed: (1) profound hypomethylation in several LRR-containing loci; (2) substantial hypermethylation of actin loci and (3) no change in methylation in the loci of repetitive elements from *N. tabacum* or 5.8S ribosomal RNA. Global genome hypermethylation of the progeny is believed to be part of a general protection mechanism against stress, whereas locus-specific hypomethylation is associated with a higher frequency of rearrangements. Increased recombination events combined with the specific methylation pattern induced by pathogen attack could be a sign of an adaptive response by plants.

INTRODUCTION

Throughout their entire life cycle plants continuously respond to stimuli that alter their physiology, morphology and development. Many of these stimuli are of an external nature and have an adverse effect on growth, development and reproduction. These external stimuli are commonly described as stresses (1,2). Constant exposure to a particular stress results in the evolutionary selection of adaptive traits beneficial to those conditions, albeit at a slow and gradual rate. Conversely, plants are able to acclimate on a reduced timescale by modifying their homeostasis and, therefore, adjusting to a frequently changing environment (3,4). This change in genotype or phenotype, respectively, represents adaptations that enhance the environmental fitness of a population of organisms. The fact that plants can successfully react to unrelated physical, chemical or temporal environmental factors suggests the existence of complex perception and response signaling pathways (3,5,6).

Such environmental influences lead to a global response which includes the processes of systemic acquired resistance (SAR) (7), systemic wound signaling (8), systemic acquired acclimation to light (9), systemic post-transcriptional RNA silencing (10,11) and the photoperiodic induction of flowering (12). These responses, including those associated with viral infection, are based on the ability of plants to recognize the stress and produce mobile signals that can activate specific responses in distant tissues.

What drives the mechanisms of acclimation and adaptation? What regulates transient or stable changes of gene expression in adapting plants? How do plants transmit this information to the next generation? These, and other, questions have yet to be answered.

The plant systemic reaction to pathogen-induced stress is a well-described mechanism involving the recognition of the pathogen avirulence (*Avr*) gene by the plant resistance (*R*) gene (7,13). This process commonly leads to a local hypersensitive response, followed by SAR to future infections by similar and even unrelated pathogens (7). As SAR is a systemic process, it is directly dependent on the spread of a signal throughout the plant. This *R–Avr*

*To whom correspondence should be addressed. Tel: +1-403 329 2579; Fax: +1-403 329 2242; Email: igor.kovalchuk@uleth.ca

gene interaction, termed incompatible, prevents the pathogen from systemically infecting the plant.

The absence of a specific *R*-gene for pathogen recognition results in a compatible interaction with the pathogen and, in the instance of a virus, the systematic spread of infection. Recent data suggests that an immediate plant response in the form of increased radical production occurs regardless of whether the plants are resistant (incompatible) or sensitive (compatible) to the pathogen (14). This initial boost of free radicals apparently triggers the production of a special warning signal. A second, larger burst of radicals occurs only if the plant has the required *R*-gene. It is possible that this second radical burst inactivates the initially generated warning signal. Plants that do not contain the appropriate *R*-gene have the warning signal systematically spread throughout the plant tissue. As plants do not have a predetermined germline, and plants cells are generally totipotent, it is likely that the changes triggered by this signal can be transmitted to the next generation, better preparing progeny plants to similar pathogen infections. Recent evidence supporting this hypothesis was the finding of a dramatic change in the transcriptome of Arabidopsis plants exposed to a compatible virus (15). This finding further supports the hypothesis of the existence of a warning signal generated upon compatible pathogen infection.

Previous work in our laboratory has shown that a compatible interaction between the pathogen '*Tobacco mosaic virus*' (TMV) and the plant *Nicotiana tabacum* (tobacco) results in the production of a signal that led to systemic changes in the frequency of somatic recombination (16). This signal, termed the systematic recombination signal (SRS), was locally generated at the site of infection and was capable of spreading faster than the virus, altering genome stability in non-infected tissue (16).

Genome instability generally refers to the susceptibility of the genome to rearrangements and activation of mobile elements, whereby a stable genome impedes these mechanisms. This stability is largely due to the addition of functional groups, most commonly methyl groups, to DNA and/or histones. The loss of methyl groups, termed hypomethylation, allows for rearrangement events, such as homologous recombination, to occur (17). Homologous recombination acts both as a double-strand break repair mechanism and the mechanism underlying crossing over events during meiosis. Hypermethylation, the addition of methyl groups, together with specific histone modifications, stabilize the genome and prevent recombination events. However, the homologous recombination mechanism can prove dangerous in cells, as it can be responsible for the induction of recessive genotypes from heterozygous loci. Thus, genome stability must be closely monitored to balance this risk with the need for genome diversity.

We have previously demonstrated that rearrangements in a transgene of infected plants could potentially be transmitted to the next generation (16). This is logical, as an increase in somatic recombination only seems sensible if the resulting changes are forwarded to the next generation. However, as these rearrangements can prove harmful, we examined the loci of several important plant genes to see if their methylation status changed in response to the SRS. We hypothesized that important housekeeping genes essential to proper plant function would remain stable, while *R*-gene loci would become unstable. If our hypothesis was correct, an increase in rearrangements in *R*-gene loci due to a compatible infection could be seen as an attempt to formulate novel *R*-genes for the next generation (18,19).

This form of adaptive response to biotic stress through genomic alterations is similar to McClintock's original model of the activation of transposable elements in response to environmental changes. Further research into this model has shown that many forms of stress stimulate transposon activity. Among these stresses are salicyclic acid, methyl jasmonate, $CuCl_2$, cell subculture, protoplast isolation, oxidative stress, wounding and pathogen attack (1). This is important as transposon activity is associated with decreased genome stability, which is further associated with hypomethylation (20,21). The link between stress exposure and hypomethylation has been established for cold stress, whereby it was shown that cold treatment promotes tissue-specific hypomethylation of defined areas of the genome, including areas specific to retrotransposon sequences (22). It remains to be established, however, whether there is a link between biotic stress, loci-specific hypomethylation and changes in the genome stability of pathogen-infected plants.

Here, we analyzed the progeny of TMV-sensitive tobacco plants (cultivar SR1) whose parental lines were infected with TMV. We found substantial alterations in the stability of the *R*-gene loci and no changes in the stability of the repetitive elements in *Nicotiana tabacum* (RENT) and 5.8S rRNA loci. These changes were paralleled by alterations in global genome- and *R*-gene-loci-specific methylation. These results support our hypothesis and lend an important insight into the ability of plants to adapt to biotic stresses.

RESULTS

Generation of progeny of plants infected with virus

Single leaves of the *N. tabacum* cultivar SR1 were inoculated with 300 ng of TMV RNA or mock treated (Figure 1; ref. 16). The upper, non-treated leaves (virus-free, checked as previously published; ref. 16) of these plants were cut 24 h after the treatment and grafted onto 10-week-old healthy plants from which the tops were previously removed (21 plants with leaves from virus-treated and 20 plants with leaves from mock-treated plants). To analyze the next generation, we collected seeds that were derived from the newly emerged tissue of the grafted plants and named them 'progeny of infected' (PI) or 'progeny of control' (PC) (Figure 1). In this article, we present the data from 21 independent PI lines (PI#1, PI#2, ...) and 20 independent PC lines (PC#1, PC#2, ...).

Stability of genomic loci

Our previous experiments showed that plants exposed to TMV had a higher frequency of

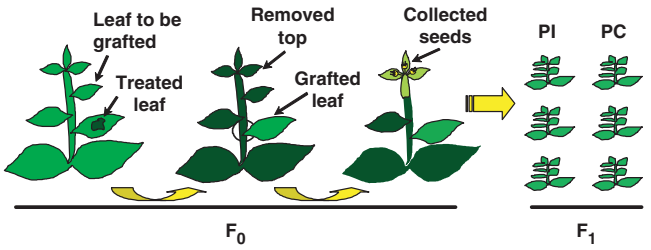

Figure 1. Analysis of the progeny of infected and progeny of control plants. Schematic representation of the experimental set up. Briefly, in a previous experiment, single leaves of 10-week-old SR1 tobacco plants were inoculated with 300 ng of TMV RNA (21 plants) or mock treated (20 plants). About 24 h after inoculation, the upper, non-treated leaves (virus-free, checked as previously published, ref. 16) from these plants were grafted onto 10-week-old healthy plants (21 plants with leaves from virus-treated and 20 plants with leaves from mock-treated plants), from which the tops were previously removed (F_0). The seeds (F_1) derived from the newly emerged tissue were collected and named 'progeny of infected' (PI) or 'progeny of control' (PC). These seeds were used to analyze global and loci-specific methylation and RFLP of various loci.

homologous recombination. The progeny of these plants (PI) also exhibited an increased frequency of meiotic recombination as measured in by luciferase homologous recombination transgene construct (16). The recombination changes in this transgene are believed to represent the changes in all other areas of the genome.

LRR-containing loci are more unstable in PI plants. It is possible that the large variety of polymorphic *R*-gene families have evolved by mechanisms such as gene and chromosomal duplications, unequal crossing over and deletions/insertions incited in plants challenged by pathogens.

To test this hypothesis, we analyzed changes in resistance genes carrying the homology to the LRR region (fourth exon) of the *N*-gene. Genomic DNA of PC or PI plants was digested with either the HindIII, EcoRI, or DraI restriction enzymes and hybridized with LRR region. Hybridization of DNA cut with HindIII revealed that the SR1 tobacco cultivar contained 30 fragments with various degrees of homology to the LRR region of the *N*-gene (Figure 2A). Hybridization of EcoRI and DraI cut DNA revealed between 28 and 35 loci, depending on length of the exposure (Figure 2A).

It was important to show that the fragments that were observed in RFLP upon the hybridization with the LRR region of the *N*-gene indeed represented homologous sequences. To verify that the SR1 tobacco cultivar contains the sequences similar to the fourth exon, we used two different strategies. First, we screened the tobacco genome database of Washington University (Saint Louis, Missouri, USA) and found more than 10 sequences with varying degrees of homology to the *N*-gene (35–64%; data not shown). Second, we designed several sets of primers based on the sequence of the fourth exon and performed PCR reactions using the genomic DNA of SR1 plants as a template (Figure 2B and C). We then cloned and sequenced several PCR products and aligned these sequences with the fourth exon containing the LRR

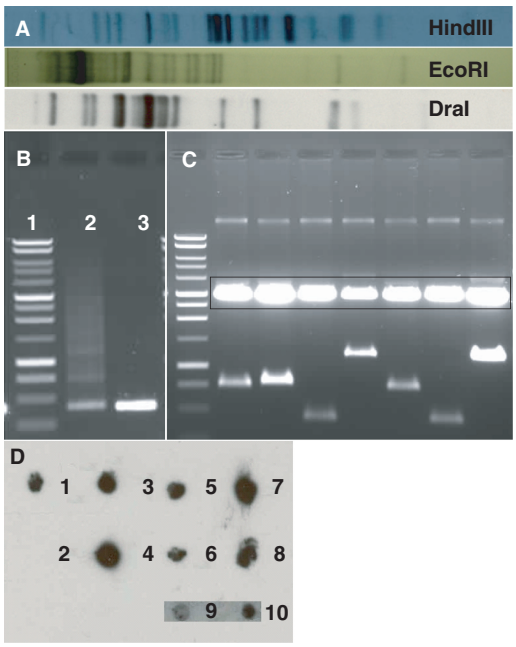

Figure 2. RFLP pattern of the *N*-gene-like *R*-genes; cloning and hybridization of the sequences with high degree of homology to the fourth exon of the *N*-gene. (**A**) RFLP analysis of *N*-gene-like loci was performed after digestion with three different enzymes, HindIII, EcoRI, DraI. (**B**) PCR amplification from the plasmid carrying the *N*-gene (3) or from SR1 plant genome (2), using primers annealing to the fourth exon of the *N*-gene; (1) is the marker. (**C**) Cloning of the PCR fragment (line 2 from Figure 2B) from SR1 plants. Digestion of the pGEM cloning vector with EcoRI shows the number of fragments of different sizes. The frame shows the band corresponding to the cloning vector; the bands of lower molecular weight represent the PCR fragments. (**D**) Hybridization of the colonies containing the PCR product (1—positive control, bacteria containing the vector; 2—negative control, empty bacteria; 3–10—clones with different degree of homology to the fourth exon. Clones 3–8 have the homology of >75%, whereas 9 and 10—only 60–65% (overexposed blot shown).

region. Alignment revealed that the clones had various degrees of homology ranging from 63 to 87% (Figure 1S).

It was then necessary to check whether these sequences can indeed be detected by hybridization. This was done by hybridizing the bacteria containing these clones with the probe and showed that they have sufficient homology for hybridization (Figure 2D). Further investigation found that out of the six possible translation frames from each of the cloned fragments, several of them gave 30–40% of similarity (data not shown). One of the clones actually contained a putative LRR domain (Figure 2S). Overall, this data demonstrates that the SR1 genome indeed contains a number of sequences with a high degree of similarity to the fourth exon of the *N*-gene. Moreover, they show that these sequences can, in fact, be detected by hybridization.

We analyzed the RFLP of these resistant-like gene loci in 149 PI and 147 PC plants digested with HindIII and found 33 and 4 fragments to be of different sizes, respectively. This corresponded to rearrangement frequency of 7.4×10^{-3} per plant for PI and 9×10^{-4} per plant for PC, resulting in a significant 8.2-fold difference in stability of LRR-containing loci ($P < 0.01$).

To substantiate the data obtained from HindIII digestions, we performed a similar analysis using the EcoRI enzyme. Hybridization of genomic DNA digested with the EcoRI enzyme revealed a similar pattern with a statistically significant 6.5-fold difference in *R*-gene loci instability (14 fragments out of 98 PI and 2 fragments out of 91 PC). Further, as it was possible that the difference in RFLP could in part be the consequence of unequal methylation at the HindIII or EcoRI recognition sites, we used the restriction enzyme DraI that does not contain a cytosine in the recognition sequence (TTT/AAA). Similarly, hybridization of genomic DNA digested with the DraI enzyme revealed 4 fragments out of 28 PI plants and none out of 28 PC plants. This corresponded to a rearrangement frequency of 4.8×10^{-3} per PI plant for both, EcoRI- and DraI-digested DNA. Examples of polymorphic loci are shown in Figure 3 A–C.

RENT and 5.8S loci have comparable stability in PI and PC plants. Although spontaneous rearrangements in *R*-gene loci resulting in changes in pathogen resistance have been shown (23), we present the first case of pathogen-induced reshuffling. The frequent rearrangements observed in the transgene (16) and *R*-gene loci would be harmful if they occurred throughout the genome. It was important to analyze the stability of the genes that were neutral to pathogen attack but essential for correct plant function. To avoid drawing false conclusions, we analyzed the genome stability of two different groups of loci, RENT and 5.8S. Both groups have multiple members and either contain moderately repetitive DNA (RENT; ref. 24) or are themselves found in clusters (5.8S; ref. 25).

RFLP of the RENT loci revealed 45 fragments of different sizes and intensities (Figure 3S). No significant difference in RFLPs was found, as there were 3 fragments of different sizes from PI, and two fragments differing from PC plants (Figure 3D). This translated to a statistically identical rearrangement frequency of 4.47×10^{-4} and 3.02×10^{-4} for PI and PC plants, respectively. The comparable stability of the RENT loci is an important finding. RENT contains cryptic gene regulatory elements that are inactive at their native locations in the genome, but have the capacity to regulate gene expression when positioned adjacent to genes (24). Thus, the increased frequency of rearrangements of RENT loci in the genome could lead to detrimental changes in the expression of neighboring genes.

RFLP analysis of the 5.8S loci revealed 52 fragments of different sizes and intensities (Figure 3S). There were two loci of very high intensity and, thus, multiple film exposures had to be done. Screening of 149 PI and 147 PC plants revealed a similar frequency of rearrangements. There were seven different fragments in PI and five different fragments in PC. This translated to a statistically indifferent rearrangement frequency of 9×10^{-4} and 6.5×10^{-4} for PI and PC, respectively.

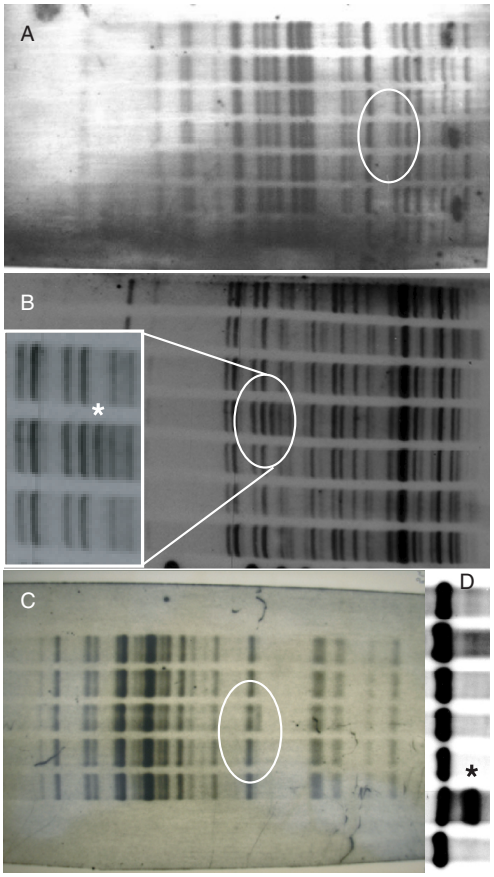

Figure 3. RFLP showed increased instability of the *R*-gene loci, and similar stability of other loci. Examples of the polymorphisms in RFLP of *N*-gene-like loci from PI plants are shown in A–C. (**A**) RFLP in the sample digested with HindIII. (**B**) RFLP in the sample digested with EcoRI. (**C**) RFLP in the sample digested with DraI. Occasional differences (**D**) were found in hybridization with 5.8S probe (PI plant). Additional examples of the RFLP are shown in Figures 6S–8S.

PI lines have globally hypermethylated genomes

The increased frequency of rearrangements at a specific locus could in part be explained by changes in the methylation status of this locus. Previous articles have indeed shown the correlation between frequency of recombination and methylation status at a particular locus, with hypermethylated loci being more stable and hypomethylated loci more unstable (17,26–28).

To test our hypothesis of whether changes in genome stability are triggered by changes in methylation, we analyzed global genome and locus-specific methylation status in PI and PC. Since the most common sites for methylation are symmetrical CpG and CpNpG sites, we used the methylation sensitive HpaII and MspI restriction enzymes that recognize the commonly methylated CCGG nucleotides. Both, external and internal cytosines at this restriction site can potentially be methylated. Methylation of the external cytosine in CCGG prevents digestion with MspI and severely impairs (~3000-fold) digestion with HpaII (29). Methylation of the internal cytosine in CCGG does not influence digestion with MspI, but prevents digestion with HpaII.

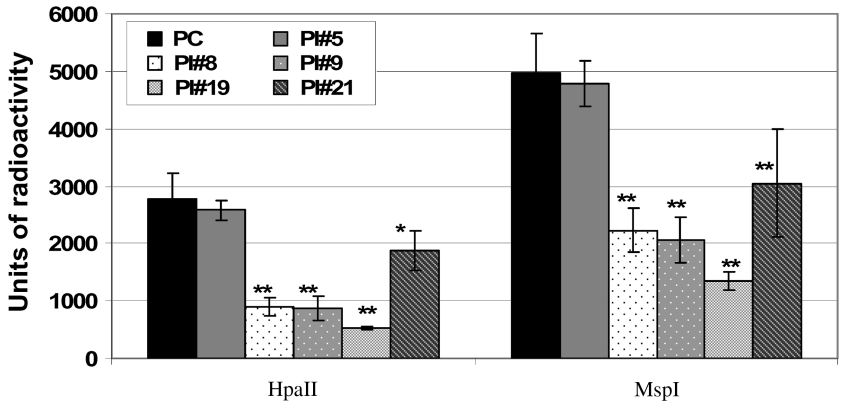

Figure 4. Global genome methylation was analyzed by ROPS assay after digestion with either HpaII or MspI. 'Y' axis shows radioactive incorporation (dpm/μg) in PC and PI lines. Each bar represents the average (with SD) from five individual assays, each representing the readings from five plants. Asterisks show the significance of the data, where one represents 95% and two represent at least 99% confidence interval.

Analysis of global genome methylation showed that out of the five PI lines tested, four had significantly ($P < 0.001$ in all cases) increased global genome methylation levels when compared to PC lines (Figure 4; Table 1S).

Specific methylation changes in PI and PC plants

Since higher methylation levels are normally associated with the lower recombination levels, we assumed that changes in the stability of LRR-containing loci were the result of loci-specific hypomethylation that was present in globally hypermethylated genomes. To test whether PI and PC plants have differences in loci-specific methylation pattern, we analyzed the methylation pattern of the LRR region of *N*-gene-like *R*-genes, actin, RENT and 5.8S rRNA loci in both progenies. Based on what we had found in the RFLP instability assays, we hypothesized that LRR-containing *N*-gene-like would have a lower methylation status, whereas the other tested loci would either have no changes or a higher methylation status.

Further, given the nature of the control loci we chose, any changes in methylation patterns that differed between these loci would lend more evidence to a locus-specific methylation mechanism as opposed to a structure-specific mechanism.

PI plants exhibit loci-specific changes in methylation: hypomethylation in LRR-containing loci and hypermethylation in actin loci. To analyze the methylation status of LRR-containing loci, genomic DNA of 20 PI and 20 PC plants was digested with HpaII, MspI, HindIII, EcoRI or DraI restriction enzymes and hybridized with a probe based on the sequence of the fourth exon of the *N*-gene. We predicted that there would be no significant difference in fragment intensity in the blots derived from HindIII, EcoRI or DraI digestions, whereas there should be some significant changes in the HpaII digestions. Indeed, it was found that fragments in HindIII, EcoRI and DraI DNA digestions were similar in intensity in PI and PC plants (see examples in Figure 2A). In contrast, we found substantial heterogeneity in the intensity of the fragments generated by HpaII or MspI digestion.

Whereas most of the fragments had similar intensity (Figure 5A), there were several loci with different intensities and, thus, different methylation (Figure 5A). We noticed that many PI plants either completely lack the fragment of ~3 kb (Figure 5A, black arrow) or retained it at a much lower intensity. At the same time, PI plants have a higher intensity of two other smaller fragments (Figure 5A, white arrows). This could be due to the loss of methylation at a HpaII recognition site allowing the cleavage of the bigger (3 kb) fragment into two smaller ones. Unfortunately, it would be difficult to perform quantification analysis on this observation as the 3-kb fragment disappears almost entirely in most of the PI plants tested.

A similar picture was observed upon cleavage with MspI. There was a lower intensity of the fragment of ~1.5 kb (Figure 5B, black arrow) and a higher intensity of the smaller fragment (Figure 5B, white arrow) in PI plants.

Similar analysis of the actin loci showed no changes in the intensity of the 13 fragments when the DNA of PI and PC plants was cut with HindIII (data not shown). However, among the fragments representing the actin loci obtained after the digestion with HpaII or MspI, there were substantial changes in fragment intensities (Figure 6). As can be seen in Figure 6A and B, PI plants have a substantially lower intensity or almost complete disappearance of two fragments (white arrows) and no appearance of any additional fragments. This would be possible only if there was hypermethylation of the HpaII recognition site in PI DNA, as, in this case, the smaller fragments cannot be formed. In contrast, methylation at HpaII in PC DNA was apparently lower and allowed the formation of these two fragments. Digestion with MspI supported this pattern, as there was a substantially higher intensity of the larger fragments (Figure 6C and D, white arrows) and substantially lower intensity of the smaller fragments in the majority of tested PI plants

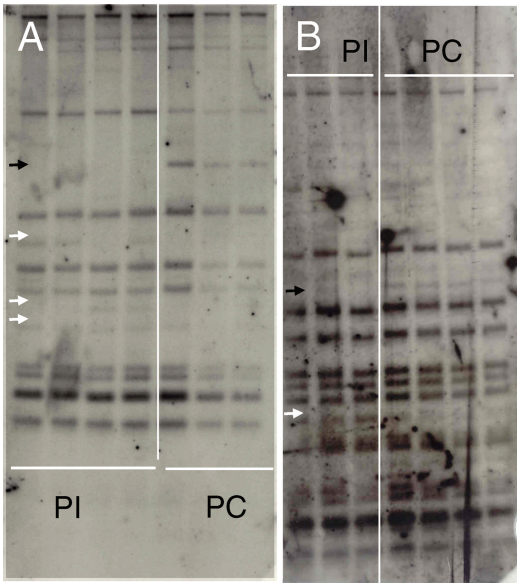

Figure 5. Analysis of methylation in LRR-containing loci. Representative gels show the DNA samples of PI and PC plants digested with HpaII (**A**) and MspI (**B**) and probed with the fourth exon of the *N*-gene. Black arrows show the fragment of substantially lower intensity (or complete disappearance), whereas the white arrows (the arrows are located just above the fragments) show the higher intensity (or appearance) of the two smaller fragments in PI samples.

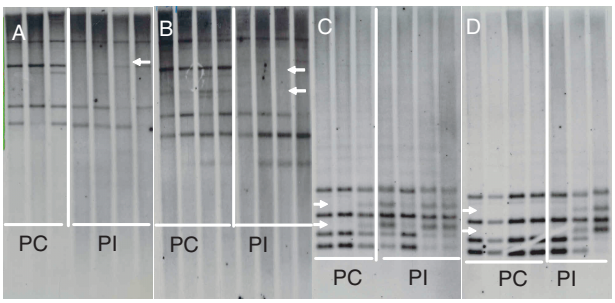

Figure 6. Analysis of methylation in actin loci. Representative gels show the DNA samples of PI and PC plants digested with HpaII (**A** and **B**) and MspI (**C** and **D**) and probed with Tob71 actin probe. In HpaII cut gels (**A** and **B**) white arrows show the absence or substantially lower intensity of the fragments in PI samples. Since there were no other fragments appearing on the gel, the only way to have the lower intensity of these fragments (white arrows in A and B) was the higher methylation in the larger (heavier) fragments. In MspI cut gels (**C** and **D**), white arrows show the higher intensity (or appearance) of the two larger fragments in PI samples. At the same time, there is a lower intensity of the smallest fragments, suggesting higher methylation in the two larger fragments (labeled with white arrow in **C** and **D**).

(Figure 6 shows two representative blots with 7 PI plants of the 20 lines tested).

These experiments suggest that the methylation status of the progeny of plants infected with the virus were significantly changed, whereby some *N*-gene-like loci were found to be strongly hypomethylated and several actin loci were found to be hypermethylated. This differential methylation could possibly allow for more flexibility in rearrangements of LRR-containing loci and for less flexibility of actin loci.

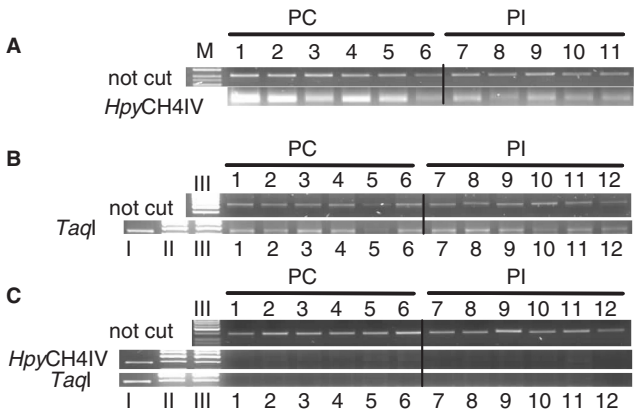

Figure 7. Methylation status of actin, RENT and 5.8S loci in PI and PC plants performed using COBRA. 'Bisulfite-treated genomic DNA from PI and PC lines was used for amplification of the actin, RENT and 5.8S loci. (**A**) Comparison of digestion patterns in actin loci of PI and PC plants after 16-h incubation with HpyCH4IV. The upper panel shows the undigested PCR product serving as a loading control. The lower panel shows the undigested fragment from the digested samples (see the entire gel in Figure 4S). Lines 1–6 represent individual PC plants; lines 7–11 represent individual PI plants (two samples from PI line #3, and one sample each from PI lines #8–10). (**B**) Comparison of digestion patterns in RENT loci of PI and PC plants after 16-h incubation with TaqI. Line 'I' is the undigested PCR product, lines 'II' and 'III' show 100- and 50-nt ladders, respectively. The upper panel shows the undigested PCR product serving as a loading control. The lower panel shows the undigested fragment from the digested samples (see the entire gel in Figure 4S). Coding is identical to that used for actin, except that there were six PI samples, lines 7–12. (**C**) Comparison of digestion patterns in 5.8S loci of PI and PC plants after 16-h incubation with HpyCH4IV or TaqI. The upper panel shows the undigested PCR product serving as a loading control. Two lower panels show the undigested fragments (completely absent) from the digested samples (see the entire gel in Figure 4S). Coding is identical to that used for actin. Heavy methylation of 5.8S loci result in complete digestion of the PCR product.

Confirmation of the methylation status of the actin, 5.8S and RENT loci using COBRA. To support our data on the epigenetic changes that were obtained using RFLP analysis based on digestion with the HpaII and MspI enzymes, we performed a combined bisulfite restriction analysis (COBRA) analysis on PI plants (lines 3, 8, 9 and 10) and PC plants.

For amplification of the actin fragment, we used bisulfite-converted genomic DNA (see materials and methods). The PCR revealed the expected fragment size of 298 nt (Figure 7A; top line). The PCR product was digested with the HpyCH4IV enzyme. The HpyCH4IV will cut this fragment at the recognition sequence ACGT when the cytosine in the original genomic sequence had been methylated, and it does not cut when the cytosine had been unmethylated and modified by bisulfite to thymine (Figure 4S). Digestion of the PCR product with HpyCH4IV would result in a lower intensity of the 298 nt band in hypermethylated DNA (Figure 7A; see Figure 4S for more details).

We found a nearly 2-fold lower intensity of the 298-nt fragment (49.7 ± 14.8 arbitrary units of intensity in PI versus 95 ± 12.2 in PC; $P < 0.01$; see quantification details in the Materials and methods section) at the actin locus.

Table 1. Summary of the experiments

	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
R-gene	3	3	2	1			5	2	5	1
GG-CH_3	n/e	n/e	n/e	n/e	=	n/e	n/e	+++	+++	n/e
R-gene-CH_3	+	++	+	n/e	=	n/e	++	+	+++	n/e
Actin-CH_3	+	+	n/e	n/e	=	+	n/e	n/e	++	n/e

	#11	#12	#13	#14	#15	#16	#18	#19	#20	#21
R-gene		3	4	3	3	5	3		4	
GG-CH3	n/e	n/e	n/e	n/e	+++	n/e	n/e	n/e	n/e	+
R-gene-CH_3	=	n/e	+	+	+	+++	++	n/e	+	n/e
Actin-CH_3	=	n/e	+	+	++	+	+	n/e	++	n/e

The table shows the comparison of PI lines. '*R*-gene' shows the number of RFLPs in a particular PI line. The numbers of RFLPs for '*R*-gene' represent data combined from HindIII and EcoRI digestions. 'GG-CH_3' and '*R*-gene-CH_3', show the intensity of changes in methylation [severe (+++), moderate (++) and weak (+)] in the global genome (GG) and the specific loci, respectively. '=' represents lines with no change in methylation. 'n/e' stands for 'not estimated'.

These results indicate a higher degree of methylation in the actin loci in the PI when compared to PC plants. Further, these results confirm the hypermethylation status of the PI actin loci obtained with the RFLP analysis of HpaII and MspI cut DNA.

COBRA performed for the RENT and 5.8S loci did not reveal any significant difference in methylation between PI and PC plants (Figure 7B and C; quantification data not shown). It was noticed, however, that the 5.8S loci was severely hypermethylated when compared to the RENT or actin loci (indicated by complete absence of an undigested fragment, Figure 7C). This was also confirmed upon RFLP analysis of the 5.8S loci after digestion of genomic DNA with the HpaII enzyme, as very little DNA was digested in both PI and PC plants (Figure 5S, part A). Digestion of the same DNA with MspI was found to be more efficient (Figure 5S, part B). The high methylation status of the rRNA-coding loci is not surprising as only a subset of the total rRNA gene pool is active at a given time (30).

This experiment supports our hypothesis that pathogen infection triggers loci-specific methylation changes in the genome of PI plants.

Changes in methylation status correspond to changes in rearrangements of *N*-gene-like loci

To analyze whether plants with methylation changes also exhibited a higher frequency of rearrangements in the *N*-gene-like loci, we plotted all the data into one table (Table 1). It became obvious that the lines with the most frequent rearrangements in the *N*-gene-like loci had significant changes in methylation. For example, all the lines that had more than two rearrangements exhibited changes in the *N*-gene-like loci methylation status. Global genome methylation was also severely affected in lines #8, #9 and #15, which corresponded to 2, 5 and 3 *N*-gene-like loci rearrangements, respectfully. Conversely, lines #21 and #5 had no *N*-gene-like loci rearrangements with little global methylation changes.

DISCUSSION

In this article, we analyzed the progeny of SR1 plants compatibly infected with TMV and found that these plants have a higher frequency of rearrangements in the loci containing LRR regions, an unchanged frequency of rearrangements among the RENT and 5.8S loci. We hypothesized that such differential stability was triggered by different levels of methylation. Indeed, we confirmed that some of the LRR-containing loci were hypomethylated. At the same time, the other loci were either hypermethylated (several actin loci) or did not change their methylation status (RENT, 5.8S).

The LRR-containing loci of PI plants are more unstable

Our experiments showed more rearrangement freedom in the LRR-containing loci of PI plants. Digestion with HindIII revealed 33 different RFLPs found in 149 PI plants and only 4 RFLPs in PC plants. Similarly, digestion with EcoRI revealed 14 RFLPs in 98 PI and 2 RFLPs in 91 PC plants. Depending on the enzyme used, we observed a 5.3–8.2-fold increase in *N*-gene-like *R*-gene loci RFLP instability.

When we compared the patterns from both the EcoRI and HindIII digestions, we found that only five PI plants had changes in RFLPs after cutting with either enzyme, whereas the majority of PI plants had changes in RFLPs only after cutting with one of the enzymes. This likely suggests that the majority of the changes experienced in the LRR regions were minor, and unable to span the distance required to alter both enzyme's restriction pattern. Larger, more complex rearrangements would be able to alter both restriction sites, resulting in changes in the same plants with both enzymes. Our data suggests that the majority of the genome changes found via the RFLP in the *N*-gene-like *R*-gene loci were most likely small events such as point mutations and small insertions/deletions. However, despite these small changes, it is apparent that these LRR-containing loci are more unstable in PI plants.

Most of the rearrangements in LRR-containing loci analyzed by RFLP included the appearance of extra fragments. This was expected, as most of the events would be occurring in only one allele and, thus, the original fragments would also be retained. In two cases, we observed the disappearance of fragments coupled by the appearance of several others. This suggests that rearrangements either occurred very early in embryo development or that rearrangements occurred in both alleles.

The increase in recombination frequency found at these loci upon pathogen infection becomes an important finding when one considers the variety of polymorphic *R*-gene families in plant genomes. In fact, much evidence has been gathered suggesting that these families have evolved through extensive rearrangement mechanisms such as gene and chromosomal duplications, unequal crossing over and deletions/insertions (31–34). We propose that the changes have been promoted by a constant exposure to various pathogens. In the current article, we only looked at the rearrangements of LRR regions. In the future, it would be interesting to look at the changes in different regions of the *R* genes, such as those coding for TIR or NBS domains, etc. The LRR regions of *R* genes mediate protein–protein interactions in the plant 'immune' system, and are thought to confer pathogen-receptor recognition specificity to each resistance protein (35–38). Indeed, mutations in this domain showed a loss of *Avr*-binding ability and, thus, a loss of resistance (37). Our sequencing data (Figure 1S) showed that the observed RFLP fragments carried substantial degree of homology to the *N*-gene LRR region. Although in SR1 plants these regions do not carry sufficient homology to confer the resistance to TMV, it is possible that all these loci are 'building blocks' of (un)successful rearrangements aiming at the 'creation' of active *R*-gene(s).

RENT and 5.8S loci showed similar stability in PI and PC plants

We found no difference in the stability of RENT- or 5.8S rRNA- coding loci in PI and PC plants. The RENT loci of PI and PC plants exhibited statistically identical rearrangement frequencies of 4.47×10^{-4} and 3.02×10^{-4} per locus per plant, respectively, while the 5.8S rRNA loci showed a statistically indifferent rearrangement frequency of 9×10^{-4} and 6.5×10^{-4} for PI and PC, respectively. These data also suggest that 5.8S loci are likely more unstable than the RENT loci.

Higher than normal recombination rates in clusters of rRNA-coding genes is a well-known phenomenon (39). In fact, it is thought that the stability of multi-copy genes is purposefully low to allow for genome evolution. However, despite the significant variations in the rDNA clusters observed in bacteria and yeasts, these loci have reasonably uniform expression levels and are not significantly influenced by stress (40). This is the principle reason these loci are frequently used as internal controls (40).

PI plants have hypermethylated genomes

The analysis of global genome methylation revealed PI plants to be hypermethylated, a phenomenon that could be a part of protective/adaptive response. It is well known that gene expression can be regulated in a genetic and epigenetic manner. The role of epigenetic control in the adaptation and acclimation process is hard to underestimate, as transgenerational changes in methylation patterns are a powerful tool to reversibly modify the expression of a number of genes. It was previously shown that the progeny of plants constantly exposed to ionizing radiation had hypermethylated genomes (26). Thus, changes in DNA methylation patterns could be considered as part of the plant protection mechanism (41). It should be noted, however, that the data on transgenerational methylation changes in stressed plants are scarce and more studies are needed to confirm this phenomenon.

As seen in Figure 4, the radionuclide incorporation pattern in HpaII and MspI is similar, with less radionuclides incorporated in HpaII cut DNA (Table 1S). Jeddeloh and Richards (42) have previously shown that over 90% of the external cytosines in CCGG sequences are methylated in tobacco. Therefore, as methylation of the external cytosine completely blocks the digestion with MspI and severely inhibits digestion with HpaII (29), these results follow. Further, less digestion with HpaII could also be due to the methylation of the internal cytosine, as this does not impair digestion with MspI.

The analysis of asymmetric cytosine methylation was not performed, as these sites are reported to be less frequent in plants. It is generally believed that the ratio of methylation at CpG:CpNpG:CpX (where CpX corresponds to methylation at asymmetrical sites) is ~4:2:1 (43). Moreover, some areas of the genome show as little as 4% of asymmetrically methylated cytosines (44). It should be noted, however, that plants with larger genomes have a higher percentage of non-canonical asymmetrically methylated cytosines (45). Thus, we cannot exclude that there could be some differences in asymmetric cytosine methylation in PI and PC plants.

PI plants exhibit a loci-specific methylation pattern

The global genome hypermethylation observed in PI plants cannot explain the higher frequency of rearrangements in LRR-containing loci. Since we observed higher frequency of rearrangements in *N*-gene-like *R*-gene loci, we hypothesized that despite the hypermethylated genome, these *R*-gene loci would have lower methylation.

The changes in methylation status were not similar in all the PI lines. One explanation for this could be that PI parental plants were differently affected by the virus, and, thus, received an SRS of different strength. For example, some parental plants (lines #9, #15, #16) may have generated a stronger SRS, which promoted more severe changes in the progeny, while other parental plants (lines #5 and #11) may have generated a SRS of much lower intensity, or failed to produce an SRS at all, failing to exhibit any changes in methylation or *R*-gene rearrangements.

From all the enzymes tested (HpaII, MspI, EcoRI, HindIII, DraI), we observed changes in methylation only after digestion with HpaII and MspI. This was expected, since both of these enzymes are sensitive to the most common methylation at CpG sites. We did not see any changes in methylation between PI and PC plants tested with DraI, EcoRI and HindIII in more than 300 plants tested. This was expected, since the recognition sequence for DraI does not contain C or G nucleotides at all, and the recognition sequence of EcoRI and HindIII does not contain a CpG sequence. However, it should be noted that digestion with EcoRI and HindIII could theoretically be methylation sensitive. The EcoRI recognition sequence, GAATTC, contains a cytosine that has been shown to be symmetrically methylated when followed by a guanine, or asymmetrically methylated when followed by thymidine or adenine. Similarly, the HindIII recognition sequence, AAGCTT, contains a cytosine that may also be methylated. However, the lack of any changes in EcoRI- and HindIII-digested DNA, together with changes in HpaII/MspI-digested DNA suggest that the changes in symmetrical methylation we observed should be the primary reason for the difference in methylation status of the *R*-gene-like loci and actin loci.

Importance of changes in methylation for plant adaptation

The methylation pattern of every individual organism is established after fertilization and is specific to each individual organism. It is possible that exposure to a stress influences the inheritance of the methylation pattern according to the organism's needs, as dictated by a particular stress. Logically, external stresses of various extensities should be able to change the plant's methylation status not only in somatic tissue, but also in tissue destined for future generations (46,47). Recently, this hypothesis was supported by Molinier *et al.* who showed the inheritance of an epigenetic signal generated by exposure of Arabidopsis plants to abiotic (UVC) and biotic (flagellin, a pathogen elicitor) stresses (48). The article reported that plants exposed to stress exhibited changes in genome rearrangements not only in treated somatic cells, but also had these changes persisted in several generations of untreated plants (48). The authors suggested that the information about stress was probably transmitted into next generation via epigenetic mechanisms as crosses between treated and non-treated plants resulted in the increase in rearrangement frequency in all progeny plants (48). Although this report supports our data, it shows the increase in rearrangements of neutral transgene loci and does not show the consequences to other loci.

Since methylation is a well-explored process of genome maintenance, whereby methyl groups tend to make chromatin less accessible to various remodeling processes, hypomethylation could be suggested as a mechanism that facilitates the rearrangement of certain loci. For example, the high rate of recombination in the V(D)J region of immunoglobulin and T-cell receptors in the mammalian immune system is regulated by DNA hypomethylation (27). Engler *et al.* quantified rearrangements in the V(D)J locus of immune cells in mice and demonstrated that demethylated loci experienced a higher frequency of rearrangement events, whereas methylated loci were highly stable (17). Studies have also shown that DNA regions of highly duplicated genes also contain a high level of methylation, and the removal of gene copies reduces the methylation density of the remaining copies (28).

Our report is not the first evidence of the existence of a specific mechanism directed toward genome rearrangements in stressed plants. Previously, a number of publications reported heritable changes in inbred flax in response to specific, defined environmental changes such as nutrient balance and temperature regimes (49,50). This appeared to originate from the activity of a transposon-like sequence, LIS1, that assembles and inserts itself into the genome of stressed flax plants (50). The new genotroph is apparently stable, as no further changes in the activity of this transposon occur in plants upon the exposure to additional stresses (50). Indeed, it would be interesting to see if a corresponding hypomethylation of LIS1 is accommodating its activation in these instances.

It remains to be determined whether changes reported in this article are virus-specific or pathogen-wide. Future research should also address whether the progeny of PI plants will again have a higher frequency of rearrangements, or whether any of the observed epigenetic and genetic changes contribute in any way to virus tolerance.

Conclusion

Our data suggests the existence of a specific, epigenetically controlled mechanism that promotes rearrangements in *R*-gene loci upon contact with a compatible pathogen. Future studies are clearly needed to understand the signal specificity and the mechanism underlying the methylation changes. To exceed natural breeding limits for plant tolerance, a better understanding of the complex interaction between plant and environment is urgently required. This research has to include the analysis of adaptive and coordinating capacities of global genome regulation.

MATERIALS AND METHODS

Generation of transgenic tobacco plants carrying a homologous recombination substrate

Generation of transgenic tobacco plants carrying the luciferase recombination substrate and used for the grafting experiments was previously described (16). Seeds from the newly emerged tissue of SR1 plants (line LU#1) grafted with leaves derived from the pathogen (TMV)-infected plants and mock-treated plants were used for the experiment in this article (Figure 1). Progeny of infected (21 different lines) were named PI and progeny of control (20 lines) were named PC.

DNA extraction

Total DNA was prepared from whole plants using Nucleon PhytoPure total DNA isolation kit

(Amersham Life Science) in accordance with the manufacturer's protocol.

RFLP of *N*-gene like resistance gene, RENT and 5.8S loci

Restriction fragment length polymorphism (RFLP) analysis for *N*-gene-like loci was performed on 149 PI plants and 147 PC plants by digesting genomic DNA (5 µg) with either HindIII or EcoRI or DraI, and probing it with the fourth exon of the *N*-gene. The frequency of rearrangements in the *R*-gene loci was calculated according to the following formula:

$$f_r = \frac{n_r}{p \times 30}$$

where f_r is the frequency of rearrangements, n_r is the number of rearranged loci, p is the number of plants screened (149 and 147) and 30 is the number of loci that carry homology to *N*-gene.

The RFLP analysis of the RENT loci was performed on 142 PI plants and 137 PC plants by digestion with HindIII or EcoRI. The DNA was probed with a 514-nt probe obtained with RENT1-specific primers (forward 5′ TCTGATTTATCCGACTCAGATC 3′, reverse 5′ TTT AGAAGGGACTTAAACAGAG 3′). This region had over 90% homology to the other known RENT genes. The RFLP analysis of the 5.8S ribosomal RNA loci was also performed on 142 PI plants and 137 PC plants, but by digestion with HindIII only (EcoRI digestion was giving smeary hybridization patterns with only a few fragments clearly visible; data not shown). Digested DNA was probed with a 370-bp fragment (forward 5′ TAAATTGA AAGCCTGCCTCTC 3′, reverse 5′ ACCACTTGTCGTG ACGTCCG 3′). Fragment intensity was quantified using 'Image J' (NIH, www.rsb.info.nih.gov/ij).

Analysis of global genome methylation

Genomic DNA was digested for 48 h with a 10-fold excess of HpaII or MspI endonucleases according to the manufacturer's protocol (New England Biolabs, Beverly, MA). An additional DNA aliquot was incubated without a restriction enzyme and served as a background control. This single-nucleotide extension reaction was performed on 2 µg of DNA, 1 × PCR bufferII, 1.0 mM $MgCl_2$, 0.25 units of Amplitaq DNA polymerase (Perkin Elmer, Foster City, CA), [^{3}H]dCTP (57.4 Ci/mmol) (NEN, Boston, MA) as previously described (51). The radioactive incorporation (Table 1) is shown in dpm/µg of DNA. Incorporation is directly dependent on the completeness of the DNA digestion with methylation-sensitive enzymes. Higher methylation is associated with a decrease in digestion rate and, thus, a lower incorporation of radioactively labeled cytosine ([^{3}H]-dCTP).

Analysis of methylation of *N*-gene-like and actin loci

Genomic DNA (10 µg) was digested with the methylation-sensitive-enzyme HpaII and probed with either the fourth exon of the *N*-gene or with the actin Tob71 probe (298 bp; 5′ TTGTGTTGGACTCTGGTGATGGTG and 3′ AAT GGTGATCACCTGCCCATCTGG primers). This region was shown to carry over 95% homology to the same region in the other tobacco actin genes such as Tac9, Tob93, Tob103, Tob54, etc. The fragment intensity inversely correlates with the methylation status at a particular locus. Tissue with hypermethylated loci have less genomic DNA cut and thus will have loci (fragments) with lower intensity. In contrast, hypomethylated loci have more genomic DNA cut and, thus, will have loci (fragments) with higher intensity. Up to 15 plants per each group were analyzed. All the data for *N*-gene and actin was plotted using the Microcal Origin 6.0. Fragment intensity was quantified using 'Image J' (NIH, www.rsb.info.nih.gov/ij).

Bisulfite treatment

DNA was treated with bisulfite to accommodate the COBRA assay as described previously (52). In brief, 1 µg of DNA was denatured in 50 µl of 0.2 mM NaOH at 37°C for 20 min and 30 µl of freshly prepared 10 mM hydroquinone (Sigma) and 520 µl of 3 M sodium bisulfite (Sigma) at pH 5 were added. Samples were incubated under a layer of mineral oil at 55°C for 16 h. The DNA was purified using the Gene Clean kit (Promega Corporation, Madison, WI, USA) and eluted with 100 µl of water. The DNA was desulfonated with 0.3 M NaOH at 37°C, followed by an ethanol precipitation. The DNA pellet was resuspended in 1 mM Tris-HCl, pH 8 and used for PCR.

Combined bisulfite restriction analysis (COBRA)

The assay was performed according to a previously published protocol (53). The assay consists of three major steps: (1) a bisulfite treatment that converts all the unmethylated cytosines to thymine, (2) a gene-specific PCR that amplifies the gene fragment to be analyzed and (3) a restriction digestion reaction that allows the identification of newly created methylation-dependent restriction sites and methylation-dependent retention of pre-existing sites (53).

In brief, sodium bisulfite-treated DNA was PCR amplified with the following sets of primers: actin, forward 5′ TTGTGTTGGACTCTGGTGATGGTG 3′, reverse 5′ AATGGTGATCACCTGCCCATCTGG 3′; RENT, forward 5′ TCTGATTTATCCGACTCAGATC 3′, reverse 5′ TTTAGAAGGGACTTAAACAGAG 3′; 5.8S, forward 5′ TAAATTGAAAGCCTGCCTCTC 3′, reverse 5′ ACCACTTGTCGTGACGTCCG 3′. To avoid bias in the amplification process we also used primers that would amplify the same fragments by annealing to the opposite strand (actin, forward 5′AACACAACCTGAGACCACT ACCAC 3′, reverse 5′ TTACCACTAGTGGACGGGTA GACC 3′; RENT, forward 5′ AGACTAAATAGGCTGA GTCTAG 3′, reverse 5′ AAATCTTCCCTGAATTTGT CTC 3′; 5.8S, forward 5′ ATTTAACTTTCGGACGGA GAG 3′, reverse 5′ TGGTGAACAGCACTGCAGGC 3′). The expected size of the product was 298, 514, 370 nt, for actin, RENT and 5.8S, respectively. The PCR products from 20 plants from PI or PC groups were digested with either HpyCH4IV or TaqI and separated on 1.5% agarose gel. Fragment's intensity was quantified

using 'Image J' (NIH, www.rsb.info.nih.gov/ij). To make up for the differences in PCR amplifications, the intensity of cut fragments was standardized to the uncut PCR fragment. The average from 20 individual plants was calculated.

Statistical treatment of the data

In all cases, the average and standard error was calculated. The statistical significance of the experiments was confirmed by performing either a Students *t*-test (two-tailed paired or non-paired) or a single factor ANOVA. Statistical analyses were performed using the MS Excel software and Microcal Origin 6.0.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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