Epigenetic mechanisms in plants and their implications in plant breeding

ATHANASIOS S. TSAFTARIS1, 2*, ALEXIOS N. POLIDOROS1, RACHEL KOUMPROGLOU1, ELENI TANI1, NIVEC KOVACEVIC2, 3 AND ELENI ABATZIDOU2

1Institute of Agrobiotechnology, CERTH, 570 01 Thermi, Greece
2Department of Genetics and Plant Breeding, AUTH, 540 06 Thessaloniki, Greece
3Present address: Department of Agronomy, University of Wisconsin-Madison, 1575 Linden Drive, Madison, WI 53706, USA

*Corresponding author: tsaft@certh.gr

Abstract

Higher organisms, including plants, use three systems to initiate and sustain epigenetic gene regulation: DNA methylation, histone modification and RNA-interference. Unraveling the relationships between these epigenetic components has led to surprising and rapidly evolving new concepts, showing how they interact and stabilize each other. These interacting systems can regulate expression or silencing of genes, resulting in epigenetically controlled phenotypes that can be meiotically or mitotically heritable. In this review we discuss issues relevant to the involvement of epigenetic inheritance as a source of polymorphism generating useful variation for selecting superior genotypes. The role of methylation in hybrid vigor and stability of performance, and aspects of epigenetic transgene silencing in elite transgenic varieties will also be addressed.

Introduction

“Epigenesis” is a term coined by Aristotle to indicate that the development of an organism evolves through a series of causal interactions between various components, in antithesis to the theory favored by other Greek philosophers, particularly Democritus and Leucippus, that the fertilized egg represented a preformed miniature model of the organism. Preformation was broadly accepted and maintained its hold for millennia until the advent of the microscope and the discovery of the germ layers that settled the issue in favour of epigenesis in the 18th century. In the 20th
century, Waddington (1942) coined the term “epigenetics” in analogy to epigenesis in order to indicate that factors above the genotype were also involved in determining a certain phenotype during development. The modern use of the term “epigenetics” defines all meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself.

Higher organisms, including plants, use three systems to initiate and sustain epigenetic gene regulation: 1) DNA methylation that historically was the earliest heritable epigenetic mark to be studied, 2) histone modification, and 3) RNA-interference. While in DNA methylation cytosine methylation is the sole epigenetic mark, histone modifications are numerous involving acetylation, methylation, phosphorylation, ribosylation, ubiquitination of mostly H3 histone, but also H4, H1, H2A as well. These constitute the “histone code” of chromatin epigenetic marks. RNA-interference that entered dynamically the field of epigenetics provides a common denominator of both DNA methylation and histone modifications.

Unravelling the relationships between these epigenetic components has led to surprising and rapidly evolving new concepts, showing how they interact and stabilize each other. Histone deacetylation and other modifications, particularly the methylation of lysine 9 within histone H3 (H3-K9) residues located in the histone tails, cause chromatin condensation and block transcriptional initiations. Histone modification can also attract DNA methyltransferases to imply cytosine methylation, which in turn can reinforce histone modification patterns conducive to silencing. Experiments in plants have clearly shown also the involvement of RNA interference in the establishment of heterochromatic states and silencing. Disruption of one or the other of these interacting systems can lead to inappropriate expression or silencing of genes, resulting in epigenetically controlled phenotypes.

We discuss here these three epigenetic mechanisms, the molecular reactions involved, their effects on plant development and particularly their multiple implications in conventional plant breeding and modern genetic engineering.

Molecular epigenetic mechanisms

**DNA methylation**

DNA methylation is a heritable epigenetic enzymatic modification resulting from the addition of a methyl group in the cyclic carbon-5 of cytosine. Methylation is primarily found as part of host defense systems in prokaryotes, but it is also present in eukaryotes performing different roles, mostly as a control mechanism for transposable elements in the genome. Levels of methylation vary greatly between organisms that display this modification. The percentage of methylated cytosines (5mC) ranges from 0-3% in insects, 2-7% in vertebrates, 10% in fish and amphibians, to more than 30% in some plants (Adams 1996). This can be attributed to several factors influencing 5mC content, the most important that methylation in mammalian
genomes is generally restricted to symmetric CpG sequences (CG islands) often associated with genes, while plant and fungal genomes have methylated symmetric cytosines in CpNpG (where N can be any nucleotide) or random asymmetric C nucleotides often associated with DNA outside nuclear genes, which in certain plant species comprises most of the cellular DNA. However, the distinction of two patterns of genomic methylation hides a variety of unique situations where exceptions occur (Colot and Rossignol 1999). Finally, variations in methylation are observed in response to endogenous or exogenous cues. In mammals, for example: (i) a highly regulated developmental process first erases and then resets the genome-wide methylation pattern during early embryogenesis, (ii) many tissue-specific genes undergo demethylation during tissue differentiation and (iii) differences in methylation exist in the sperm and oocyte (Brandes et al. 1993). Tissue and developmental stage-specific as well as stress-induced variation in DNA methylation have been recorded also in plants (Messeguer et al. 1991; Tsaftaris and Polidoros 1993, 2000; Tsaftaris et al. 1997, 2000).

DNA methylation is catalyzed by a family of conserved DNA methyltransferases (MTases), which points to an ancestral origin of this form of DNA modification. Different types of DNA MTases include: a) maintenance methylases, which maintain stable cytosine methylation patterns through successive cell generations, b) de novo methylases able to transfer methyl groups to cytosines of unmethylated DNA and c) domain-rearranged methylases (DRMs) that are directed from short RNAs and specifically methylate homologous genes in a process termed RNA-directed DNA methylation (RdDM). *Arabidopsis* contains at least ten different members of MTases and mutations of each compromise normal development or other features of the plant (Tariq and Paszkowski 2004).

Methylation can be removed from DNA by either passive or active mechanisms. Passive demethylation can occur when methylated cytosines are replaced with non-modified cytosines during DNA replication (Tsaftaris and Polidoros 2000). In active demethylation the participation of specific proteins that demethylate DNA sequences has been reported in mammals and birds (Vairapandi and Duker 1993; Jost et al. 1995, 1999). Two *Arabidopsis* mutations, one interfering with maternal expression of an imprinted gene (Choi et al. 2002) and the other inducing transcriptional gene silencing (TGS) of transgenes and endogenous homologous loci (Gong et al. 2002), were mapped to the genes DEMETER (DME1) and ROS1, respectively, which encode DNA glycosylases that remove 5mC from DNA.

The most prominent roles that have been proposed for methylation are: (i) to provide a heritable epigenetic mark that could direct the developmental program of the organism (Holliday and Pugh 1975; Regev et al. 1998; Wolffe and Matzke 1999), (ii) to provide a means of genome defense against the activity of parasitic mobile elements (Yoder et al. 1997), (iii) to reduce background transcriptional noise in organisms that have a large number of genes (Bird 1995) and (iv) to “memorize” patterns of gene activity by stabilizing gene silencing brought about
by other mechanisms (Bird 2002). These hypotheses may be not mutually exclusive and may all be true, depending on the organisms considered. In fact, if methylation is an evolutionary tool (Colot and Rossignol 1999) it would be expected to serve a variety of functions and to play different roles both within and between organisms.

DNA methylation exerts its effects through repression of gene expression. Studies of numerous tissue-specific genes using different techniques have shown a clear correlation between methylation status and gene activity. In plants, abundant evidence supporting a role of methylation in suppression of gene expression comes from studies on regulation of transposable elements (Fedoroff 1996; Martienssen 1996) and transgene silencing in genetically-engineered plants (Morel et al. 2000; Paszkowski and Whitham 2001; Fojtova et al. 2003; Matzke et al. 2004).

**Histone modifications**

Histone modifications have also been defined as epigenetic modifiers. Post-translational modifications of histones, including acetylation, methylation, phosphorylation, ribosylation, ubiquitination of conserved lysine residues on the amino-terminal tail domains, have been studied closely over the past few years. Generally, the acetylation of histones marks active, transcriptionally competent regions, whereas hypoacetylated histones are found in transcriptionally inactive euchromatic or heterochromatic regions. In addition, histone methylation can be a marker for both active and inactive regions of chromatin. Methylation of lysine 4 of histone H3 (H3-K4) leads to activity and is found predominantly at promoters of active genes (Lachner and Jenuwein 2002). Methylation of lysine 9 on the N terminus of histone H3 (H3-K9) is a marker of silent DNA and is found throughout heterochromatic regions such as centromeres and telomeres. Lysine methylation can be monomeric, dimeric or trimeric; histones may also be subject to other post-translational modifications such as phosphorylation, ribosylation and ubiquitination. This enormous variation leads to a multiplicity of possible combinations of different modifications, representing a “histone code” (Strahl and Allis 2000) which can be read and interpreted by different cellular factors.

Important links between histone modifications on one hand and DNA methylation on the other have been found in H3-K9 methylation, a prerequisite for DNA methylation (Tamaru and Selker 2001; Jackson et al. 2002; Malagnac et al. 2002) while DNA methylation can also trigger H3-K9 methylation (Johnson et al. 2002; Soppe et al. 2002; Lehnertz et al. 2003; Tariq et al. 2003). It has been mentioned that histone deacetylases (HDACs), histone methyltransferases and methylcytosine-binding proteins (Nan et al. 1998; Fuks et al. 2003) lead to the recruitment of DNA methyltransferases (Fuks et al. 2000), although it is not yet clear what initiates the recruitment of the different epigenetic modifiers to their specific target sequences.
Multimeric protein complexes have being identified that function as “cellular memory keys” that “lock” gene expression states and enable their inheritance over many cell mitotic or meiotic division cycles (Narlikar et al. 2002). The multiprotein complexes involved in the regulation of developmental stages were originally identified and studied in *Drosophila*. Two such main complexes were originally described in *Drosophila*: the Polycomb (PcG) and the trithorax groups (trxG) (Mahmoudi and Vernijzer 2001). Both of these are known for their role in controlling the expression of homeotic genes but with opposite effect: Polycomb group maintains them in an inactive state while the trithorax group maintains their active state. Similar complexes were recently identified to execute conserved functions in plants, the PcG protein group in plants being the most studied. The PcG complex is involved in the control of the expression of homeotic genes that are essential transcriptional factors for proper plant development. Although there is no structural relevance between the PcG target genes in *Drosophila* and *Arabidopsis*, they are functionally similar and control developmental processes. A main component of the PcG complex in plants are, again, methylotransferases like the MEDEA protein of *Arabidopsis* that specifically methylates H3-K27 leading to a silent state of chromatin (Reyes and Grossniklaus 2003).

**RNA interference**

The central player in RNA-mediated gene silencing is a double-stranded RNA (dsRNA) that is chopped into tiny RNAs (siRNA 21-22 nucleotides-long hairpin or miRNA 22-24 nucleotides-long single strand RNA) by the enzyme Dicer. dsRNA could be the product of antisense transcription (in addition to sense), RNA viral duplication by RdRp and/or intramolecular RNA loops from transcripts derived from DNA with terminal inverted repeats, intron removal etc. The tiny RNAs associate with various silencing effector complexes and attach to homologous target sequences (RNA or DNA) by base pairing. Depending on the protein composition of the effector complex and the nature of the target sequence, the outcome can be either mRNA degradation, translational repression leading to PTGS (post-transcriptional gene silencing) and mRNA alternative splicing or genome modification leading to TGS, all of which silence gene expression. In fact, RNAi was discovered in experiments designed to compare the silencing activity of single-stranded RNAs (ssRNAs; antisense or sense) with their dsRNA hybrid. While only marginal silencing of a target gene was achieved after injecting *caenorhabditis elegans* with the individual strands, injection of a sense-antisense mixture resulted in potent and specific silencing (Fire et al. 1998). This unequivocally fingered dsRNA as the trigger of silencing. Shortly thereafter, dsRNA was shown to provoke gene silencing in other organisms, including plants (Waterhouse et al. 2001).

Following is a short unfolding of the discovery of TGS and PTGS in plants as lucidly recited by two of the pioneers in the discovery of these phenomena, Matzke and Matzke (2004). TGS was revealed when two different transgene complexes
were introduced in sequential steps into the tobacco genome. Each complex encoded different proteins but contained identical gene regulatory regions. The first transgene complex, which was stably active on its own, often became silenced in the presence of the second. The silenced transgenes acquired DNA methylation, a genome modification frequently associated with silencing, while silencing and methylation were abolished when the transgene complexes segregated from each other in progeny (Matzke et al. 1989; Park et al. 1996).

PTGS was discovered in two ways. One involved experiments to evaluate antisense suppression, a promising approach at the time for selectively silencing plant gene expression. In theory, antisense RNA encoded by a transgene should basepair to the complementary mRNA of a plant gene, preventing its translation into protein. Although the control “sense” transgene RNAs are unable to basepair to mRNA and hence should not induce silencing, they often inexplicably did (Smith et al. 1990). In a different experiment, efforts to enhance floral coloration in petunia by overexpressing a transgene encoding a protein involved in pigment synthesis led to partial or complete loss of colour. This resulted from coordinate silencing (“cosuppression”) of both the transgene and the homologous plant gene (Napoli et al. 1990; van der Krol et al. 1990), later shown to occur at the posttranscriptional level (de Carvalho et al. 1992; van Blokland et al. 1994). Similarly to TGS, PTGS was often associated with DNA methylation of transgene sequences (Ingelbrecht et al. 1994).

RNA-mediated gene silencing pathways have essential roles in plant development, chromosome structure and virus resistance. Although the mechanistic details are still under investigation, RNA-mediated silencing has already provided a powerful tool for studying gene function.

Epigenetic inheritance and plant breeding

Epigenetic changes in chromosomal proteins and DNA methylation (termed epimutations; Martienssen and Colot 2001) occurring in organisms have important phenotypic consequences. Careful observation and study of epigenetic plant phenotypes directed plant geneticists to recognize the role of epigenetic inheritance systems in plant evolution (Kaliz and Purugganan 2004), domestication and breeding (Tsafarlis and Polidoros 2000). The ubiquity and significance of epigenetic inheritance in plants has been gradually recognized over the past 50 years and has been founded on the grounds of the fundamental discoveries that epigenetic phenotypes can result from: (1) activation, excision and translocation of transposon elements (McClintock 1951), (2) allelic interactions known as paramutations (Brink 1956), (3) transgene silencing in plants (concurrently observed from several groups working with transgenic plants in the late 1980s; for a review see Matzke and Matzke 2004) and (4) epialleles of endogenous plant genes that control floral induction and morphogenesis, seed development and parental imprinting (Jacob-
sen and Meyerowitz 1997; Luff et al. 1999; Melquist et al. 1999). Excellent reviews covering these topics have been recently published (Martienssen 1998; Chandler et al. 2000; Kakutani 2002) and the reader is referred there for a more detailed analysis. In an attempt to discuss issues more pertinent to plant breeding, the following paragraphs will review reports relevant to the involvement of epigenetic inheritance as a source of polymorphism generating useful variation for plant breeding. The role of methylation in hybrid vigor and stability of performance, and aspects of epigenetic transgene silencing in contemporary elite transgenic varieties will be covered as well.

**Epigenetic mechanisms and genetic variation**

Epigenetic states in plants, once established, can be inherited through the transmission of epigenetic alleles (epialleles) over many generations (Kakutani 2002). Such heritable epigenetic alleles can be considered as a new source of polymorphism and may produce novel phenotypes. This could have significant implications in plant breeding. Heritable phenotypic variation within populations is the basis for selection and breeding. The genetic causes of phenotypic variation are attributable to mutations that create allelic variation and recombination that alters the genetic structure in which alleles are expressed, offering new backgrounds for epistatic interactions. Now, in addition to mutations that create genetic variation underlying phenotypic traits, epialleles have been found to produce a new source of variation for selection. Most importantly, epigenetic alleles can result as a genome response to stressful environments and enable plants to tolerate stress (Tsaftaris and Polidoros 2000; Finnegan 2001; Sherman and Talbert 2002; Steward et al. 2002). Comparison of these two mechanisms generating polymorphism (mutations and epialleles) has been presented (Tsaftaris and Polidoros 2000). DNA methylation as a generator of epialleles, could have important implications for the breeder. Epialleles could emerge at high frequency in a single generation, by far exceeding the rate of mutational events giving rise to new alleles. Their reversion rate is by far higher and that will interfere in heritability estimation. Their emergence is highly affected by plant growth conditions while random mutational events are largely considered independent of growth conditions. DNA methylation, by its mutational role, gives rise to more permanent mutant alleles at a locus, while mutations only rarely lead to new epialleles (when, by chance, critical C-residues in methylation sites are eliminated or generated). Assessing the importance of methylated epialleles in plant breeding requires the determination of: (i) the extent of variation in methylation patterns among individuals within the selection population; (ii) the degree to which methylation patterns affect phenotypes; and (iii) the extent to which methylation variants linked to superior phenotypes are stably inherited. These are challenging tasks, but the technical potential exists to assess methylation pattern differences between individuals and thus, estimate the levels of methylation-associated epiallelic diversity and its related phenotypic diversity. A better understanding of the role
and significance of this new source of polymorphism in plants will be achieved as more data accumulate for the role of DNA methylation in plant evolution, domestication and breeding.

**DNA methylation and somaclonal variation**

Somaclonal variation is generally attributed to tissue-culture induced, heritable genetic changes (Kuckuck et al. 1991; Phillips et al. 1994) rather than preexisting genetic or even epigenetic variation in the somatic cells of the explant. Distinguishing between tissue-culture induced and preexisting genetic or epigenetic variability is difficult unless the exact genetic or epigenetic makeup of individual cells in the explant was known prior to its culture.

A somatic cell or small group of cells, having a unique genetic constitution or epigenetic modification, may reproduce preferentially in culture. Any plant eventually regenerated from this cell(s) would probably be scored as a variant acquired during the culture period, although in reality it comprised a minuscule, possibly even undetectable, proportion of the cells in the original explant (Matzke and Matzke 1995). As it is becoming more apparent that somatically-acquired epigenetic modifications in plants can be mitotically stable and meiotically heritable, more emphasis is given to variation in DNA methylation as a source of somaclonal variation.

The first such evidence involving DNA methylation originated from studies of regenerants from crown-gall tumor lines where changes in T-DNA methylation were associated with phenotypic variation (John and Amasino 1989) as well as from demethylation, thus reactivation of silent \(Ac\) elements following tissue culture (Brettell and Dennis 1991; Peschke et al. 1991). The most compelling evidence suggesting that a substantial proportion of somaclonal variation might be due to diverse, preexisting epigenetic states that are mitotically and meiotically stable in plants regenerated from individual somatic cells, was provided by Neuhuber et al. (1994) who demonstrated that variation in antibiotic resistance markers in regenerated double transformants was due to the presence of the transgene complex in the original line as an epigenetic mosaic resulting from partial methylation.

To the extent that these data with transgenes will be verified with endogenous plant genes, this would reveal considerable variation among individual somatic cells. Newly developed technology should allow analysis of individual somatic cells (Schindler 1998; Schutze and Lahr 1998); this new source of variation, when generated in the meristematic cells of a plant could be of importance since some of these meristematic cells will become gametes.

Kaeppler et al. (2000), also mentioned that variations in both total methylation levels and in methylation of specific sites occur during culture process. Interestingly, they suggested that methylation levels in tissue culture decrease. Moreover, data from various studies support the idea that developmental timing may effect variation in methylation levels and patterns.
DNA methylation, heterosis and hybrid breeding

Quantitative variation in gene expression is responsible for variation in different biochemical/physiological processes and consequently, is essential for the manifestation of phenotypic diversity and phenomena linked to phenotypic expression of genetic differences such as heterosis and hybrid vigor. Variability in gene expression can be assessed through polymorphism of individual RNA amount (RAP) and through the polymorphism of individual protein amount (PAP). Numerous significant correlations between PAP indices and hybrid vigor for agronomic traits have been reported (Leonardi et al. 1988, 1991). Results suggest that genes controlling protein amounts and enzyme activities, and particularly those with multiple effects, directly affect the expression of hybrid vigor. Moreover, results obtained for polymorphism of individual RAP were similar to those of PAP. In one such RAP analysis (Tsaftaris and Polidoros 1993), expression of many genes in different maize tissues and developmental stages was measured. In a more detailed analysis of the data, the deviation in quantity of each RNA of every one of the two hybrids at the same mRNA in the two parental hybrids at the same developmental stage was examined. The heterotic hybrid had a significant number of genes expressed over the quantity of the better parent at three stages. Moreover, a recent work by Song and Messing (2003) provides evidence for altered regulatory effects in hybrids. The above data with PAP, enzyme amounts and RAP indicate that quantitative variation in the expression of certain loci may be important in vigor manifestation, and underline again the significance of regulatory mechanisms involved in the quantitative modulation of gene expression in manifesting vigor.

DNA methylation could be considered as a genome-wide general regulatory mechanism that affects the expression of many genes important for the manifestation of heterosis (Tsaftaris and Kafka 1998; Tsaftaris et al. 1998). To obtain more evidence for the possible involvement of methylation, Tsaftaris and Polidoros (1993) used the RAP analysis described above to obtain a gross estimation of genome activity, and HPLC chromatography to estimate DNA methylation. They found a significant negative correlation between genome activity and total DNA methylation. Studies were also conducted for monitoring the pattern of DNA methylation in maize parental inbreds and their hybrids using two additional techniques: the CRED-RA technique that was used to estimate the effects of planting density on methylation (Tani et al. 2005), and the RLGS technique that allowed to estimate the methylation pattern of CpG islands in almost a thousand NotI landmarks of the maize genome (Kovačević et al. 2005). Like the results of isoschizomeric enzymes and HPLC (concerning total methylation), the CRED-RA and RLGS analyses indicated that: (1) hybrids in general are less methylated than their parental inbreds; (2) heterotic hybrids are less methylated than related non-heterotic hybrids; (3) old, low-yielding inbreds are highly methylated; (4) more modern inbreds, especially those selected for high and stable yield under spacing
in the isolation environment, have lower percentages of methylation in comparison
with old progenitor lines. These findings support the hypotheses made by several
researchers (reviewed by Stuber 1998) that systematic selfing for isolation of
inbreds, with emphasis only on combining ability of inbreds (leading to line
inbreeding depression), is also leading to a gradual accumulation of more methy-
lated sites, which then could be released and/or repatterned when the selfed lines
are crossed to generate hybrids. Shifting emphasis to line performance per se during
selection, though, could moderate line inbreeding depression.

Contemporary F1 hybrids produce high but most importantly stable yield while
growing in different fields and in different years. Genetic stability (homeostasis)
refers to reduced genotype × environment interaction, particularly under stress. Pro-
duction of contemporary hybrids with high and stable yield was made possible by
efficient multi-year and multi-site testing of the genetic material. Different years and
different sites impose different kind of stresses (water, nutritional, light, pH, tem-
perature, etc.) on the tested material. What is the role of methylation in a hybrid’s
genomic DNA, especially in tolerating stress, leading to more stable yield? Data have
been presented showing that F1 hybrids are in general less methylated than their
parental inbreds. The possible role of methylation in the expression of maize genes
and performance of hybrids under different growth conditions has been examined
in experiments with maize inbreds and hybrids grown under different plant densi-
ties (Tsaftaris and Kafka 1998; Tsaftaris et al. 1998; Tani et al. 2005). We can con-
clude that the genotype, the developmental stage, and conditions of growth affect
the methylation status of genomic DNA. Stressful growth conditions result in more
methylated DNA (less expressed) and, in general, vigorous hybrids are more resis-
tant to such density-induced methylation and suppression of genome activity in
their genomic DNA. This resistance of the hybrid genome to genome methylation
under different stresses, and consequently, avoidance of suppression of many of its
genes could be at the core of high F1 yield and, more importantly, F1 stable yield. As
emphasis in line selection has been shifting to more productive lines that could gen-
erate high-yielding hybrids with stable yield in higher densities (substituting double
and triple hybrids with single F1 seeds), there was a concomitant shift in parental
lines giving less heterosis, and inbreeding depression and with less methylation.

Manipulation of parental imprinting

Many laboratories are currently interested in manipulating the development of
endosperm through the epigenetic mechanisms that control parental imprinting.
As studies in Arabidopsis showed, removal of the parental imprinting leads to over-
proliferation of the endosperm, which is a desirable trait for seed crops (Berger
2003). Exploring the epigenetic mechanisms of seed development will eventually
reveal the mysteries behind apomixis, i.e. the production of fertile plant progeny
without double fertilization and this identical to the mother plant (Koltunow et al.
2003). In case this mechanism can be applied to commercial crops, hybrids can be regenerated indefinitely, thus overcoming the current limitations of plant breeding to maintain hybrid vigor for more than one single generation.

**Avoiding transgene silencing in GM plants**

In plants, transgenes can be silenced at both the transcriptional and post-transcriptional levels (Matzke and Matzke 2004). Silencing is correlated with methylation of the corresponding transgenes. Methylation of the transgene promoter correlates with transcriptional gene silencing (Matzke et al. 1989; Park et al. 1996) whereas methylation of the coding sequence is associated with post-transcriptional gene silencing (Ingelbrecht et al. 1994). However, recent evidence suggests that a unifying mechanism based on RNA interference is underlying both processes (Matzke and Matzke 2004; Matzke et al. 2004). Silencing of the introduced transgenes has frequently been observed in plants, constituting a major commercial risk and hampering the general economic exploitation of transgenic plants. There is of course tremendous commercial interest for avoiding this epigenetically-imposed transgene silencing. Until now, the most efficient strategy to avoid transgene silencing involves careful design of the transgene construct and thorough analysis of transformants at the molecular level (De Wilde et al. 2000).

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