South African Journal of Botany 2003, 69(2): 117–128
Printed in South Africa — All rights reserved

SOUTH AFRICAN JOURNAL
OF BOTANY
ISSN 0254–6299

Minireview

Genetic and epigenetic aspects of somaclonal variation: flower colour bud sports in azalea, a case study

S de Schepper[1]*, P Debergh[1], E van Bockstaele[1], M de Loose[2], A Gerats[3] and A Depicker[4]

[1] *Department of Plant Production, Ghent University, Coupure Links 653, 9000 Ghent, Belgium*
[2] *Department of Plant Genetics and Breeding, Centre of Agricultural Research-Gent, Caritasstraat 21, 9090 Melle, Belgium*
[3] *Katholieke Universiteit Nijmegen, Postbus 9102, 6500 HC Nijmegen, The Netherlands*
[4] *Department of Molecular Genetics, Ghent University, K. Ledegankstraat 35, 9000 Gent, Belgium*
* *Corresponding author, e-mail: sandra.deschepper@UGent.be*

Received 6 January 2003, accepted in revised form 27 January 2003

Bud sporting is the consequence of sudden variations in gene expression of somatic cells, leading to the occurrence of phenotypically altered shoots on plants. This phenomenon can be observed in most vegetatively propagated plant species and finds its origin in genetic as well as epigenetic events. In azalea the frequent occurrence of flower colour sports is appreciated as a valuable additional source of variation; more than half of the commercial assortment consists of flower colour bud sports. However, when clonal uniformity is desired for registration (Distinctness Uniformity Stability, DUS) and mass propagation, this somaclonal variation is perceived as a hindrance. Insight into the molecular mechanism(s) underlying the induction of bud sporting might improve breeding strategies, towards the appropriate choice of cultivars in hybridisation experiments. The complex beauty of sport phenotypes clearly indicates interactions within and between genetic and epigenetic mechanisms. Therefore, using azalea somaclonal variation as a model, a review of the literature on various genetic and epigenetic phenomena that regulate or influence gene expression, as well as on the links that exist between them is presented.

Introduction

Belgian pot azaleas (*Rhododendron simsii* hybrids) belong to the *Tsutsusi* subgenus of *Rhododendron* within the family *Ericaceae* (Chamberlain and Rae 1990, Cullen 1991). The subgenus *Tsutsusi* comprises 66 species, with semi-evergreen spring leaves and persistent summer leaves. Within the *Rhododendron* genus, breeding barriers among species limit the assessable gene pool. Fortunately, in the vegetatively propagated azalea, the sporting phenomenon provides a useful additional source of variation (Heursel 1999, De Schepper 2001).

Sport induction is the consequence of a sudden variation in gene expression in somatic cells, and results in plants having shoots with a different phenotype, e.g. leaf shape or colour, growth habit, flower colour or form. Because of their clonal origin, sports can also be referred to as somaclonal variants. In azalea, the appearance of somaclonal variants depends on the intensity of propagation of the parent plant (De Schepper *et al.* 2001a). The frequency of sporting increases in old varieties and after tissue culture (Samyn *et al.* 2002). However, some cultivars never sport.

Our objective is to unravel the molecular background of the sporting phenomenon (De Schepper 2001). The origin of sports, however, is unclear. Not only somatic 'mutations' (Horn 1968, Bauer 1993), but also 'transposition of mobile genetic elements' (Bino *et al.* 1984, Doodeman and Bianchi 1985, Phillips *et al.* 1994) as well as 'epigenetic' switches (Grunewaldt and Duneman 1991, Coccilione and Cone 1993, Kaeppler *et al.* 2000) have been put forward as possible mechanisms behind sport phenotypes.

The qualitative and quantitative determination of flavonoids in *Rhododendron simsii* flowers was investigated by De Cooman *et al.* (1993) and De Loose (1969, 1979). Azalea flower colour sports are characterised by variegated corollas. Variegation types in general are categorised as either cell lineage vs non-cell lineage and patterned vs non-patterned (Marcotrigiano 1997). The azalea sport collection harbours three different sport types (Figure 1) (De Schepper 2001, De Schepper *et al.* 2001a).

In the first category shoots sport to a different but uniform flower colour, e.g. the switch from carmine red to brick red (Figure 1A) and no flower colour variegation can be observed. The second type of sports are characterised by a

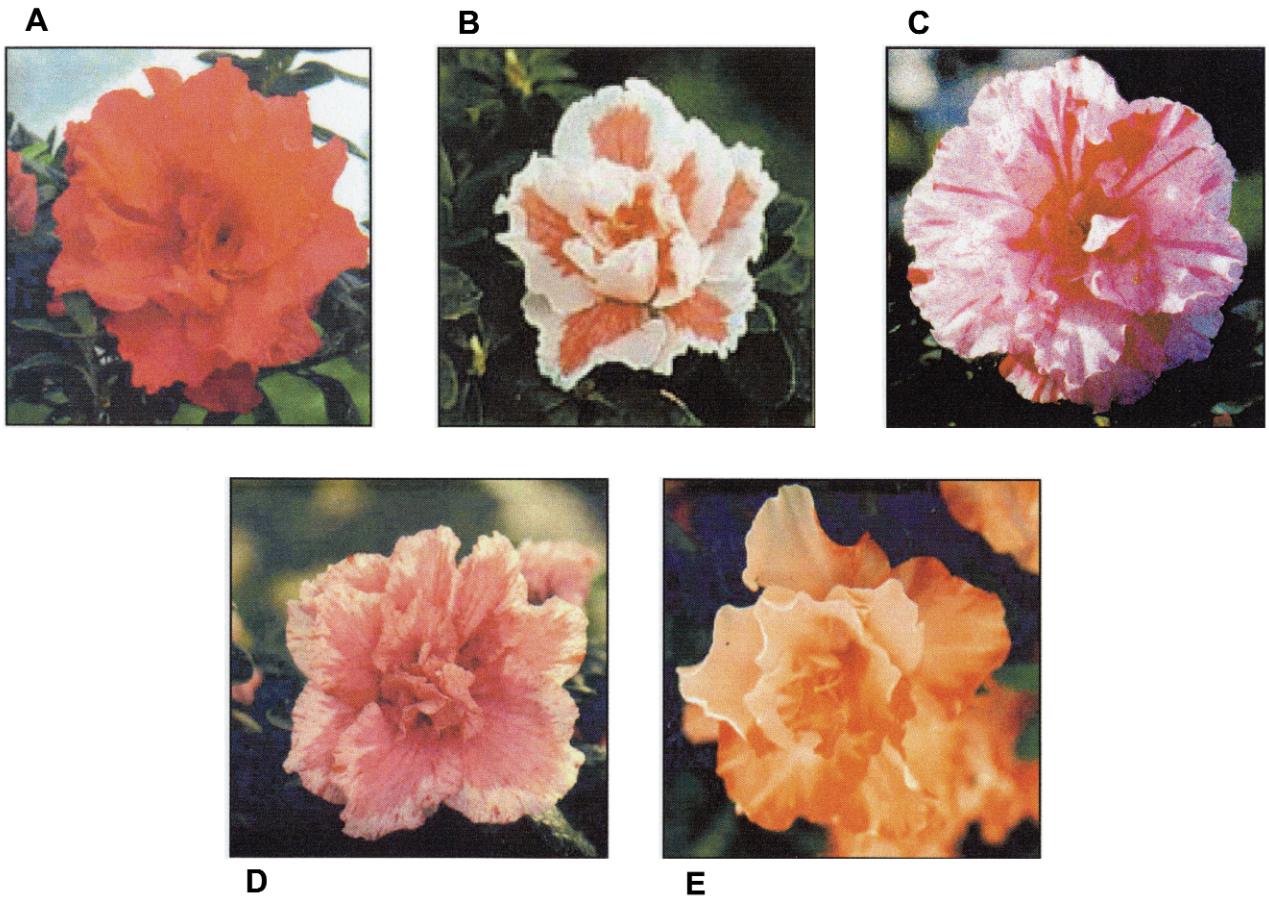

Figure 1: Azalea flower colour sport phenotypes: (A) uniform sport, (B) picotee sport, (C) flecked sport, (D) complex phenotype: superimposition of flecked and picotee coloration pattern, (E) complex phenotype: sectored picotee coloration

bicoloured corolla (Figure 1B). This phenotype has been termed 'picotee' or 'Cossack dancer' (Jorgensen *et al.* 1996, Marcotrigiano 1997). The key descriptive term for this patterned, non-cell lineage, or non-cell autonomous, variegation is petal border or margin. The third azalea sport phenotype shows very irregular flower coloration (Figure 1C). Petals are marked by spots, stripes, flecks or sectors of one colour on the background of another. Upon careful inspection these markings can be seen to follow cell lineage (Martin and Gerats 1993).

According to the variegation category, the molecular mechanism(s) behind the sport phenotype is more likely situated at a genetic or at an epigenetic level (Figure 2). The 'genetic' components of our model are flavonoid structural and regulatory genes, mutation, chimerism, polyploidy and transposition. Regulation of gene expression at the 'epigenetic' level comprises phenomena such as DNA methylation (DNA- as well as RNA-directed), chromatin remodelling and RNA silencing. Aberrant nucleotide structures such as direct and inverted repeat structures (DR, IR), aberrant RNA (aRNA), double-stranded RNA (dsRNA), micro RNAs (miRNAs), mediate interactions between the genetic and epigenetic phenomena. Arrows in Figure 2, connecting those components, point to the potential (mutual) relationships between these factors, which will be discussed in the final part of this review.

Genetic origins of sport induction

Mutations

Before considering 'genetic' origins of sport induction, the heritability of sport phenotypes must be assessed. The general rule for the inheritance of pigments in azalea is that the presence of anthocyanins and flavonols is dominant to their absence (Heursel and Horn 1977, Heursel 1999). The most intense colour is purple, which is dominant over carmine red, red, pink and white. However, this rule does not apply to sports.

The general rule is that the colour and/or variegation of a sport are not transmittable to progeny. Sports behave in crosses as having the genotype of the parent plant. This implies that the 'sporting event' does not affect their generative tissues (De Schepper 2001). The genetic information of the germinal LII layer of sports remains identical, 'mutation-free'. Because picotee sports never yield a picotee offspring a 'picotee-sporting gene' does not exist (De Schepper 2001). Picotee patterns in azalea sports always originate somatically, and are lost after hybridisation. This is in con-

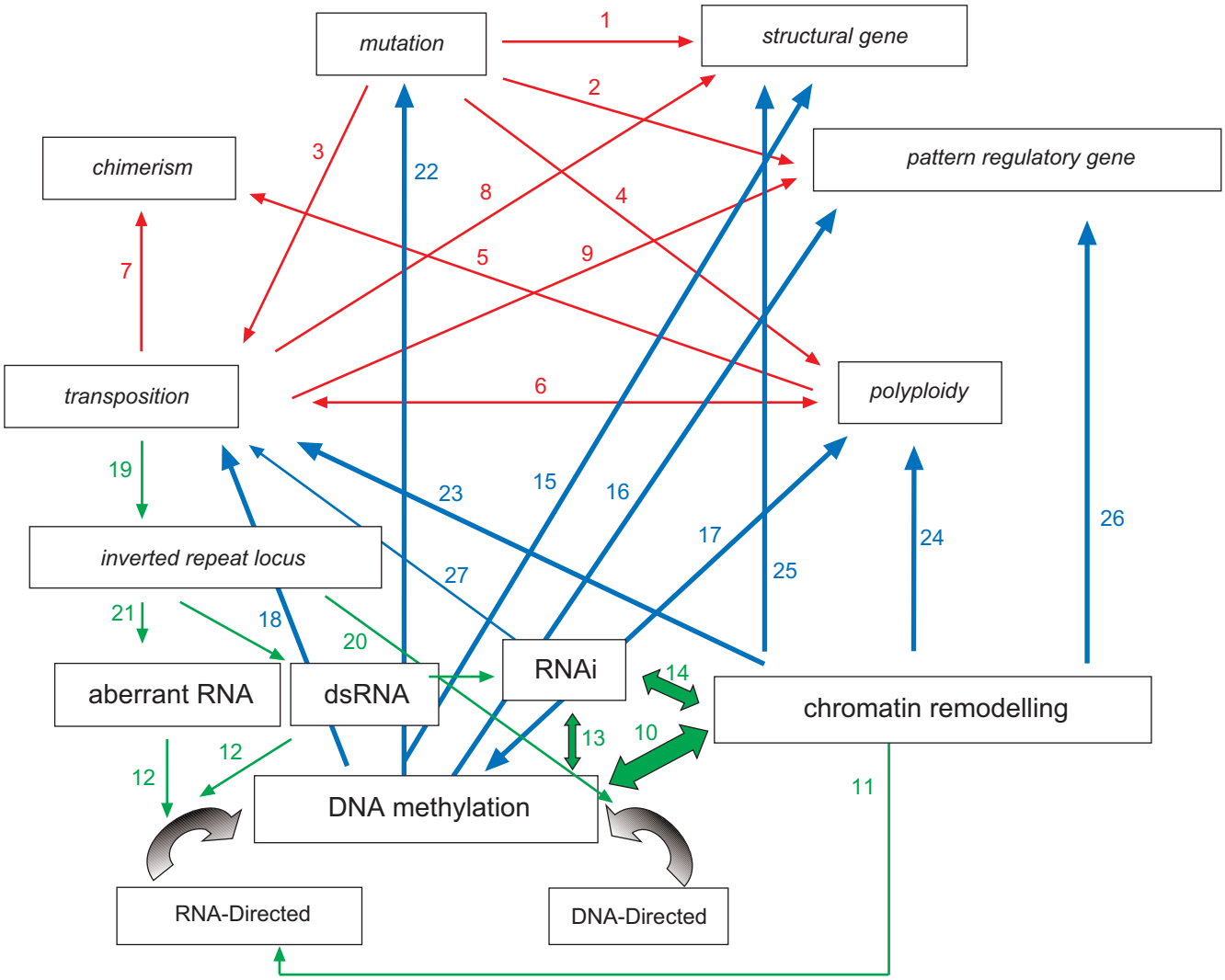

Figure 2: Theoretical model comprising genetic (in italics) and epigenetic phenomena potentially playing a role in somaclonal variation. Numbered arrows point to the (mutual) relationships, which exist both within and between the genetic and epigenetic mechanism level (see text)

trast with species expressing 'pattern genes' such as described for snapdragon (Coen *et al.* 1988, Martin and Gerats 1993).

There are a few exceptions to the general rule. The first is the completely white sport, which passes on the genetic information for lack of anthocyanins. The second exception is the flecked sport. The majority of their progeny is flecked as well.

However, while for most sport types 'germinal' mutations are ruled out, 'somatic' mutations remain a possible mechanism underlying sport induction. Although mitotically stable, the altered DNA sequence is lost after (meiotic) gamete formation. We do not rule out the possibility that some sports indeed arose through somatic mutation, although a genetic AFLP profile of the 'Hellmut Vogel' sport family did not reveal any DNA polymorphisms (De Riek *et al.* 1999). This result indicates that no gross structural DNA rearrangements occurred. However, small changes, such as point mutations

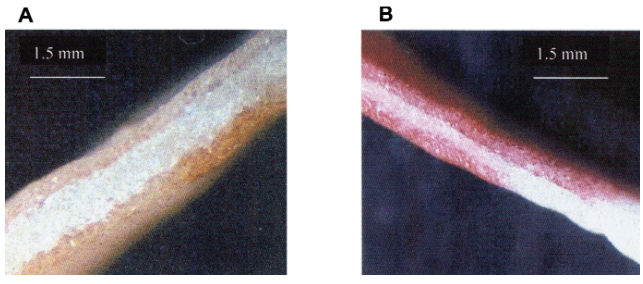

Figure 3: Microscopic analysis of the distribution of flavonoid pigments in azalea petals of the 'Hellmut Vogel' sports 'Hector' (A) and 'Marcella' (B). **A**: Predominant epidermal location of anthocyanins. **B**: Transition from red to white occurs in the epidermal layer in white-edged picotee sports

or frameshifts, can remain undetected in this type of experiment. As sports differ from their parental cultivar in only one specific trait, we could imagine that a subtle change affecting one target locus might be sufficient to cause the phenotypic switch. Although somatic mutations are often involved in somaclonal variation (Kaeppler *et al.* 1998), the frequency of sporting to novel types as well as the reversion frequency cannot be ascribed solely to these events.

Flecked azalea sports and the presence of transposable elements

The somatic instability in flower colouration is a hallmark of flavonoid genes fallen victim to transposable elements (TEs) (Nevers *et al.* 1986). Barbara McClintock (1947) inspired by the concept of 'unstable' or 'mutable' genes and 'ever-sporting' varieties (De Vries 1905), discovered transposable elements more than 60 years ago in maize. Since then a wealth of data has accumulated on the variety and distribution of TEs in both animal and plant populations. The unpredictable, somatic unstable but heritable nature of flecked sports concurs well with transposable element behaviour.

Molecular analyses were carried out to investigate the presence of TEs in azalea (De Keukeleire 2000, De Schepper 2001). As no previous sequence information was available, a homology-based isolation strategy was chosen. Based on sequence similarities, several superfamilies of evolutionary related transposable elements can be defined (Capy *et al.* 1996). One such superfamily is the *hAT*-group, named after its first isolated members: *hobo* of *Drosophila melanogaster* (fruit fly), *Ac* of *Zea mays* (maize), and *Tam3* of *Antirrhinum majus* (snapdragon) (Atkinson *et al.* 1993). With representatives presently identified in over 30 species, including fungi, algae, flies, moths, nematodes, fish, mammals, monocots and dicots, the *hAT* superfamily appears to be widely distributed in all eukaryotic kingdoms (for reviews, see Kempken and Windhofer 2001, Rubin *et al.* 2001). Because of the universal presence of those *hAT*-group-members, homology-based isolation of *hAT*-group-related sequences in azalea was attempted, using a polymerase chain reaction (PCR) approach (De Keukeleire 2000).

Using degenerate primers, two major amplification products could be identified in all the sports analysed of a *Rhododendron simsii* hybrid (De Schepper 2001). Three different sequences were cloned from this PCR mixture. The amino acid sequences were 32% identical to the homologous region of the *Z. mays Ac* transposase, and 21% identical to the *A. majus Tam3* transposase. This level of identity is comparable to the level (25%) that exists between the *Ac* and *Tam3* elements in this region (De Keukeleire 2000). These findings indicate that the forward/reverse primer pair amplifies *hAT*-group-related, transposase-like sequences from genomic DNA of the *R. simsii* hybrids. However, in contrast to, for instance, transposons-trapping strategies, the method provides no information on their functionality. To associate the somatic instability in flower coloration in azalea with the presence of the *hAT*-like elements, the location and organisation of possible elements need to be determined. In future iPCR and anchored-PCR approaches will be set up to thoroughly characterise the isolated fragments and their surrounding sequences.

Picotee variegation: periclinal chimerism versus topographic determination

Variegation patterns of picotee flowers have been attributed to periclinal chimerism in different cases (Tilney-Bassett 1963, 1986). Chimerism in plants leads to the formation of specific types of genetic mosaics, genotypically altered in a specific histological layer. When the epidermal layer loses its ability to synthesise pigment, petal margins, comprising only lower- and upper-epidermis, become white (or palely pigmented). These periclinal chimeras never yield variegated offspring, a feature that concurs well with the azalea picotee behaviour. However, when investigating microscopically the distribution of flavonoid pigments in azalea petals, we did not recognise the white epidermis over a coloured core (De Schepper *et al.* 2001b). First of all, pigments are located predominantly in the epidermal layer (Figure 3A). Second, picotee petal margins comprise several cell layers. Finally, the epidermal layer becomes pigmented at the transition from white to red (Figure 3B). Picotee coloration therefore seems to be positionally determined: petal margin vs mid-region. An argument that supports the non-periclinal chimeric nature of picotee sports is that their regeneration does not lead to a strict chimeral separation (Mertens *et al.* 1996).

Picotee variegation: somatic polyploidy and topographic determination

The importance of a positional determinant in picotee phenotypes was confirmed when we tackled the sport induction phenomenon from a more cytological angle (De Schepper *et al.* 2001b, 2001c)

Aside from the benefit of the obtained colour range, we investigated whether ploidy changes within sport series are not another valuable asset available for breeding purposes. Flow cytometric analysis of leaf tissue proved that the entire 'Hellmut Vogel' family is diploid (De Schepper *et al.* 2001c). However, when petals, the tissue where the sporting event is phenotypically expressed, were analysed, surprising results were obtained. Picotee petals exhibiting a broad petal margin (width >7mm) appeared to be tetraploid in this margin, while diploid in the remainder of the flower tissue. Neither flower variegation, nor polyploidy were periclinal chimeric in origin (De Schepper *et al.* 2001c). Furthermore, the transition diploid/tetraploid corresponded exactly with the crossing of the colour boundary. This again demonstrates the importance of topographic determination in picotee phenotypes: positional information clearly distinguishes petal marginal versus central cells; both polyploidisation and coloration is non-cell autonomously determined. Importantly, this somatic polyploidy is only observed in broad-edged picotees. Narrow-edged sports, which comprise the majority of the picotees in our breeding collection, are completely diploid. Molecular and cytological research is momentarily conducted in our lab to investigate the mechanism of this topographically determined polyploidisation.

Epigenetic origins of somaclonal variation

Epigenetic regulation of gene expression: DNA methylation, chromatin remodelling and RNA silencing

None of the aforementioned 'genetic' mechanisms can fully

account for the very frequent, mitotically more or less stable (although reversion occurs), but meiotically non-heritable sporting event. These remarkable features, however, are marked characteristics of 'epigenetic' mechanisms (Holliday 1990). Epigenetic phenomena can add another level of variation in expression, distinct from the alteration of the actual gene sequence, thereby contributing to the phenotypic diversity (Holliday 1990). The non-chimeric, non-cell lineage variegation observed in azalea picotees has been reported as the most common form of variegation and can be attributed in many cases to differences in gene expression (epigenetic regulation) that are positionally rather than genotypically dependent (Marcotrigiano and Stewart 1984, Marcotrigiano 1997).

Epigenetic aspects of somaclonal variation have been reviewed by Kaeppler *et al.* (2000). DNA methylation, chromatin remodelling and RNA silencing are global mechanisms that contribute to the regulation of gene expression (Finnegan *et al.* 2000, Morel *et al.* 2000, Habu *et al.* 2001, Plasterk 2002). They have been reported to be involved in tissue- and stage-specific gene expression, (trans)gene silencing, and paramutation. Epigenetic regulation of gene expression can occur at the transcriptional or at the post-transcriptional level (De Picker and Van Montagu 1997).

In eukaryotes the methylation of cytosines at position five is the most common post-replicative DNA modification (Jost and Saluz 1993). The widespread occurrence of cytosine methylation in higher organisms indicates that this form of DNA modification is ancestral. However, the biological significance of DNA methylation remains elusive although numerous reports have demonstrated the involvement of cytosine methylation in different biological processes. Two major roles are proposed for DNA methylation: 1) the regulation of natural developmental processes (Richards 1997, Colot and Rossignol 1999), 2) the inactivation of invasive sequences, which can be subdivided in the protection against parasitic mobile elements and viruses, and the epigenetic silencing of (trans)genes (Flavell 1994, Matzke *et al.* 2000). The importance of DNA methylation in normal plant development (Richards 1997) has been demonstrated by the serious developmental abnormalities associated with a general diminishing of methylation (Finnegan *et al.* 1996, Ronemus *et al.* 1996). Normal development requires stable epigenetic repression of genes not required in specific cell types (Wolffe and Matzke 1999). A large body of evidence has accumulated in recent years that for animal, viral and plant genes there is an inverse correlation between DNA methylation and the expression of genes (Razin and Riggs 1980, Doerfler 1983, Paszkowski and Whitham 2001, Attwood *et al.* 2002).

DNA methylation is rare or absent — probably lost — in several fungal and animal cell lineages that include *Saccharomyces ceverisiae*, *Drosophila melanogaster* and *Caenorhabditis elegans*. In these organisms epigenetic control of gene expression is attributed to chromatin configuration (Wolffe and Guschin 2000, Jenuwein and Allis 2001, Meyer 2001). The 'epigenome' has been defined as the collection of biochemical modifications to chromatin, such as acetylation, phosphorylation, poly(ADP-ribosylation), ubiquitination and methylation of the amino-terminal tails of histones, that indexes genetic information (Wolffe and Hayes 1999, Jenuwein and Allis 2001, Rice and Allis 2001, Jenuwein 2002). The pleiotropic effects of blocking histone deacetylation on gene regulation and development illustrate the essential roles played by histone acetylation and deacetylation in eukaryotic gene regulation (Tian and Chen 2001). The neutralisation of positive lysine residues in the histone tails of nucleosomes through acetylation decondenses the chromatin configuration and renders promoter regions accessible to the transcriptional machinery. Alternatively, heterochromatin, the inactive condensed form of chromatin, is characterised by high-density methylation of the histone H3 protein at amino acid lysine 9 (H3–K9) (Rea *et al.* 2000, Nakayama *et al.* 2001, Noma *et al.* 2001). Chromatin is also remodelled by ATP-dependent assemblies typified by the yeast SWI/SNF complex, which is targeted to promoters via direct interactions with transcription activators and alters nucleosome structure (Peterson and Workman 2000, Sudarsanam and Winston 2000). Although recent studies on plant gene regulation prove the importance of chromatin remodelling (Chen and Pikaard 1997, Habu *et al.* 2001, Tian and Chen 2001), research on the histone code is more advanced in the vertebrate kingdom because the (universal) presence of methylation in plants (Finnegan *et al.* 1998) is easier to monitor than changes in chromatin configuration (Matzke *et al.* 2000, Meyer 2001).

Aside from the mechanisms of DNA methylation and chromatin remodelling, an RNA silencing mechanism has emerged in recent years, that is conserved among species from different kingdoms (fungi, worms, flies, mammals and plants) (Fire 1999, Bass 2000, Cogoni and Macino 2000, Carthew 2001, Hammond *et al.* 2001, Sharp 2001, Plasterk 2002). RNA interference (RNAi), also termed quelling in fungi and co-suppression or Post-Transcriptional Gene Silencing (PTGS) in plants, is the process by which double stranded RNA (dsRNA) silences gene expression. The mechanism by which dsRNA silences gene activity is not completely understood although it has been discovered to involve cleavage of longer dsRNA molecules and the corresponding mRNA into short interfering RNAs (siRNAs) by an ATP-dependent ribonuclease called 'Dicer' (Bernstein *et al.* 2001, Nykänen *et al.* 2001). These siRNAs are then transferred to a second enzyme complex, the RNA-Induced Silencing Complex (RISC), which contains an endoribonuclease that is distinct from Dicer (Hammond *et al.* 2000, 2001, Nykänen *et al.* 2001). This endoribonuclease uses the sequence encoded by the antisense siRNA strand to find and destroy mRNAs of complementary sequence (Zamore 2002). The siRNAs thus act as guide and sequence specificity is guaranteed. RNAi is believed to act as the 'immune system' of the genome (Plasterk 2002).

Epigenetic regulation of azalea pigment gene expression

Our search for epigenetic aspects of sport induction required the possession of azalea pigment gene sequences (De Schepper *et al.* 2001a). We focussed on the flavonoid *chalcone synthase* (*chs*) and *dihydroflavonol 4'-reductase* (*dfr*) genes, based on the numerous reports concerning epigenetic silencing (and transposition) of these loci (Mol *et al.* 1983, Coen *et al.* 1986, Nevers *et al.* 1986, Napoli *et al.*

1990, Bollmann *et al.* 1991). Both azalea cDNA clones were isolated through heterologous cDNA screening (De Schepper *et al.* 2001a).

Our first approach to find evidence for epigenetic gene regulation was to visualise expression of the genes in different sport phenotypes. After the identification of the optimal flower bud stage for the detection of expression, both cDNA clones were probed against RNA from different sports of the 'Hellmut Vogel' group (De Schepper 2001). Expression of both genes was correlated with pigment intensity. With the exception of high (colourless-) flavonol-related *chs* expression in the white sport, the quantitative colour differences between the sports were correlated with differences in mRNA levels of the two structural genes. This co-ordination of expression indicates that, at least a part or maybe the entire flavonoid pathway is under control of a common regulatory mechanism. There are many reports describing regulatory genes activating several or all the flavonoid biosynthetic genes. It is possible that in azalea sports, such regulation exists in different epigenetic states, which lead to varying degrees of gene (de)activation and subsequently result in different shades and pigment intensities, i.e. the gradual colour differences observed between sport flower colours. A dynamic regulatory mechanism and metastable gene expression states have been argued by Jorgensen (1995) to account for the diversity and variability of petunia pigment co-suppression patterns. Instead of the strict 'on' versus 'off' situation, multiple stable states of activator concentration lead to corresponding states of transcript concentration. Isolation and characterisation of other structural flavonoid genes and regulatory loci in azalea sports should further substantiate this assumption.

In the specific case of picotee sport induction we observed a morphological determination: petal margin versus petal mid-region. These features of petal morphology are believed to reflect underlying pre-patterns of gene transcription (Jorgensen 1995, Vaucheret *et al.* 1998). Many silencing events are related to threshold induced transcript turnover. If transcription reaches a threshold concentration, RNA molecules are degraded. This could explain the white-edged azalea picotees. Due to a gradient in expression, a transcription factor increasingly activates a biosynthetic gene, leading to its suppression towards the petal margin. In cells located at the boundary from red to white petal tissue, the threshold is reached. Depending on the start situation, the reverse coloration pattern could be established. In these petals transcript or transcription factor levels are perhaps not abundant enough at the petal base, but increase toward the petal margins.

To search for epigenetic silencing in picotee sports, we analysed *chs* expression in differently coloured sectors of picotee petals, because of the reported *chs* PTGS in petunia picotees (Mol *et al.* 1983, Napoli *et al.* 1990, Jorgensen *et al.* 1996). In two azalea sports analysed, however, we did not find evidence for absolute (100%) silencing of this flavonoid gene in the white areas (De Schepper 2001). The high colourless flavonol content in the 'Hellmut Vogel' sports requires high *chs* expression. Therefore, the loss of pigment (anthocyanins) in the petal margin must be due to a block in flavonoid synthesis at a later, maybe anthocyanin-specific step of the pathway.

As a second approach to identify epigenetic regulation in azalea bud sports we chose to analyse the methylation state of different sports of the 'Hellmut Vogel' family (De Schepper 2001). In general, techniques to analyse DNA methylation can be divided into two categories. One leads to sequence-unspecific results and quantifies the global amount of modified bases in an organism; the second allows the specific location of methylated sites within a target sequence. Our first priority was to search for evidence of epigenetic differences between sports. Hence, we chose to compare global levels of DNA methylation of different 'Hellmut Vogel' sports with their parental cultivar. The technique used is called the '*SssI-methylase*-accepting assay' (*SssI*-MAA) (Schmitt *et al.* 1997, Jaligot *et al.* 2000), a reverse dosage method involving the *in vitro*-saturation of all unmethylated CpG-sites with tritium labelled methyl residues using *SssI methylase*. Through the measurement of incorporated methyl groups, the proportion of sites that is methylated or unmethylated is quantified in comparison with a reference sample. The parent plant 'Hellmut Vogel' was chosen as standard, and genomic leaf and flower DNA of 18 of its sports was used as a template. Leaves are not the organs where the sporting phenomenon is phenotypically expressed. However, regeneration of azalea from leaf explants enhances and fastens the formation of sports (Mertens *et al.* 1996, Samyn *et al.* 2002), indicating that all the information required to sport (the 'sporting capacity signal'?) is present in leaves.

The equation used for the calculation of the Normalised Methylation Index (NMI) is

$$NMI = (Q_{METH}{}^{sample}/[DNA]^{sample})/(Q_{METH}{}^{standard}/[DNA]^{standard})$$

where Q_{METH} represents the amount of [^{3}H]-methyl groups incorporated in sample or in standard DNA and [DNA] the corresponding concentration of DNA. According to this formula, a NMI less than one corresponds to an increase of CG methylation for the sample relative to the standard.

The results obtained from the analysis of leaf tissue reveal a tendency towards hypermethylation of the colour sports compared to their parent plant: only four of the 18 sports analysed appeared to be hypomethylated (De Schepper 2001). When measuring methylation in flower DNA, the majority of the sports again appeared hypermethylated. However, some sports appeared hypermethylated in leaves whereas hypomethylated in flowers, and vice versa. An *SssI-methylase*-accepting assay proved the 'Hellmut Vogel' sports to be polymorph in their methylation state (De Schepper 2001). Global values of DNA methylation, however, are not linked with specific sport phenotypes. A direct cause-effect relationship could thus not be established because of the variable intensity and direction (hypo- and hypermethylation) of the NMI-values. This can be explained by several of the restrictions inherent to the technique (Rival A, France, pers. comm.).

To prove the connection between sport phenotypes and epigenetic DNA modification, precise target sequences should be subjected to methylation analysis (transposable elements, flavonoid genes). The meiotic instability of sport patterns suggests that methylation at non-conventional asymmetric sites is probably involved (Diéguez *et al.* 1997). Different studies have already proven the correlation

between the extent of methylation and the level of phenotypic variation (Ronchi *et al.* 1995, Jacobsen and Meyerowitz 1997, Jeddeloh and Richards 1998, Melquist *et al.* 1999, Bartee and Bender 2001). We can imagine that the differential methylation of a few sites in a limited number of loci, leads to the above mentioned multiple stable states and results in the subtle, quantitative colour differences between some sports. Similarly, differential methylation of genes responsible for pigment synthesis in defined areas of the petal could lead to the appearance of variegation patterns. These subtle methylation changes will go unnoticed in the global approach described here.

Relations between the genetic and epigenetic aspects of somaclonal variation: a working model

The genetic or epigenetic cause of certain sport types is very straightforward, e.g. true flecked sports are clearly affected by transposition in a flavonoid gene. The classification of sports into sport categories (i.e. uniform, flecked and picotee) is, however, not always so evident (Figure 1D–E). Picotee and flecked patterns can be superimposed in one flower (Figure 1D) and picotee coloration sometimes occurs sectored (Figure 1E). In these cases, there is not just one mechanism responsible for the coloration pattern. We have therefore created a model theoretically linking both genetic and epigenetic components, some already reported to be involved spontaneous in somaclonal variation (Kaeppler *et al.* 2000), others more belonging to the field of transgenetics (Figure 2).

Interconnecting 'genetic' mechanisms

First of all, besides affecting structural (Figure 2: 1) and regulatory genes (Figure 2: 2) of the pathway, mutations can alter transposon sequences rendering them 'immobile' (Figure 2: 3). Secondly, one can imagine that a 'mutation' specifically affecting cell cycle genes, could lead to (somatic) polyploidy (Figure 2: 4). In particular cases the outcome is the formation of a cytochimera (Satina *et al.* 1940), explaining the connection between polyploidy and chimerism (Figure 2: 5). Polyploidy in turn has been associated with buffering genomes leading to an enhanced tolerance to parasitic mobile elements (Vanderwiel *et al.* 1993, Wessler 1998, Matzke *et al.* 2000), providing a link between polyploidy and transposition (Figure 2: 6).

Any genetic change in one or more cells in a multicellular shoot meristem can result in the generation of a chimera. One possible mechanism inducing such a genetic alteration is the activity of transposable elements (Marcotrigiano 1997). When a somatic transposition event occurs early in development, it may give rise to a branch in which a whole layer of the meristem is mutant, i.e. a periclinal chimera (Figure 2: 7). Examples of chimeras, which arose by transposition, have been described in snapdragon, morning glory, petunia and maize (Imai 1934, Doodeman and Bianchi 1985, Carpenter and Coen 1995).

A question that arises is whether TEs, apart from causing 'flecked', non-patterned phenotypes, also can be responsible for a 'patterned' form of colour variegation? Plant TEs are well known for their ability to cause chromosomal rearrangements, including deletions, inversions and duplications. In snapdragon, the *nivea* gene codes for the structural flavonoid gene *chs*. Insertion of *Tam3* in the *chs* gene gives rise to 'flecked' flowers (Figure 2: 8) (Coen *et al.* 1986). However, excision of *Tam3* in one particular inversion allele, *nivrec:531*, placed new sequences (derived from the inversion) upstream of the *niv* TATA-box and coding region (Lister *et al.* 1993). This resulted in a novel distribution of anthocyanin pigment in the flower tube, caused by the interaction of new sequences with the remnant of the *niv* promoter. This novel pattern of expression, responding to flower polarity, appeared to be under the control of the gene *cyclodea radialis*, which is required for bilateral symmetry of the flower.

Not only structural flavonoid genes can be affected by transposition. One example concerns the regulatory, 'pattern' locus *R* from maize (Figure 2: 9). Also, in this specific case the rearrangement of sequences resulted in the acquisition of new expression patterns. The net effect was a novel distribution of anthocyanin pigments in the maize aleurone (May and Dellaporta 1998). These examples illustrate the power of transposons to mediate rearrangements of genes (structural as well as 'pattern' genes) and confer new splicing and expression patterns. This has led to a diversity of new, 'patterned', pigmentation forms.

Interconnecting 'epigenetic' mechanisms

The identification of mutants in the *DDM1* (*DNA DEMETHYLATION 1*) gene of *Arabidopsis* (Jeddeloh *et al.* 1999, Kakutani *et al.* 1999) provided a first direct link between DNA methylation and chromatin configuration (Figure 2: 10). The gene encodes a plant homologue of the SWI2/SNF2 protein, a component of chromatin remodelling complexes. Another recently isolated plant homeodomain (PHD)-like protein, ATRX, provides a similar connection with the methylation machinery (Gibbons *et al.* 2000). Another link is provided by the fact that heterochromatic H3-K9 histone methylation directs DNA methylation in *Arabidopsis thaliana* (Jackson *et al.* 2002) (Figure 2: 10). These findings suggest that proteins involved in changing chromatin configuration are required for the correct levels and allocation of methylation (Paszkowski and Whitham 2001).

On the other hand, DNA methylation is reported to play a role in the pattern of chromatin modification (Ng and Bird 1999) (Figure 2: double arrow 10). MeCP2 is a chromosomal protein that binds specifically to methylated DNA both *in vitro* and *in vivo*, via its methyl-CpG binding domain (Nan *et al.* 1998). In addition, MeCP2 can 'invade' chromatin in a methylation-dependent manner. Its recent discovered interaction with the Sin3/histone deacetylase co-repressor complex provides a mechanistic explanation for the relationship between both processes (Ng and Bird 1999). Not only methyl binding proteins such as MeCP2, MBD2 and MBD3, but also the DNA methyltransferase *Dnmt 1* is observed to associate with histone acetylase, again connecting DNA methylation and chromatin modifications (Dobosy and Selker 2001, Urnov and Wolffe 2001, Wade 2001).

Another link lies in the discovery of a set of *Arabidopsis* genes encoding a plant-specific group of methyltransferases that contain a chromodomain motif, the chromomethylases (Henikoff and Comai 1998). The recent finding that chro-

modomains bind to RNA (Akhtar *et al.* 2000) presents the interesting idea that chromomethylases are involved in RNA-Directed DNA methylation (RdDM) (Figure 2: 11) (Habu *et al.* 2001). The interacting RNA species in RdDM could be either dsRNAs or siRNAs derived from the initiator of silencing (Wassenegger 2000) (Figure 2: 12). Because of this relationship, it is apparent that there are connections between RNA silencing and DNA methylation, and RNA silencing and chromatin remodelling (Figure 2: 13–14).

RNAi has been proposed to involve chromatin because RNAi-like phenomena require proteins predicted to interact with chromatin- a SWI2/SNF2 component, a DNA methyltransferase, and a DNA helicase-like protein (Fagard *et al.* 2000, Morel *et al.* 2000, Wu-Scharf *et al.* 2000, Dalmay *et al.* 2001, Dudley *et al.* 2002) — as well as proteins predicted to interact with RNA (Fagard *et al.* 2000, Mourrain *et al.* 2000). Volpe *et al.* (2002) also discovered a mechanistic link between RNAi and chromatin configuration in *Schizosaccharomyces pombe*. By deleting part of the RNAi machinery they proved that it is necessary for the initiation, but not the maintenance of heterochromatin formation (Volpe *et al.* 2002). In plants, RNAi is thought to guide DNA methylation, which then represses transcription (Hamilton and Baulcombe 1999, Jones *et al.* 1999, Dalmay *et al.* 2000, Mette *et al.* 2000, Matzke *et al.* 2001). Other researchers proved that in plants spreading of RNA silencing is accompanied by DNA methylation (Vaistij *et al.* 2002).

Connecting 'genetic' and 'epigenetic' mechanisms

Despite the association between the advent of transgene technology and the discovery of epigenetic phenomena, it is unlikely that silencing mechanisms did only materialise through the introduction of T-DNA constructs (Matzke *et al.* 2000, Urnov and Wolffe 2001). The unifying concept is that epigenetic control mechanisms have arisen through the recruitment of the genome defence system that targets invasive sequences. It is the discovery of DNA methylation and its dual role in normal gene regulation (Figure 2: 15–16) and genome defence that provided a first link between 'genetic' and 'epigenetic' events. DNA methylation is involved in the heritability, i.e. 'epigenetic inheritance' (Holliday 1990), and flexibility of epigenetic states.

Studies concerning the distribution of DNA methylation in eukaryotic genomes demonstrate that repeats are prime targets for methylation in many organisms (Yoder *et al.* 1997). Examples of inter-species variation in the level of cytosine methylation, e.g. *Arabidopsis* (4.6%) versus maize and rye (33%), are correlated with differences in repeat content (Bennetzen *et al.* 1994). This particular feature led to the proposal that a primary role of DNA methylation might be to reduce background transcriptional noise. The three different targets for this repression of expression are polyploid genomes (Figure 2: 17), repetitive DNA sequences (Figure 2: 18) and transgenes.

Considering our interest in somaclonal variation, let us first consider endogenous, naturally occurring events. During evolution, eukaryotic genomes expanded in size, both through polyploidisation and the accumulation of repetitive DNA sequences (TEs and remnants of TEs) (Matzke *et al.* 2000).

First of all, homology-based inactivation of duplicated loci could have played a role in the genetic diploidisation of polyploid genomes, contributing to their successful establishment during plant and vertebrate evolution (Gastony 1991, Bird 1995, Galitski *et al.* 1999, Matzke *et al.* 1999, Wolffe and Matzke 1999). Considering the above, DNA methylation provides the link between polyploidy and 'epigenetic' silencing (Figure 2: 17) (Mittelsten-Scheid *et al.* 1996). This connection can be applied to the broad-edged picotees displaying somatic polyploidy. A change of ploidy is known to influence gene expression (Mittelsten-Scheid *et al.* 1996, Galitski *et al.* 1999, Matzke *et al.* 1999), and this might provide the link between epigenetic regulation of pigment genes and the somatic polyploidy. Threshold induced transcript turnover might lead to the suppression of pigment gene expression in the tetraploid petal margin. On the other hand, the gene dosage effect could be operating in the true sense of the concept in dark-edged picotees: transcript levels are not abundant enough at the diploid level, but by doubling of the genome, pigment production becomes visible. Whether colour and ploidy are cause-effect related or determined independently by some kind of superimposed spatial signal remains to be elucidated. Secondly, taking the increasing threat accumulating TEs exert on the plant genome into consideration, the natural plant defence mechanism must have assumed an increasingly prominent role. Yoder *et al.* (1997) have even proposed that DNA methylation is restricted almost entirely to TEs. The primary role of DNA methylation might thus be to prevent transposition (Yoder *et al.* 1997), which would otherwise be expected to 'lacerate' the genome. A large number of studies implicate DNA methylation in the suppression of transposition (Figure 2: 18) (Schwartz and Dennis 1986, Yoder *et al.* 1997, Martienssen 1998, Miura *et al.* 2001).

Finally, the prominent role of DNA methylation in the field of silencing in transgenetics (TGS as well as PTGS) (Jacobsen 1999, Paszkowski and Whitham 2001), provides the third connection between genome defence and 'epigenetic' regulation: transgenes become 'epigenetically silenced' because they activate a genome defence system that is normally triggered by TEs and other invasive sequences (endogenous 'genetic' components) (Matzke *et al.* 2000).

The proposed widespread role of transposons in provoking epigenetic events extends from the legacy of McClintock (1947) who discovered transposable elements, and detailed their epigenetic behaviour in plants. TEs have been proven to be implicated in different types of epigenetic silencing. An example is the potential role of TEs in paramutation, an important type of endogenous silencing (Patterson *et al.* 1995, Martienssen 1996, Matzke *et al.* 1996).

Transposition, generating Inverted Repeats (IRs) (Figure 2: 19) has been proposed to be involved in epigenetic silencing of the *nivea* and *PAI* loci (Bollmann *et al.* 1991, Jacobsen 1999, Luff *et al.* 1999, Muskens *et al.* 2000). IRs are prime targets for *de-novo* methylation, methylation occurring via DNA hairpin structures (Figure 2: 20) or mediated by aberrant or dsRNA transcripts derived from IR structures (Wassenegger 2000) (Figure 2: 12, 18 and 21). The important role of RNA in directing or mediating DNA methylation in

TGS as well as PTGS is the subject of recent studies and reviews (Wassenegger 2000, Paszkowski and Whitham 2001, Matzke *et al.* 2001, Zamore 2002).

Besides the 'epigenetic' regulation, DNA methylation can induce direct 'genetic' changes. Despite the existence of an active DNA repair pathway that corrects G/T-mismatches, arising through the spontaneous hydrolytic deamination of 5-methylcytosine back to G/C, the sites of cytosine methylation remain significantly mutagenic (Figure 2: 22) (Wiebauer *et al.* 1993). Moreover, genome-wide demethylation has been reported to lead to elevated mutation rates and is proposed to be a step in carcinogenesis (Chen *et al.* 1998).

Because of the importance of the role of DNA methylation in plants as an endogenous gene regulatory mechanism, the focus of this final part of the review is mostly on the DNA methylation machinery. However, relations exist between DNA methylation, chromatin remodelling and RNA silencing and the role of the latter two processes is as prominent in defending the genome and in regulating gene expression (Figure 2: 23). Whether the non-cell autonomous gene expression in picotee sports is due to the action of the DNA methylation machinery, chromatin-remodelling systems, RNA silencing, or to a (sequential) co-occurrence of these mechanisms, remains to be elucidated.

Chromatin remodelling has been suggested to be a cause of cytological ploidy aberrations in somaclonal variants obtained through tissue culture (Figure 2: 24) (Kaeppler *et al.* 2000) and, as for DNA methylation, to establish 'heritable' imprints to epigenetically regulate gene expression (Figure 2: 25–26) (Meyer 2001, Selinger and Chandler 2001). Enhanced TE mobilisation in RNAi deficient mutants has also been reported (Ketting *et al.* 1999, Allshire 2002) (Figure 2: 27). Furthermore, eukaryotic heterochromatin is characterised by a high density of repeats and transposons (Volpe *et al.* 2002). It is proven that H3–K9 is conserved in plant heterochromatin and is preferentially associated with transposable elements (Gendrel *et al.* 2002). These TEs can regulate neighbouring genes by conferring the same oppressed epigenetic expression state and it has been proposed that many of the properties of heterochromatin stem from these elements (Martienssen and Colot 2001) (Figure 2: 23).

Sport induction, and somaclonal variation in general, is a complex event, in which many different phenomena can play a role. Differences in sports' phenotypes and behaviour convinced us of a diverse mechanistic background. This phenomenon needs to be approached in a multidisciplinary fashion. Because of the visible complexity of this form of somaclonal variation, we aimed at making an inventory of genetic and epigenetic mechanisms that regulate gene expression, not only restricted to somaclonal variation but in the domain of virus-induced and transgene silencing as well.

In the future the well-characterised flavonoid pathway will continue to prove an elegant and powerful tool in unravelling these molecular interactions. Understanding the sporting event in molecular terms might in turn lead to the identification of 'appropriate' varieties (e.g. high or low TE-copy number lines) required for breeding purposes.

Acknowledgements — SDS is supported by grants from the Flemish Fund for Scientific Research (FWO) and the DWTC/IUAP.

References

Akhtar A, Zink D, Becker PB (2000) Chromodomain proteins are protein-RNA interaction modules. Nature **407**: 405–409

Allshire R (2002) RNAi and heterochromatin — a hushed-up affair. Science **297**: 1818–1818

Atkinson PW, Warren WD, O'Brochta DA (1993) The hobo transposable element of *Drosophila* can be cross-mobilized in houseflies and excises like the Ac element of maize. Proceedings of the National Academy of Sciences USA **90**: 9693–9697

Attwood JT, Yung RL, Richardson BC (2002) DNA methylation and the regulation of gene transcription. Cellular and Molecular Life Sciences **59**: 241–257

Bartee L, Bender J (2001) Two *Arabidopsis* methylation-deficiency mutations confer only partial effects on a methylated endogenous gene family. Nucleic Acids Research **29**: 2127–2134

Bass BL (2000) Double-stranded RNA as a template for gene silencing. Cell **101**: 235–238

Bauer H (1993) Identification of 'flower-sports' of pelargoniums and roses by phenolic compounds. Gartenbauwissenschaft **58**: 82–89

Bennetzen JL, Schrick K, Springer PS, Brown WE, SanMiguel P (1994) Active maize genes are unmodified and flanked by diverse classes of modified, highly repetitive DNA. Genome **37**: 565–576

Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature **409**: 363–366

Bino RJ, Bianchi F, Wijsman HJW (1984) Periclinal chimerism in *Petunia* demonstrated by regeneration of plants from the mutated epidermal layer. Heredity **52**: 437–441

Bird AP (1995) Gene number, noise reduction and biological complexity. Trends in Genetics **11**: 94–100

Bollmann J, Carpenter R, Coen ES (1991) Allelic interactions at the nivea locus of *Antirrhinum*. The Plant Cell **3**: 1327–1336

Capy P, Vitalis R, Lagin T, Higuet D, Bazin C (1996) Relationships between transposable elements based upon the integrase-transposase domains: is there a common ancestor? Journal of Molecular Evolution **42**: 359–368

Carpenter R, Coen ES (1995) Transposon induced chimeras show that floricaula, a meristem identity gene, acts non-autonomously between cell layers. Development **121**: 19–26

Carthew (2001) Gene silencing by double-stranded RNA. Current Opinion in Cell Biology **13**: 244–248

Chamberlain DF, Rae SJ (1990) A revision of *Rhododendron* IV subgenus *Tsutsusi*. Edinburgh Journal of Botany **47**: 89–200

Chen RZ, Pettersson U, Beard C, Jackson-Grusby L, Jaenisch R (1998) DNA hypomethylation leads to elevated mutation rates. Nature **395**: 89–93

Chen ZJ, Pikaard CS (1997) Epigenetic silencing of RNA polymerase I transcription: a role for DNA methylation and histone modification in nucleolar dominance. Genes and Development **11**: 2124–2136

Coccilione SM, Cone KC (1993) Pl-Bh, an anthocyanin regulatory gene of maize that leads to variegated pigmentation. Genetics **135**: 575–588

Coen ES, Almeida J, Robbins TP, Hudson A, Carpenter R (1988) Molecular analysis of genes determining spatial patterns in *Antirrhinum majus*. In: Verma DPS, Goldberg RB (eds) Temporal and Spatial Regulation of Plant Genes. Springer-Verlag, Wien, pp 63–78

Coen ES, Carpenter R, Martin C (1986) Transposable elements generate novel patterns of gene expression in *Antirrhinum majus*. Cell **47**: 285–296

Cogoni C, Macino G (2000) Post-transcriptional gene silencing

across kingdoms. Current Opinion in Genetics and Development **10**: 638–643
Colot V, Rossignol JL (1999) Eukaryotic DNA methylation as an evolutionary device. BioEssays **21**: 402–411
Cullen J (1991) The logic of *Rhododendron* classification. Royal Horticultural Society London **44**: 14–19
Dalmay T, Hamilton A, Meuller E, Baulcombe DC (2000) Potato virus X amplicons in *Arabidopsis* mediate genetic and epigenetic gene silencing. Plant Cell **12**: 369–379
Dalmay T, Horsefield R, Braunstein TH, Baulcombe DC (2001) SDE3 encodes an RNA helicase required for posttranscriptional gene silencing in *Arabidopsis*. EMBO Journal **20**: 2069–2078
De Cooman L, Everaert ESW, Faché P, Van de Casteele K, Van Sumere CF (1993) Flavonoid biosynthesis in petals of *Rhododendron simsii*. Phytochemistry **33**: 1419–1426
De Keukeleire P (2000) Transposable Elements in *Petunia hybrida*. PhD Thesis, Ghent University, Belgium, 135pp
De Loose R (1969) The flower pigments of the Belgian hybrids of *Rhododendron simsii* and other species and varieties from *Rhododendron* subseries *obtusum*. Phytochemistry **8**: 253–259
De Loose R (1979) Characterisation of *Rhododendron simsii* Planch. cultivars by flavonoid and isozyme markers. Scientia Horticulturae **11**: 175–182
De Picker A, Van Montagu M (1997) Post-transcriptional gene silencing in plants. Current Opinion in Cell Biology **9**: 373–382
De Riek J, Dendauw J, Mertens M, De Loose M, Heursel J, Van Bockstaele E (1999) Validation of criteria for the selection of AFLP markers to assess the genetic variation of a breeders' collection of evergreen azaleas. Theoretical and Applied Genetics **99**: 1155–1165
De Schepper S (2001) Molecular Analysis of the Induction of Sports in Azalea (*Rhododendron simsii* hybrids). PhD Thesis, Ghent University, Belgium, 173pp
De Schepper S, Debergh P, Van Bockstaele E, De Loose M (2001a) Molecular analysis of sport induction in azalea (*Rhododendron simsii* hybrids). Acta Horticulturae **552**: 143–149
De Schepper S, Leus L, Mertens M, Debergh P, Van Bockstaele E, De Loose M (2001b) Somatic polyploidy and its consequences for flower coloration and flower morphology in azalea. Plant Cell Reports **20**: 583–590
De Schepper S, Leus L, Mertens M, Heursel J, Debergh P, Van Bockstaele E, De Loose M (2001c) Flow cytometric analysis of ploidy in *Rhododendron* subgenus *Tsutsusi*. HortScience **36**: 125–127
De Vries H (1905) Species and Varieties: Their Origin by Mutation. Open Court Publishing, Chicago
Diégeuz MJ, Bellotto M, Afsar K, Mittelsten-Scheid O, Paszkowski J (1997) Methylation of cytosines in nonconventional methylation acceptor sites can contribute to reduced gene expression. Molecular Genetics and Genomics **253**: 581–588
Dobosy JR, Selker EU (2001) Emerging connections between DNA methylation and histone acetylation. Cellular and Molecular Life Sciences **58**: 721–727
Doerfler W (1983) DNA methylation and gene activity. Annual Reviews in Biochemistry **52**: 93–124
Doodeman M, Bianchi F (1985) Genetic analysis of the instability in *Petunia* hybrids. 3. Periclinal chimeras resulting from frequent mutations of unstable alleles. Theoretical and Applied Genetics **69**: 297–304
Dudley NR, Labbé JC, Goldstein B (2002) Using RNA interference to identify genes required for RNA interference. Proceedings National Academy of Sciences USA **99**: 4191–4196
Fagard M, Boutet S, Morel JB, Bellini C, Vaucheret H (2000) AGO1, QDE-2, and RDE-1 are related proteins required for post-transcriptional gene silencing in plants, quelling in fungi, and RNA interference in animals. Proceedings National Academy of Sciences USA **97**: 11650–11654
Finnegan EJ, Genger RK, Peacock WJ, Dennis ES (1998) DNA methylation in plants. Annual Review of Plant Physiology and Plant Molecular Biology **49**: 223–247
Finnegan EJ, Peacock WJ, Dennis ES (1996) Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. Proceedings of the National Academy of Sciences USA **93**: 8449–8454
Finnegan EJ, Peacock WJ, Dennis ES (2000) DNA methylation a key regulator of plant development and other processes. Current Opinion in Genetics and Development **10**: 217–223
Fire A (1999) RNA-triggered gene silencing. Trends in Genetics **15**: 358–363
Flavell RB (1994) Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proceedings of the National Academy of Sciences USA **91**: 3490–3496
Galitski T, Saldanha AJ, Style, CA, Lander ES, Fink GR (1999) Ploidy regulation of gene expression. Science **28**: 251–254
Gastony GJ (1991) Gene silencing in a polyploid homosporous fern: paleopolyploidy revised. Proceedings of the National Academy of Sciences USA **88**: 1602–1605
Gendrel AV, Lippman Z, Yordan C, Colot V, Martienssen RA (2002) Dependence of heterochromatic histone H3 methylation patterns on the *Arabidopsis* gene DDM1. Science **297**: 1871–1873
Gibbons RJ, McDowell TL, Raman S, O'Rourke DM, Garrick D, Ayyub H, Higgs DR (2000) Mutations in ATRX, encoding a SWI/SNF-like protein, cause diverse changes in the pattern of DNA methylation. Nature Genetics **24**: 368–371
Grunewaldt J, Duneman F (1991) Variation and selection *in vitro*. Gartenbauwissenschaft **56**: 1–5
Habu Y, Kakutani T, Paszkowski J (2001) Epigenetic developmental mechanisms in plants: molecules and targets of plant epigenetic regulation. Current Opinion in Genetics and Development **11**: 215–220
Hamilton AJ, Baulcombe DC (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286**: 950–952
Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature **404**: 293–296
Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ (2001) Argonaute2, a link between genetic and biochemical analyses of RNAi. Nature **293**: 1146–1150
Henikoff S, Comai L (1998) A DNA methyltransferase homologue with a chromodomain exists in multiple polymorphic forms in *Arabidopsis*. Genetics **149**: 307–318
Heursel J (1999) Azalea's Oorsprong, Veredeling en Cultivars. Lannoo-Terra, Tielt, Belgium, 194pp
Heursel J, Horn W (1977) A hypothesis on the inheritance of flower colours and flavonoids in *Rhododendron simsii* Planch. Zeitschrift für Pflanzenzücht **79**: 238–249
Holliday R (1990) Mechanisms for the control of gene activity during development. Biological Reviews **65**: 431–471
Horn W (1968) Genetische Ursachen der Variation bei Zierpflanzen. Gartenbauwissenschaft **33**: 317–333
Imai Y (1934) On the mutable genes Pharbitis, with special reference to their bearing on the mechanism of bud-variation. Journal of the College of Agriculture Imperial University Tokyo **12**: 479–523
Jackson JP, Lindroth AM, Cao X, Jacobsen SE (2002) Control of CpNpG DNA methylation by the KRYPTONITE histone H3 methyltransferase. Nature **416**: 556–560
Jacobsen SE (1999) Gene silencing: maintaining methylation patterns. Current Biology **9**: 617–619
Jacobsen SE, Meyerowitz EM (1997) Hypermethylated Superman epigenetic alleles in *Arabidopsis*. Science **277**: 1100–1103

Jaligot E, Rival A, Beulé T, Dussert S, Verdeil JL (2000) Somaclonal variation in oil palm (*Elaeis guineensis* Jacq.): the DNA methylation hypothesis. Plant Cell Reports **19**: 684–690

Jeddeloh JA, Richards EJ (1998) ^{m}CCG methylation in angiosperms. Plant Journal **9**: 579–586

Jeddeloh JA, Stokes TL, Richards EJ (1999) Maintenance of genomic methylation requires a SWI2/SNF2-like protein. Nature Genetics **22**: 94–97

Jenuwein T (2002) An RNA-guided pathway for the epigenome. Science **297**: 2215–2218

Jenuwein T, Allis CD (2001) Translating the histone code. Science **293**: 1074–1080

Jones L, Hamilton AJ, Voinnet O, Thomas CL, Maule AJ, Baulcombe DC (1999) RNA-DNA interactions and DNA methylation in post-transcriptional gene silencing. The Plant Cell **11**: 2291–2301

Jorgensen RA (1995) Cosuppression, flower colour patterns and metastable gene expression states. Science **268**: 686–691

Jorgensen RA, Cluster PD, English J, Que Q, Napoli CA (1996) Chalcone synthase cosuppression phenotypes in petunia flowers: comparison of sense vs. antisense constructs and single copy vs. complex T-DNA sequences. Plant Molecular Biology **31**: 957–973

Jost JP, Saluz HP (eds) (1993) DNA Methylation: Molecular Biology and Biological Significance, Birkhäuser Verlag, Basel, 572pp

Kaeppler SM, Kaeppler HF, Rhee Y (2000) Epigenetic aspects of somaclonal variation in plants. Plant Molecular Biology **43**: 179–188

Kaeppler SM, Phillips RL, Olhoft P (1998) Molecular basis of heritable tissue culture-induced variation in plants. In: Jain SM, Brar DS Ahloowalia BS (eds) Somaclonal Variation and Induced Mutations in Crop Improvement. Kluwer Academic Publishers, Dordrecht, pp 465–484

Kakutani T, Munakata K, Richards EJ, Hirochika H (1999) Meiotically and mitotically stable inheritance of DNA hypomethylation induced by ddm1 mutation of *Arabidopsis thaliana*. Genetics **151**: 831–838

Kempken F, Windhofer F (2001) The *hAT* family: a versatile transposon group common to plants, fungi, animals and man. Chromosoma Focus **110**: 1–9

Ketting RF, Haverkamp TH, Van Luenen HG, Plasterk RH (1999) mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RnaseD. Cell **99**: 133–141

Lister C, Jackson D, Martin C (1993) Transposon-induced inversion in *Anthirrinum* modifies *nivea* gene expression to give a novel flower color pattern under the control of *Cyclodea radialis*. The Plant Cell **5**: 1541–1553

Luff B, Paszkowski J, Bender J (1999) An inverted repeat triggers cytosine methylation of identical sequences in *Arabidopsis*. Molecular Cell **3**: 505–511

Marcotrigiano M (1997) Chimeras and variegation: patterns of deceit. HortScience **32**: 773–784

Marcotrigiano M, Stewart RN (1984) All variegated plants are not chimeras. Science **23**: 505

Martienssen RA (1996) Epigenetic phenomena: paramutation and gene silencing in plants. Current Biology **6**: 810–813

Martienssen RA (1998) Transposons, DNA methylation and gene control. Trends in Genetics **14**: 12–13

Martienssen RA, Colot V (2001) DNA methylation and epigenetic inheritance in plants and filamentous fungi. Science **293**: 1070–1074

Martin C, Gerats AGM (1993) Control of pigment biosynthesis genes during petal development. The Plant Cell **5**: 1253–1264

Matzke MA, Matzke AJM, Eggleston W (1996) Trans-gene silencing and paramutation: a common response to invasive DNA? Trends in Plant Sciences **1**: 382–388

Matzke MA, Matzke AJM, Pruss G, Vance VB (2001) RNA-based silencing in plants. Current Opinion in Genetics and Development **11**: 221–227

Matzke MA, Mette MF, Matzke AJM (2000) Transgenic silencing by the host genome defense: implications for the evolution of epigenetic control mechanisms in plants and vertebrates. Plant Molecular Biology **43**: 401–415

Matzke MA, Mittelsten-Scheid O, Matzke AJM (1999) Rapid structural and epigenetic changes in polyploid and aneuploid genomes. BioEssays **21**: 761–767

May BP, Dellaporta SL (1998) Transposon sequences drive tissue-specific expression of the maize regulatory gene R-s. Plant Journal **13**: 241–247

McClintock B (1947) Cytogenetic studies of maize and *Neurospora*. Carnegie Institute Washington Yearbook **46**: 146–152

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

Mertens M, Werbrouck S, Samyn G, Botelho dos Santhos Moreira de Silva H, Debergh P (1996) *In vitro* regeneration of evergreen azalea from leaves. Plant Cell Tissue Organ Culture **45**: 231–236

Mette MF, Aufsatz W, Van der Winden J, Matzke MA, Marzke AJ (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. EMBO Journal **19**: 5194–5201

Meyer P (2001) Chromatin remodelling. Current Opinion in Plant Biology **4**: 457–462

Mittelsten-Scheid O, Jakovleva L, Afsar K, Maluszynska J, Paszkowski J (1996) A change of ploidy can modify epigenetic silencing. Proceedings of the National Academy of Sciences USA **93**: 7114–7119

Miura A, Yonebayashi S, Watanabe K, Toyama T, Shimada H, Kakutani T (2001) Mobilization of transposons by a mutation abolishing full DNA methylation in *Arabidopsis*. Nature **411**: 212–214

Mol JNM, Schram AW, De Vlaming P, Gerats AGM, Kreuzaler F, Hahlbrock K, Reif HJ, Veltkamp E (1983) Regulation of flavonoid gene expression in *Petunia hybrida*: description and partial characterisation of a conditional mutant in chalcone synthase gene expression. Molecular Genomics and Genetics **129**: 424–429

Morel JB, Mourrain P, Béclin C, Vaucheret H (2000) DNA methylation and chromatin structure affect transcriptional and post-transcriptional transgene silencing in *Arabidopsis*. Current Biology **10**: 1591–1594

Mourrain P, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Jouette D, Lacombe AM, Nikic S, Picault N, Remoue K, Sanial M, Vo TA, Vaucheret H (2000) *Arabidopsis* SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance Cell **101**: 533–542

Muskens MWM, Vissers APA, Mol JNM, Kooter JM (2000) Role of inverted DNA repeats in transcriptional and post-transcriptional gene silencing. Plant Molecular Biology **43**: 243–260

Nakayama J, Rice JC, Strahl BD, Allis CD, Grewal SIS (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. Science **292**: 110–113

Nan X, Ng HH, Johnson CA, Laherty CD, Turner BM, Eisenman RN, Bird AP (1998) Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. Nature **393**: 386–389

Napoli CA, Lemieux C, Jorgensen RA (1990) Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. The Plant Cell **2**: 279–289

Nevers P, Shepherd NS, Saedler H (1986) Plant transposable elements. Advances in Botanical Research **12**: 103–203

Ng H, Bird AP (1999) DNA methylation and chromatin configuration. Current Opinion in Genetics and Development **9**: 158–163

Noma K, Allis CD, Grewal SIS (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. Science **293**: 1150–1155

Nykänen A, Haley B, Zamore PD (2001) ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell **107**: 309–321

Paszkowski J, Whitham SA (2001) Gene silencing and DNA methylation processes. Current Opinion in Plant Biology **4**: 123–129

Patterson GI, Kubo KM, Shroyer T, Chandler VL (1995) Sequences required for paramutation of the maize b gene to a region containing the promoter and upstream sequences. Genetics **141**: 1389–1406

Peterson CL, Workman JL (2000) Promoter targeting and chromatin remodelling by the SWI/SNF complex. Current Opinion in Genetics and Development **10**: 187–192

Phillips RL, Kaeppler SM, Olhof P (1994) Genetic instability of plant tissue cultures: breakdown of normal controls. Proceedings of the National Academy of Sciences USA **91**: 5222–5226

Plasterk RHA (2002) RNA silencing: The genome's immune system. Science **296**: 1263–1269

Razin A, Riggs AD (1980) DNA methylation and gene function. Science **210**: 604–610

Rea S, Eisenhaber F, O'Carroll N, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases Nature **406**: 593–599

Rice JC, Allis CD (2001) Histone methylation versus histone acetylation: new insights into epigenetic regulation. Current Opinion in Cell Biology **13**: 263–273

Richards EJ (1997) DNA methylation and plant development. Trends in Genetics **13**: 319–322

Ronchi A, Petroni K, Tonelli C (1995) The reduced expression of endogenous duplications (REED) in the maize R gene family is mediated by DNA methylation. EMBO Journal **14**: 5318–5328

Ronemus MJ, Galbiati M, Ticknor C, Chen J, Dellaporta SL (1996) Demethylation-induced developmental pleiotropy in *Arabidopsis*. Science **273**: 654–657

Rubin E, Lithwick G, Levy AA (2001) Structure and evolution of the *hAT* transposon superfamily. Genetics **158**: 949–957

Samyn G, De Schepper S, Van Bockstaele E (2002) Regeneration of explants of several azalea (*Rhododendron simsii*) cultivars in view of the appearance of sports. Plant Cell Tissue Organ Culture **70**: 223–227

Satina S, Blakeslee AF, Avery AG (1940) Demonstrations of the three germ layers in the shoot apex of *Datura* by means of induced polyploidy in periclinal chimeras. American Journal of Botany **27**: 895–905

Schmitt F, Oakeley E, Jost JP (1997) Antibiotics induced genome wide hypermethylation in cultured *Nicotiana tabacum* plants. Journal Biological Chemistry **272**: 1534–1540

Schwartz D, Dennis ES (1986) Transposase activity of the Ac controlling element in maize is regulated by its degree of methylation. Molecular Genomics and Genetics **205**: 476–482

Selinger DA, Chandler VL (2001) B-Bolivia, an allele of the maize b1 gene with variable expression, contains a high copy retrotransposon-related sequence immediately upstream. Plant Physiology **125**: 1363–1379

Sharp PA (2001) RNA interference. Genes and development **15**: 485–490

Sudarsanam P, Winston F (2000) The SWI/SNF family of nucleosomes-remodelling complexes and transcriptional control. Trends in Genetics **16**: 345–351

Tian L, Chen ZJ (2001) Blocking histone deacetylation in *Arabidopsis* induces pleiotropic effects on plant gene regulation and development. Proceedings of the National Academy of Sciences USA **2**: 200–205

Tilney-Bassett RA (1963) The structure of periclinal chimeras. Heredity **18**: 265–285

Tilney-Bassett RA (1986) Plant Chimeras. Edward Arnold Publishers, London. 199pp

Urnov FD, Wolffe AP (2001) Above and within the genome: Epigenetics past and present. Journal Mammary Gland Biology Neoplasia **6**: 153–167

Vaistij FE, Jones L, Baulcombe DC (2002) Spreading of RNA targeting and DNA methylation in RNA silencing requires transcription of the target gene and a putative RNA-dependent RNA polymerase. The Plant Cell **14**: 857–867

Vanderwiel P, Voytas DF, Wendel JF (1993) Copia-like retrotransposable element evolution in diploid and polyploid cotton (Gossypium L). Journal of Molecular Evolution **36**: 429–447

Vaucheret H, Béclin C, Elmayan T, Feuerbach F, Godon C, Morel JB, Mourrain P, Palauqui JC, Vernhettes S (1998) Transgene-induced gene silencing in plants. Plant Journal **16**: 651–659

Volpe TA, Kidner C, Hall IM, Teng G, Grewal SIS, Martienssen RA (2002) Regulation of heterochromatic silencing and histone H3 Lysine-9 methylation by RNAi. Science **297**: 1833–1837

Wade PA (2001) Methyl CpG binding proteins: coupling chromatin architecture to gene regulation. Oncogene **20**: 3166–3173

Wassenegger M (2000) RNA-directed DNA methylation. Plant Molecular Biology **43**: 203–220

Wessler SR (1998) Transposable elements associated with normal plant genes. Physiologia Plantarum **103**: 581–586

Wiebauer K, Nedderman P, Hughes M, Jiricny J (1993) The repair of 5-methylcytosine-deamination damage. In: Jost JP, Saluz P (eds) DNA Methylation: Molecular Biology and Biological Significance, Birkhäuser Verlag, Basel, pp 510–568

Wolffe AP, Guschin D (2000) Review: chromatin structural features and targets that regulate transcription. Journal of Structural Biology **129**: 102–122

Wolffe AP, Hayes JJ (1999) Chromatin disruption and modification. Nucleic Acids Research **27**: 711–720

Wolffe AP, Matzke MA (1999) Epigenetics: regulation through repression. Science **286**: 481–486

Wu-Scharf D, Jeong B, Zhang C, Cerutti H (2000) Transgene and transposon silencing in *Chlamydomonas reinhardtii* by a DEAH-Box RNA helicase. Science **290**: 1159–1162

Yoder JA, Walsh CP, Bestor TH (1997) Cytosine methylation and the ecology of intragenomic parasites. Trends in Genetics **13**: 335–340

Zamore PD (2002) Ancient pathways programmed by small RNAs. Science **296**: 1265–1269

Edited by J van Staden and CH Bornman