2

Epigenetics and Plant Breeding

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Abbreviations

5-AzaC	5-azacytidine
5mC	5 methyl cytosine
mRNAs	messenger RNAs
MTases	Methyltransferases
8-oxoG	8-hydroxyguanisine
CHS	chalcone synthase
CLF	curly leaf
CMT	chromomethylase (chromomelthyltransferase)
CMV	cucumber mosaic virus
CTR	constitutive triple response
DCL	Dicer-like protein
DME	Demeter
DnMT	DNA methyl transferase
DRM	domain-rearranged methylase (methyltransferase)
dsDNA	double-stranded DNA
dsRNA	double-stranded RNA
EIS	epigenetic information systems
Esc	extra sex combs
E(z)	enhancer of Zeste
FBP26	flowering binding protein 26
FIE	fertilization-independent endosperm
FIS	fertilization-independent seed
FLC	Flowering Locus C
GMPs	genetically modified plants
GUS	b-glucuronidase
H3-K9	histone 3-lysine 9
HATs	histone acetyltransferases
HcPro	Hc-protease
HPLC	high-performance liquid chromatography
HTH	hothead
KYP	kryptonite
MARs	matrix attachment regions

MBPs	methylation binding proteins
MEA	MEDEA
MET1	methyltransferase1
mRNA	messenger RNA
MSI1	multicopy suppressor of ira
MT or Mtase	methyltransferase
PcG	polycomb group
PDR	pathogen-derived resistance
PFG	petunia flowering gene
PHE	pheres
PSTVd	potato spindle tuber viroid
PTGS	post transcriptional gene silencing
Rboh	respiratory burst oxidase homolog
RdDM	RNA-directed DNA methylation
RdRP	RNA-dependent RNA polymerase
RE	repeated element
RISC	RNA-induced silencing complex
RNAi	RNA interference
ROS	repressor of silencing *or* reactive oxygen species
S-AdoMet	S adenosylmethionine
siRNAs	small interfering RNAs
sRNAs	small RNAs
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
Su(z)12	suppressor of Zeste
TE	transposable element
TGS	franscriptional gene silencing
VRN	vernalization
VIGS	virus-induced gene silencing
VIN3	Vernalization-insensitive 3

I. Introduction

The concept of epigenesis has its roots in ancient Greece, where it was first proposed by Aristotle to differentiate from preformation, a theory favored by other philosophers, such as Democritus and Leucippus, in order to explain the development of a new organism. According to preformation, in the beginning there was an individual of each species of animal or plant that contained, within it, as nested miniatures, all the other individuals of the species that would ever live. In epigenesis, there were not such preformed miniatures and new structures arose

progressively by a process that metaphorically was linked by Aristotle to the ''knitting of a net.'' Preformation was the prevailing theory for millennia until the invention of the microscope and the discovery that living organisms, including embryos, are composed of cells. Development was then recognized as being epigenetic since during this process, division of the egg generates many new cells that differentiate to form different tissues. Later, in the middle of the twentieth century, Waddington (1942) developed the term *epigenetics,* a derivative of Aristotle's *epigenesis,* aiming to synthesize preformation and epigenesis into a single theory by combining genetics—the study of the hereditary material (preexisting complexity) found in the zygote—with developmental biology—the study of changes undergone by the zygote (epigenesis).

Holliday (1994), describing in molecular terms DNA methylation as an epigenetic mechanism, defined epigenetics as the study of the changes in gene expression, which occur in organisms with differentiated cells, and the mitotic inheritance of given patterns of gene expression. Following the proof that epigenetic mechanisms, leading to changes of gene functions, are not only mitotically stable during development but are also meiotically heritable led to the definition of epigenetics as the study of mitotically and meiotically heritable changes in gene expression that do not entail a change in DNA sequence (Wu and Morris 2001). As mentioned by Zilberman and Henikoff (2005), the major reason for changing views of epigenetics is that the original concept was meant to be a broad theoretical framework to guide studies in an area in which not only were the mechanisms not understood but the nature of the processes was unknown. In this spirit, Timothy Bestor (Zilberman and Henikoff 2005) offered the following light-hearted description at the 1995 Gordon Research Conference on Epigenetics: molecular biology: known gene, known product; genetics: known gene, unknown product; biochemistry: unknown gene, known product; epigenetics: unknown gene, unknown product.

In recent years, both genetic and biochemical studies have greatly advanced our knowledge of epigenetic processes involving sRNA-mediated gene silencing and chromatin-based inheritance of gene activity states through DNA methylation and/or modifications of histones. What has begun to emerge under the almost mystical epigenetic umbrella is a picture of an ancient system of cellular and genomic immunity predating the divergence of plants, ciliates, animals, and fungi. Much of the epigenetic machinery of higher eukaryotes, including plants, appears to be directed at silencing viruses, TEs, and other REs, with epigenetic developmental regulation of endogenous genes being mostly derived later from such processes (Martienssen 1996; Lippman

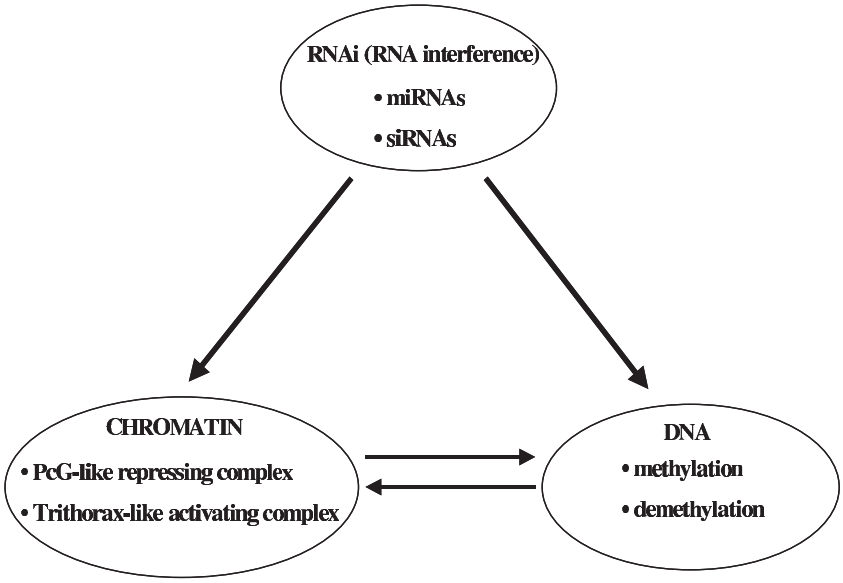

Fig. 2.1. The triangle of the three epigenetic information systems (EIS) and their interactions. DNA methylation is required for chromatin modifications and vice versa, and sRNA-based mechanisms (RNAi) regulate both.

et al. 2004). The field of epigenetics is currently enjoying a meteoric rise, after the successful sequencing of a plethora of different genomes and the turn toward functional genomics. In order to understand the role of different parts of the genome, epigenetics definitely appears to hold one of the master keys to unlock and understand these roles (Beck and Oleck 2003; Mattick 2004).

Three such epigenetic information systems (EIS)—DNA methylation, histone modification, and RNA interference—have been described so far, along with their mechanisms of action (Fig. 2.1). DNA methylation was historically the first and more extensively studied chromatin epigenetic mark, and covalent modifications of the different histones were defined as major epigenetic marks conveying epigenetic information by changing chromatin states and consequently DNA function. While cytosine methylation is the only epigenetic mark in DNA, histone modifications are numerous involving methylation, acetylation, phosphorylation, ribosylation, ubiquitation of mostly H_3 histone, but H_4, histones H_2A, H_2B, H_1 as well. This constitutes the ''histone code'' of chromatin epigenetic marks (Turner 2002). Finally, during the last few years, arguably the most important advance in biology has been the discovery that small RNA molecules can regulate the expression of genes. For years, RNAs were

thought to have just two broad functions in cells. Single-stranded mRNAs were considered vital intermediaries in gene expression, transmitting information between DNA and protein, while ribosomal and transfer RNAs were playing structural and information-decoding roles in the process of protein synthesis. This dogma is largely true for bacterial cells with their compact genome (Mattick 2004). This picture started changing after the discovery that RNAs could play different catalytic roles (Chech 1986). Furthermore, data from recent studies in different fields, including among others viral resistance, animal and plant development, and transgene silencing in plants, supported additional roles for RNA molecules. These data showed that sRNA molecules of 21 to 24 nucleotides long could interfere with the translation or the degradation of complementary mRNA molecules. Drs. Melo and Fire were honored with the 2006 Nobel Prize in physiology or medicine for their contribution in understanding the role of sRNA. In addition, sRNA molecules could lead to chromatin modifications of both DNA and histones, leading to transcriptional arrest of certain partially complementary genes. This phenomenon, termed *RNA interference* (RNAi), placed sRNAs in a central role among epigenetic mechanisms and was recognized as a third EIS. According to Mattick (2004), the current conception of how genetic information is encoded and transmitted in higher organisms will need to be reassessed. The main output of the genome of complex organisms is genetically active but non-protein coding RNA. Researchers now realize that there are numerous layers of biological information in DNA, interspersed between, or superimposed on, the passages written in the triplet code of protein coding (Pearson 2006). The three EIS are not independent from each other; rather they form a triangle of interdependent interactions. Unraveling the relationships between these epigenetic components led to rapidly evolving new concepts that reveal how, by interacting, they complement, reinforce, and stabilize the effects of each other. Histone deacetylation and other modifications, particularly the methylation of lysine 9 within histone H_3 (H_3K_9) of histone tails, cause chromatin condensation and block transcription initiation. In addition, histone modification can also attract DNA MTases to initiate 5mC formation, which in turn can reinforce histone modification patterns conducive to silencing (Fuks et al. 2003b; Tariq and Paszkowski 2004). The opposite is also true; that is, DNA methylation reinforces silencing by the attraction of histone modifications (methylation and deacetylation), leading to a more compact and thus inactive chromatin (Tamaru and Selker 2001; Tariq and Paszkowski 2004). With the recent advent of RNA interference in the epigenetic field, it became clear almost from the start that among their other roles, sRNAs also provide a common denominator of both

DNA methylation and/or histone modifications. Experiments in plants have clearly shown the involvement of sRNAs in the establishment of heterochromatic states and silencing (Wassenegger et al. 1994; Matzke et al. 2001). Disruption of one of these interacting systems can lead to expression or silencing of genes, resulting in epigenetically controlled phenotypes. Thus, their significance as new sources of epigenetically controlled variation with multiple concomitant implications in conventional plant breeding and modern genetic engineering is consolidated.

The epigenetic mechanisms and molecular machineries of their manifestation will be described in Section II. Recent studies suggest that far more numerous pathways involved in the manifestation of epigenetic phenomena and their developmental role is by far more critical than it was anticipated even a few years ago. In particular, research on the mechanisms of RNA interference proceeded at a staggering pace. It is now clear that this is part of an evolutionarily ancient mechanism of genome defense against any ''parasitic'' nucleic acid, both in the cytoplasm and in the nucleus. In parallel, RNAi became an extremely useful experimental tool not only for learning what genes do by knocking out their activity but also for generating useful genetic plant variants. The three EIS will be described briefly since extended recent reviews on each of them have been presented (Tsaftaris and Polidoros 2000; Matzke et al. 2001; Bender 2004; Bannister and Kouzarides 2005; Tsaftaris et al. 2005). The emphasis in Section II will be the integration of very recent data and the description of their interrelationships.

Section III describes epigenetic involvement in a number of biological phenomena in plants, some that were known for many years, such as paramutation, parental imprinting, and transposition of elements, amd more recently recognized ones, such as transgene silencing, viral-induced silencing, and genomic effects of different biotic and abiotic stresses. Studies of these phenomena are revealing not only for the molecular mechanisms involved but also for discovering and understanding the details of all three EIS.

The involvement of epigenetic mechanisms in controlling plant development is the subject of Section IV. It is now well established that epigenetic mechanisms are involved in the control of major developmental pathways in plants, the function of meristems, the formation of different organs, and the genomic response of plants to different environmental conditions.

Section V describes the multiple implications of EIS in conventional plant breeding and modern genetic engineering. Emphasis is given to their role and involvement as an extra source of polymorphism-generating epialles and useful variation that is inherited between generations.

The particular involvement of EIS in plant genomic responses (in addition to the known physiological responses) to different kinds of abiotic and biotic stresses, and in hybrid vigor of the modern F_1 hybrids, is described. Robustness—that is, stable yield and better exploitation of resources—distinguish modern successful F_1 hybrids and other recently released cultivars (Janick 1999; Duvick et al. 2001, 2004; Fasoula and Fasoula 2002; Tollenaar and Lee 2002; Tsaftaris et al. 2005). Understanding the molecular mechanisms behind these agronomic characteristics of elite cultivars will help to increase plant breeding efficiency in conventional breeding and assist conventional breeding with the use of molecular markers or transgenic technologies. Different aspects of the roles of EIS in modern genetic engineering are discussed. Understanding and avoiding stress-induced transgene silencing in certain transgenic cultivars when genetically modified plants (GMPs) are transferred out of the laboratory or greenhouse into the open field is one of the many reasons for serious investments in EIS research. The involvement of EIS in issues related to human health, such as stem cells and stem cell therapy (Sell 2006), cancer and other human diseases is another (see Walter and Paulsen 2003; Jiang et al. 2004; Fenech 2005). In addition, following the developments of RNAi use as an experimental tool, efforts to knock out individual genes, and mainly parallel efforts and results to generate new plant genotypes using this technology, are also presented.

This review has three main objectives. The first is to briefly review recent progress with the three EIS—particularly their interactions—and expose the agricultural community, particularly plant breeders, to this new and fast-evolving field of epigenetic research, the molecular mechanisms involved, and the methodologies used. It is now certain that many of the open questions, problems, and goals of plant agronomists and breeders—such as viral diseases, tolerance of plants to certain viruses and other pathogens, plant tolerance to abiotic stresses, somaclonal variation, polyploidy, interspecific hybridization, and robustness, homeostasis or stability of performance of conventional or genetically modified cultivars, as well as numerous plants biological phenomena such as paramutation, cycling of transposable elements (TEs), parental imprinting, transgene silencing—have an epigenetic dimension in addition to a genetic one. Understanding, for instance, transcriptional and posttranscriptional silencing is not only a matter of basic research but is also of special interest to the agrobiotech industry after the release of a GMP.

The second objective is to make familiar the problems, questions, and challenges of the agricultural and plant breeding community to plant

molecular biologists, particularly those working in molecular epigenetic mechanisms. Of paramount importance is the exploration of the possibilities offered by new knowledge and methods, particularly of modern genomic approaches (Zhang et al. 2006) for understanding these phenomena and helping to overcome the problems and challenges faced. In return, the numerous genotypes or cultivars, both domesticated and bred by many years of work by farmers and breeders, could be very useful materials for molecular epigenetic analysis, study, and understanding of EIS. In other words, this second aim is to interconnect the communities of plant molecular epigenetics and breeding.

The third objective is to uncover the role of EIS in plant domestication and plant breeding. Research on evolutionary developmental genetics (Evo-Devo) has revealed that the amazing diversity of multicellular organisms is the result of flexibility of small number of building blocks used during development, connecting evolution with development. Hence, the last objective of this review is to extend the intercalation of plant development and developmental genetic mechanisms to plant domestication and breeding research.

For some topics or subtopics of this review, there are excellent recent review articles: Matzke et al. 2001; Bender 2004; Ringrose and Paro 2004; Bannister and Kouzarides 2005; Tsaftaris et al. 2005. Journal issues include *FEBS Letters* 579(26) edited by S.-W. Ding; and books: Matzke and Matzke 2000; Engel and Antonarakis 2002; Beck and Olek 2003; Galun 2003, 2005; Hannon 2005; Jablonka and Lamb 2005. However, this is the first integrated examination of the role and significance of the different EIS and their concerted interaction in plant gene interaction and plant development and of their multiple implications in plant breeding. A glossary of terms used in this review is included in Table 2.1. Certain statements made by Eva Jablonka and Marion Lamb (2005) in ''Evolution in Four Dimensions'' reflect the philosophy of this article: ''There is more to heredity than genes. . . . Organisms have at least two systems (genetic and epigenetic) of heredity. . . . EIS play a double role, being both response systems and systems of transmission. . . . They are additional transmission technologies, transmitting interpretations of information in DNA.''

II. Molecular Epigenetic Mechanisms

A. DNA Methylation

DNA methylation is a conserved heritable epigenetic modification resulting from the enzymatic addition of a methyl moiety to DNA.

Table 2.1. Glossary of terms

ARGONAUTE (AGO). A family of evolutionarily conserved genes; their protein products are involved in various RNA interference (RNAi) processes being part of the RNA interference and silencing complex (RISC).

Chromatin remodeling. A change in chromatin structure that is achieved by the action of ATP-dependent remodeling complexes.

CpG site. A DNA site at which C (cytosine) is followed by G (guanine); p denotes the phosphate group, so that C is at the 5' position relative to G.

Dicer. The term was initially coined by Greg Hannon to describe a drosophila multidomain enzyme of the RNase III family. Drosophila Dicer cuts long dsRNA into small dsRNAs of 21 to 23 nucleotides with a 30 overhang, known as siRNAs. In plants, at least two types of detectable Dicer activity are responsible for the production of siRNAs with distinct sizes of 21 and 24 nucleotides. These plant enzymes are known as Dicer-like enzymes. Because plants have several Dicer homologs, they are sometimes referred to individually as Dicer-like-I, Dicer-like-II.

Epigenetic information systems. A mitotically and/or meiotically heritable trait that is not accompanied by a change in the DNA sequence.

Epigenetic mark. Covalent modifications of DNA or chromatin proteins that affect gene expression, which is mitotically and/or meiotically heritable.

Epimutation. A heritable change in phenotype that is not the result of an altered DNA base sequence.

Homeostasis. The maintenance of relatively steady states in an organism through internal regulatory mechanisms, despite variations in internal and external conditions.

Imprinting. An epigenetic mechanism that determines expression or repression of genes according to their parental origin.

Methyl-transferase (MT). The enzyme that catalyses the addition of methyl groups to DNA.

MicroRNA (miRNA) pathway. miRNAs are an abundant class of noncoding small RNAs (of 21 to 24 nucleotides) that are present in diverse eukaryotes and formed by Dicer or Dicer-like enzymes. In animals, most miRNAs function by repressing the translation of specific target mRNAs, but most plant miRNAs function like natural siRNAs to target specific mRNAs for cleavage. The only difference between plant and animal miRNAs is the extent of their complementarity to target mRNAs. Whereas plant miRNAs have extensive complementarity to their target mRNAs, animal miRNAs are much less homologous and, when paired to their targets, form bulges that are proposed to block the translation of the target mRNA.

miRNA precursor. An imperfect stem–loop RNA structure generated from a long transcript, known as primary miRNA, that is encoded by a nonprotein coding region in the genome. Dicer or Dicer-like enzyme cleaves a miRNA precursor and produces a mature miRNA.

Paramutation. A term coined by Brink for the process whereby one allele in a heterozygote alters the heritable properties of the other allele.

Post Transcriptional Gene Silencing (PTGS). A targeted RNA degradation mechanism in plants. PTGS may be induced by transgenes or viral infection and causes the degradation of RNAs with homology or complementarity to the transgene transcript or viral genome. The signal to degrade the specific RNA sequence is transmitted throughout the plant.

Table 2.1. (Continued)

RNA-dependent RNA polymerase (RdRP). An RNA polymerase that is involved in RNA silencing in plants, worm, Neurospora and Dictyostelium, but not in drosophila or human cells. RdRPs have been proposed to use cellular aberrant RNAs as templates and to copy them into cRNAs to form dsRNA. This newly synthesized dsRNA is thought to act as a substrate for Dicer-like enzymes.

RNA-induced silencing complex (RISC). A siRNA– or miRNA–protein complex that acts as an endonuclease and cleaves the complementary target mRNA, or as a repressor and blocks the target mRNA translation.

RNA interference (RNAi). A specific form of RNA silencing that reflects posttranscriptional RNA degradation induced by exogenous dsRNA. The term *RNAi* **is now used widely to describe RNA silencing in both plants and animals.**

RNA silencing. A group of related phenomena in diverse eukaryotes in which aberrant or dsRNA triggers a marked reduction in either transcription of the corresponding gene or direct degradation of the corresponding mRNA.

Small interfering RNA (siRNA). Small RNAs (21 to 25 nucleotides) that are produced from long dsRNA by Dicer or Dicer-like enzymes or chemically synthesized, and can be recruited by multiple cellular proteins to form an RNA-induced silencing complex (RISC) that interferes with mRNA stability or mRNA translation.

SUPERMAN. A floral homeotic gene, mutations of which affect flower development in *Arabidopsis*. **Hypermethylated SUPERMAN alleles clarkkent (clk) were used for genetic screens that led to the isolation of mutations in KRYPTONITE (KYP), CHROMOMETHYLASE3 (CMT3), and domain-rearranged methyltransferases (DRMs).**

Transcriptional Gene Silencing (TGS). Epigenetic silencing of transgenes or endogenous genes at the level of transcription.

Transgene/transgenic organism. Newly integrated DNA within an organism is a transgene, while the transformed organism is a transgenic organism.

The methyl moiety on a DNA base generally contributes to transcriptional repression by preventing activators from binding to their target or favoring the formation of inactive chromatin. In eukaryotes, DNA methylation that occurs on cytosine plays important roles in gene repression, genome organization and stability, heterochromatin formation, transgene silencing, genomic imprinting, X-chromosome inactivation, inactivation of TEs and REs, and stem or meristematic cell formation or maintenance, differentiation, and developmental control. Aberrant methylation patterns of tumor suppressor genes leading to their silence constitute a common feature of many human and animal cancers (Jiang et al. 2004). Methylation is primarily found as part of host defense systems in prokaryotes and plays a role in repair and replication. Methylation is detected in all four groups of eukaryotes, namely protists, plants, fungi, and animals, although it is not constantly detectable in every organism. In general, methylation is preferentially targeted

to heterochromatin consisting of RE including centromere-associated repeats, ribosomal RNA-encoding repeats, and TE sequences (Lippman et al. 2004). Levels of methylation vary greatly between organisms that display this modification. This can be attributed to several factors influencing 5mC content, including the RE and TE content of the genome. Thus, the overall 5mC content of a plant genome correlates with the repeated sequence content of that genome. As mentioned, the majority of methylated residues in plants are found in repetitive DNA associated with heterochromatin, but several genes in euchromatic regions have also been shown to be methylated. Although methylation at symmetric cytosines CpG and CpNpG is most common in plants, it can occur in any sequence context, particularly by RNA-directed DNA methylation (Matzke et al. 2001). Repeated HPLC measurements of different parental inbreeds and hybrids in maize show that 22 to 25% of the cytosines in the 2500 Mb maize genome are methylated (Tsaftaris et al. 1999; Tsaftaris and Polidoros 2000). Vertebrates are depleted in CpG dinucleotides and consequently show low levels of 5mC (Razin and Riggs 1980).

The distribution of methylation on DNA sequences also varies between eukaryotes. Mammals and other vertebrates display a genome–widespread pattern of methylation including coding sequences of genes (Lander et al. 2001). In contrast, invertebrates, fungi, plants, and protists appear to show primarily a fractional pattern of methylation confined to only part of the genome, and almost invariably located outside cellular genes (Bennetzen et al. 1998). The distinction of two patterns of genomic methylation between animals and plants could be the consequence of the preference of animal TEs to transpose mainly within the transcribed part of the genes, primarily inside introns, while in plants, TEs belonging to different families show a preferential transposition outside the transcribed part of genes, sometimes in their regulatory vicinity and preferentially within each other (Bestor 2003). Recently Tran et al. (2005) found a new type of DNA methylation in *Arabidopsis*, which consisted of dense CG methylation clusters found at scattered sites throughout the genome. These clusters lack non-CG methylation and are preferentially found in genes, although they are relatively deficient toward the 5' end. CG methylation clusters are present in lines derived from different accessions and in mutants that eliminate *de novo* methylation, indicating that CG methylation clusters are stably maintained at specific sites. Because 5mC is mutagenic, the appearance of CG methylation clusters over evolutionary time predicts a genome-wide deficiency of CG dinucleotides and an excess of C(A/T)G trinucleotides within transcribed regions, implying that CG methylation clusters have

contributed profoundly to plant gene evolution. The authors suggest that CG methylation clusters silence cryptic promoters that arise sporadically within transcription units. Lippman et al. (2004), using microarray analysis, also showed earlier that sporadic TE-derived tandem repeats within euchromatic areas could provide targets of DNA

methylation (through siRNA originated from the repeats, see Section III.D). They also showed that TE could regulate genes epigenetically when they are inserted within or near the promoter of a gene.

Finally, variations in methylation are observed in response to endogenous or exogenous cues. In mammals, for example: (1) a highly regulated developmental process first erases and then resets the genome-wide methylation pattern during early embryogenesis, (2) many tissue-specific genes undergo demethylation during tissue differentiation, and (3) differences in methylation exist in sperm and oocyte (Brandeis et al. 1993). Similarly in plants, tissue- and developmental stage–specific as well as stress-induced variation in DNA methylation has been recorded (Messeguer et al. 1991; Tsaftaris and Polidoros 1993; Kovačević et al. 2005). Unlike mammals, which erase and reset genomic methylation patterns early in embryogenesis, plants can inherit epigenetic changes through meiosis. ''Epiallels"—that is, phenotypic variants that are epigenetically rather than genetically different from their parents—are frequently discovered in plants and in certain cases found to be inherited to the next progeny generation (Martienssen and Colot 2001; Kakutani 2002; Tani et al. 2005). Perhaps what is revealing for the inherited stability of epialles is the *peloric* mutant (change from bilateral to radial flower symmetry), found both in *Antirrhinum* and *Linaria,* which was originally described by Linnaeus and whose phenotype remained stable for 250 years. Findings by Hoekenga et al. (2000) suggest that changes in genomic DNA methylation and local chromatin structure can also be due to developmental changes. Therefore, the vegetative phase seems to regulate in a highly specific manner the methylation changes of the maize epigenetic allele *Pl-Blotched*. Furthermore, during the juvenile- to-adult vegetative transition, the level of DNA methylation and the extent of the compact chromatin domain increase and reach their maximum in adult leaves. Similar to the vegetative phase change, the methylation changes and the modifications in chromatin structure of this locus are reset at each generation. These findings,led Hoekenga et al. (2000) to propose that the developmental regulation of *Pl-Blotched* is controlled by signals that also control vegetative phase change.

DNA methylation is catalyzed by a family of conserved DNA MTases, which points to an ancestral origin of this form of DNA modification

(see Goll and Bestor 2005 for a review). Two different types of DNA MTases activities are establishing DNA methylation patterns: (1) maintenance MTases, which maintain stable 5mC patterns through successive generations, methylating C in proximity with 5mC on the complementary strand: and (2) *de novo* MTases that are able to transfer methyl groups to C of completely unmethylated double stranded DNA. On the basis of sequence and structural similarities, four groups of DNA MTases have been recognized: Dnmt1, Pmt1/Dnmt2, Dnmt3 and CMT. The mammalian *Dnmt3*, fungal *Masc1,* and plant *DRMs* have been shown to encode for *de novo* MTases. The *Arabidopsis* genome encodes for two related *de novo* MTases genes, *DRM1* and *DMR2*. The *drm1/drm2* double mutants are blocked in TGS at some loci and completely abolish de novo methylation at CG, CNG, and asymmetric sites. Members of the mammalian Dnmt1 and plant MET1 class of enzymes serve primarily as maintenance MTases. Mutations in the *Arabidopsis MET1* gene cause a global reduction of 5mC throughout the genome and a number of developmental abnormalities. Loss of CG methylation in *met1* has also been shown to abolish the heterochromatic mark H3K9 at loci that remain transcriptionally silent (Tariq and Paszkowski 2004). The CMT class of enzymes appears to be specific to plants. Methylation profiling of mutants suggested that CMT3 preferentially methylates TE-related sequences. Interestingly many of these targets are shared between CMT3 and MET1, suggesting that CG and non-CG methylation systems might function redundantly for regulating TEs. CMT3 and DRMs also act in a partially redundant and locus-specific manner to control asymmetric and CNG methylation (Tariq and Paszkowski 2004). It was shown recently that loss-of- function mutations in *MET1* and *CMT3* lead to arabidopsis embryos with abnormal development and reduced viability (Xiao et al. 2006), pointing to the critical role of DNA methylation in proper embryogenesis.

Methylation can be removed from DNA by either passive or active mechanisms. Passive demethylation can occur when 5mC is replaced with nonmodified cytosines during DNA replication (Tsaftaris and Polidoros 2000). Apparently this is a dynamic process. As DNA replication, DNA remethylation and cell division have their own duration time. Factors speeding up or slowing down these procedures will have an effect on the capacity of MTases to complete their job in preserving DNA methylation patterns. For instance, conditions promoting fast DNA synthesis and cell division, such as plant cell cultures in vitro, plant cell division in meristems or in mammalian cancers, could affect the time and efficiency of preserving DNA methylation patterns (Klein 2005). Thus, for example, numerous somaclonal variants involving

changes in DNA methylation have been described in asexually propagated progeny plants that originated from in vitro cell cultures (see Section III.C) while significant changes in DNA methylation have been reported for many human cancers (Walter and Paulsen 2003; Jiang et al. 2004). Furthermore, cytosine MTases, described above for DNA methylation, and histone MTases required for methylating new nucleosomal histones after DNA replication both use S-AdoMet as their methyl donor. Plants, in particular, require extra amounts of this important compound since S-AdoMet serves also as methyl donor for their cell-wall lignin biosynthesis. Consequently, at a certain point of their cell cycles, cells require relatively high amounts of S-AdoMet or of its precursor methionine and by extension other molecules involved in S-AdoMet metabolism, in order to preserve in a timely fashion both DNA and histone methylation marks (Paz et al. 2002; Fenech 2003). According to Mull et al. (2006), for instance, a histone methylation-dependent DNA methylation pathway is uniquely impaired by deficiency in arabidopsis 5-adenosylhomocysteine hydrolase activity needed to metabolize the by-product of *trans*- methylation reactions. While the availability of these cofactors during plant growth, particularly under different kinds of stresses, remains to be studied, parallel studies in mammalian cancers revealed the significant role played by S-AdoMet metabolism in preserving methyl marks in both DNA and histones (Fenech 2003; Jiang et al. 2004) and as a consequence a whole new field of epinutrition, based on epigenetic-related aspects of nutrition that is emerging (Aggarwal and Shishodia 2006).

In active demethylation, the participation of specific enzymes demethylating DNA sequences is anticipated. Removal of 5mC as a free base from DNA by the enzyme 5mC-DNA glycosylase has been reported in mammals and birds (Vairapandi and Duker 1993; Jost et al. 1999). Also, the excision of nucleotides containing 5-mC from DNA has been reported (Swisher et al. 1998). Either mode of removal of 5mC from DNA would, of necessity, be followed by synthesis of a DNA repair patch containing unmethylated cytosines. A link between these mechanisms, with removal of 5mC by a DNA glycosylase, requiring an RNA cofactor that presumably guides the enzyme to the specific substrate locus, has been demonstrated in the chick embryo system (Jost et al. 1999). Until recently there was no direct in vivo evidence for active demethylation in plants. Two *Arabidopsis* mutations, the one identified by inducing TGS of transgenes and of endogenous homologous loci (Gong et al. 2002) and the other, by its interference with maternal expression of an imprinted gene (Choi et al. 2002), were mapped to genes that encode the DNA glycosylases, repressor of silencing (ROS1) and Demeter

(DME), respectively. According to their catalytic activity, DNA glycosylases can be classified into two subgroups: monofunctional DNA glycosylases that catalyze only hydrolysis of the glycosylic bond or bifunctional DNA glycosylase/lyase with associated lyase activity that cleaves the DNA backbone at the site where the base has been removed. The bifunctional DNA glycosylase/lyases belong to two broad classes, based on their reaction mechanisms. *ROS1* encodes a nuclear protein of that induces strand breaks in DNA containing 5mC, suggesting that ROS1 may be directly involved in DNA demethylation through a base excision repair mechanism. Kapoor et al. (2005) isolated the *ROS1* gene and showed that it encodes a nuclear protein with bifunctional DNA glycosylase/lyase activity against methylated but not unmethylated DNA. ROS1 participates in active DNA demethylation by a base-excision pathway, suggesting that active DNA demethylation is important in pruning the methylation patterns of the genome, and even the normally ''silent'' transposons are under dynamic control by both methylation and demethylation (Zhu et al. 2007). This dynamic control may be important in keeping the plant epigenome plastic so that it can efficiently respond to developmental and environmental cues. The genome of *Arabidopsis* encodes several other proteins belonging to family of DNA glycosylases, all of them with similar DNA repair activities to homologs found in bacteria, fungi or animals. The second glycosylase gene is *DME*. cDNAs (of this gene) from *Arabidopsis* and rice have been isolated and encode 1729 and 1952 aa proteins, respectively (Choi et al. 2002), and a cDNA of the same gene from barley has been cloned in our laboratory (Kapazoglou et al. 2004). Choi et al. (2002) found that *DME* is expressed primarily in the central cell of the female gametophyte, the progenitor of endosperm. *DME* is required for maternal allele expression of the imprinted gene *MEDEA (MEA)*, a PcG gene encoding a histone 3 lysine 27 (H_3K_{27}) methyltransferase enzyme in the central cell (described in detail in the next subtopic). Ectopic *DME* expression in endosperm activates expression of the normally silenced paternal *MEA* allele. In leaf, ectopic *DME* expression induces *MEA* and nicks the *MEA* promoter. According to Choi et al. (2002), DME activates maternal expression of imprinted genes like *MEA* in the central cell. The active MEA, in turn, controls the expression of the type I MADS-box gene *PHERES1 (PHE1)* presumably by H_3K_{27} methylation of its histones. Suppressing PHE1 ensures proper central cell and endosperm development (Köhler et al., 2003b). (see Section IV.D for more details of the interrelationships of the two epigenetic mechanisms and Section III.D for parental imprinting.) Another studied target of DME in the endosperem is the *FWA* gene (Kinoshita et al. 2004). FWA was initially

identified in *Arabidopsis* from late-flowering mutants that show ectopic *FWA* expression due to heritable hypomethylation of repeats around transcription start site. *FWA,* like *MEA,* displays maternal imprinting in the endosperm with its imprinting depending on DNA methyltransferase MET1. But as reported by Kinoshita et al. (2004), its specific maternal gametophyte activation, like the activation of *MEA*, depends on the activity of *DME* in the central cell. It is not clear how *DME* is regulated. There is a wealth of evidence that miRNAs regulate a plethora of genes involved in developmental processes. A search for putative miRNA targets along the sequence of the *Arabidopsis* DME mRNA detected six different miRNAs targets. The presence of potential miRNA targets in the barley *DME* is also being studied (Kapazoglou et al. 2004).

The first genome-wide high-resolution mapping and functional analysis of DNA methylation in arabidopsis has ben reported by Zhang et al. (2006). Their results show that pericentromeric heterochromatin, repetitive sequences, and regions producing small interfering RNAs are heavily methylated. Unexpectedly, over one-third of expressed genes contain methylation within transcribed regions, whereas only 5% of genes show methylation within promoter regions. Interestingly, genes methylated in transcribed regions are highly expressed and constitutively active, whereas promoter-methylated genes show a greater degree of tissue-specific expression. Indicative of the specific importance of the role of promoter methylations in regulating the expression of downstream structural genes, whole-genome tiling-array transcriptional profiling of DNA methyltransferase null mutants identified hundreds of genes and intergenic noncoding RNAs with altered expression levels, many of which may be epigenetically controlled by DNA methylation.

In conclusion, it appears that 5mC was initially and still used as a heterochromatic mark to suppress different forms of genomic ''parasitic'' DNA or to lower transcriptional noise, alleviating in part the enormous problem of complexity in transcription regulation. Thus it contributes in this way to the genome robustness and stability of performance at genome level. It seems that later this mark was adapted to a fast, reversible, and influenced by both external and internal signals, system of gene regulation (see Bestor 2003 for details on DNA methylation and epigenetics).

DNA methylation, in addition to being the cause of epigenetic variation, is the cause of mutations and the generation of genetic variation. Methylated C is a hot spot for mutations since 5mC frequently deaminates to T (Coulondre et al. 1978; Jones et al. 1992). The mutability of 5mC was first demonstrated in *E. coli* (Coulondre et al. 1978). C bases that were methylated in the *lacI* gene of *E. coli* were found to be hot spots

for spontaneous base substitution mutations, and the hot spots disappeared when the same sites were unmethylated, which was the reason for this increase. Whereas C deaminates to uracil (U), 5mC deaminates to T, which is a normal DNA base and therefore inherently more difficult to repair (Duncan and Miller 1980). In vertebrates, the presence of high levels of CG methylation was associated with significant deamination of 5mC to T, a change that was incompletely or inefficiently repaired (Bird 1986; Shen et al. 1994). Thus, where a 5mCG dinucleotide pair was initially present in a gene, the deamination process would convert this into a TG/CA dinucleotide pair. Currently, mutation at CG sites continues to play a significant role in the formation of new germ-line mutations contributing to genetic disease. Cooper and Krawczak (1990), in a survey of a wide variety of genetic diseases, found that 44 of 139 (32%) point mutations were C to T or G to A transitions occurring at CG dinucleotides. The isolation of tumor-suppressor genes and the detection of mutations within them in somatic cells has led to the realization that 5mC is a frequent contributor to mutations relevant to human carcinogenesis (Jones et al. 1992). Moreover, as stressed by Matzke and Matzke (1996), the C methylation pathway is inherently mutagenic, particularly under conditions in which the methyl donor S-AdoMet is limiting. Such a low in S-AdoMet environment permits the accumulation of an intermediate in the C methylation pathway (5,6-dihydrocytosine), which has a 10^4-fold higher rate deamination than 5-methylcytosine, implying that epinutrition in organisms is not only related to epigenic but extends to genetic (mutational) effects too.

B. Modifications of Histones

Histone modifications have also been defined as epigenetic modifiers. Certain histone marks disseminate heterochromatin, the condensed and ''transcriptionally'' silent chromatin in eukaryotic genomes along with the presence or absence of C methylation, while others relate to euchromatic status (Grewal and Jia 2007). In its ''naked'' form, DNA is unwieldy and unmanageable for a cell to package. A eukaryotic genome that is average in size is approximately 2m of DNA, which needs to be constrained in a typical 10çm (i.e. 10^{-6}m) diameter nucleus; it also needs to be functional. This problem is solved by histones, which compact and control DNA. Histone octamers, consisting of two subunits each of histones H_2A, H_2B, H_3, and H_4, comprise the fundamental protein unit of chromatin that facilitates compaction of DNA into the nucleus. Approximately 150 bp of DNA wraps around the histone octamer to form the nucleosome, and then this DNA/protein complex is further

compacted by higher-order folding. Reasoning from first principles, it seems extremely unlikely that cells would have evolved systems to accomplish this extraordinary feat of packaging were it not at the same time used to regulate the function of DNA. Indeed, it is difficult to imagine a packaging system that would not inevitably exert a major functional effect. There is now a rapidly growing body of experimental evidence to show that packaging of DNA and its organization within the cell nucleus play central, sometimes dominant, roles in regulating its different functions (Fuchs et al. 2006).

Many histone sites, particularly at the N-terminal tails extending outward from the nucleosome core, can be posttranslationally modified at several different lysine, serine, and arginine residues. Keeping in mind the eight histone molecules, the several different lysine, serine, or arginine residues that could be modified, and the many different types of modifications like methylations, acetylations, phosphorylations, and ubiquitinations, the number of different combinations of histone modifications becomes enormous and the different ways of controlling the status of DNA unimaginable (Bannister and Kouzarides 2005). Counting only the methylation of histones, Bannister and Kouzarides (2005) calculated 3×10^{11} distinct methylation states, taking into consideration the 24 known methylation sites (17 lysine and 7 arginine residues) and the fact that lysine residues may be mono-, di-, or tri- methylated whereas the arginine chain may be mono- or (symmetrically or asymmetrically) dimethylated.

Altogether the many different types of histone modifications constitute the newly emerged epigenetic ''histone codes,'' which suggests that specific combination of histone modifications dictate specific transcriptional responses and cellular functions (Turner 2002). The detailed analysis of these modifications, their metabolism, and the enzymatic mechanisms involved is enormous and out of scope for this review, and the reader should refer to elsewhere (Turner 2002; Fischle et al. 2003; Rusche et al. 2003; Fuchs et al. 2006). In this subsection we focus primarily on histones H_3 and H_4 methylation marks and secondly on their acetylation. The modifications of these two histones demonstrated the power of changes onto DNA-based functions, regulating fundamental processes such as gene transcription, DNA repair, replication, and recombination (Bannister and Kouzarides 2005) and also extend to DNA methylation.

The most studied methylation marks of H_3 are lysine 4 (H_3K_4), lysine 9 (H_3K_9), lysine 27 (H_3K_{27}), and lysine 79 (H_3K_{79}). For H_4, lysine 20 (H_4K_{20}) is the more thoroughly studied, not only in yeast, drosophila, and mammals including humans, but also in plants (Rusche et al. 2003; Ringrose and Paro 2004).

Chromatin modulation by histone methylation involves multimeric protein–protein complexes containing, among other proteins, enzymes with histone MTase activity. All MTases contain a SET domain, a 130- to 160-amino acid motif named after the gene (Su-var3–9), Enhancer of zeste (E-z), and Trithorax (Trx) (see Section II.B). Perhaps what could be indicative for the significant role played by these enzymes is the finding of Baumbusch et al. (2001) that even the small genome of *Arabidopsis* contains at least 29 active genes encoding SET-domain proteins that can be assigned to four evolutionarily conserved classes. This high number of SET-domain genes stresses their significant epigenetic control on chromatin functions during plant development. The identification of putative nuclear localization signals and AT-hooks in many of the SET proteins studied supports their epigenetic role in the nucleus. The finding by these researchers that eight of the Su-var3–9 type genes lack introns indicates evolution of new SET genes by retrotransposition.

Generally speaking, inactive chromatin (heterochromatin) is more methylated in both histones and DNA in comparison to active euchromatin, which is generally less methylated. This is particularly true for H_3K_9, H_3K_{27}, H_3K_{79}, and H_4K_{20} methylations (Fig. 2.2). Then there are differences in heterochromatization of chromosomal areas depending

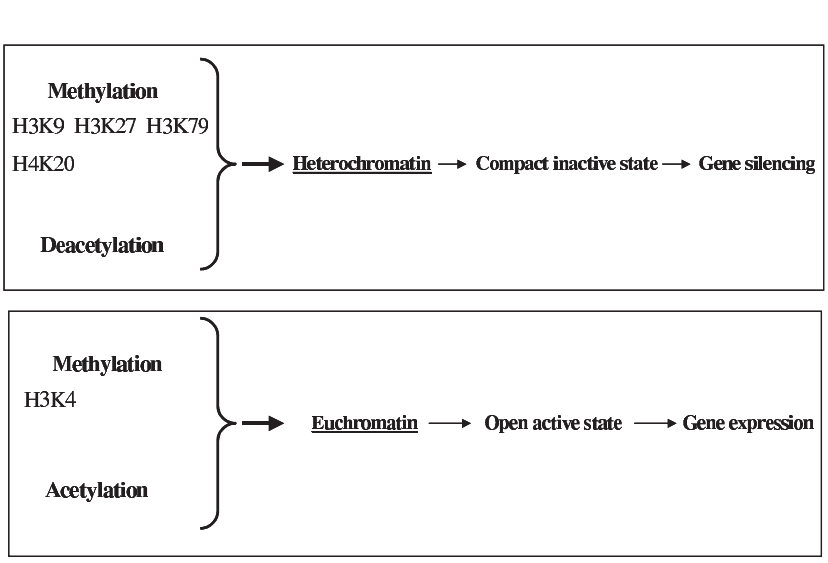

Fig. 2.2. Different modifications of histones lead to different states of chromatin architecture, which affects gene expression.

on the number of methylated histones, the number of methylated lysines in each histone, and the degree of their methylation (mono-di-tri-), as well as the extent to C methylation of the nucleosomal DNA. But the general rule holds true with the exception of H_3K_4. Methylation of H_3K_4, and particularly the trimethylated form of this lysine, leads to activated chromatin (Lachner and Jenuwein 2002). In fact, this methyl marks act in synergy with histone acetyltransferases (HATs), pointing to the convergence of the two histone epigenetic marks leading to euchromatic status. In summary, it is well established today, in all higher eukaryotes, that transcriptionally active, euchromatic regions are marked by hyperacetylation of all four histones and di-or-tri methylation of H_3K_4; on the contrary, gene-poor, transcriptionally inactive heterochromatin regions exhibit (1) hypoacetylation generated by HDACs, (2) demethylation of H_3K_4, and (3) hypermethylation of the rest of the targets mentioned (Vaquero et al. 2003; Dou et al. 2005).

It is possible that histone methylation can induce alterations in chromatin architecture, either condensing or relaxing its structure. However, a methyl group is relatively small and its addition to lysine or arginine residues does not neutralize their charge, so it is unlikely that methylation alone will significantly affect chromatin structure. It is more likely that it creates binding sites for regulatory proteins that contain specialized binding domains that recognize methylated lysines or arginines. Indeed, it is found that repressive proteins, such as heterochromatin protein 1 (HP1), not only depend on chromatin condensing, it also attracts cytosine MTases leading to DNA methylation (Tamaru and Selker 2001; Jackson et al. 2002; Malagnac et al. 2002).

Conversely, DNA methylation can also trigger H_3K_9 methylation (Johnson et al. 2002; Soppe et al. 2002; Lehnertz et al. 2003; Tariq et al. 2003). It has also been found that HDACs, histone MTases, and methylhistone-binding proteins lead to the recruitment of DNA MTases (Nan et al. 1998; Fuks 2003a, b; for a detailed genome-wide analysis of HDACs, see Ekwall [2005]). In conclusion, this not only links the two histone-based mechanisms of epigenetic marks (histone H_3K_9 methylation and deacetylation) with DNA methylation, but also indicates their convergence and cooperation for a tighter and perhaps more permanent and secure silencing of the DNA under these epigenetic controls.

Multimeric protein complexes have been 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, where two such main complexes were originally

described in this insect: the Polycomb (PcG) and the Trithorax (Trx) complexes (Mahmoudi and Vernijzer 2001). Both are known for their role in controlling the expression of homeotic genes but with opposite effects; PcG maintains them in an inactive state while the Trx leads them to an active state. Similar complexes were recently identified to execute conserved functions in plants, with the PcG protein group in plants being the most studied (Alvarez-Venegas et al. 2003). The PcG complex is involved in the control of the expression of homeotic genes that are essential for proper plant development transcription factors (primarily plant MADS-box genes). Although there is no structural relevance between the target genes of PcG complexes in animals (Homeobox genes) and plants (MADS-box genes), the PcGs are functionally similar in controlling developmental processes (Fig. 2.3). Built on a skeletal protein with many WD-40 repeats (WD-40 repeats facilitate

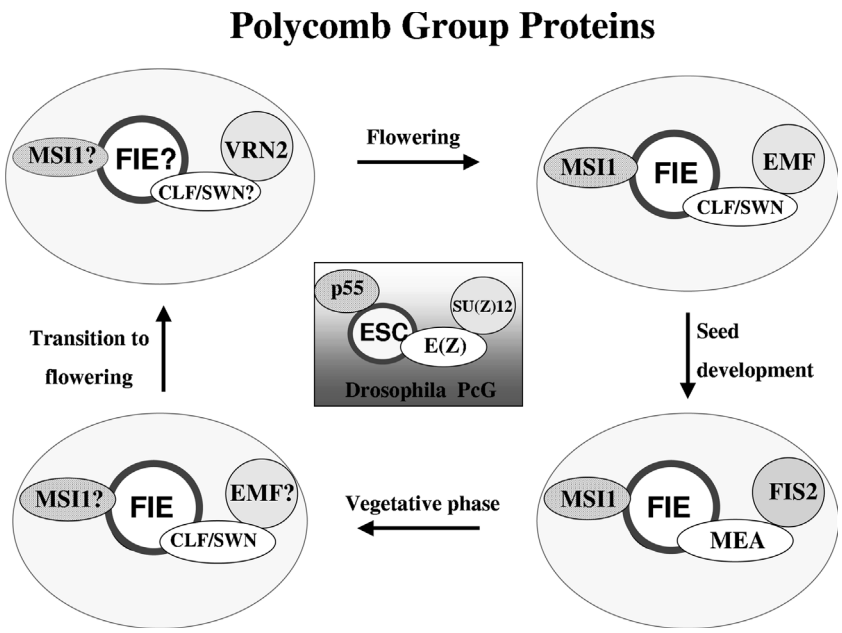

Fig. 2.3. A scheme showing a model of similar PcG complexes acting at different stages in plant development. The drosophila PcG counterpart is shown boxed in the middle. FIE: fertilization-independent endosperm; MEA: Medea; FIS2: fertilization-independent seed 2; MSI1: multicopy supressor of IRA; EMF: embryonic flower; CLF: curly leaf; SWN: Swinger; VRN2: Vernalization 2; ESC: extra sex combs; E(Z): enhancer of Zeste; SU(Z)12: suppressor of Zeste 12. Plant FIE and its drosophila homolog, ESC, comprise the core subunit of the complex around which the other PcG subunit homologs are bound.

protein–protein interactions taking a propeller-type form), thus allowing many different proteins operating on chromatin, such as the H_3K_{27} MTases MEA, and HDACs among others, to form different PcG complexes. Through the H_3K_{27} methylation and HDAC activities that are associated with DNA methylation, PcGs keeps the chromatin condensed and any respective gene included in the area inactive, thus silent. *Arabidopsis* MEA was the first plant H_3K_{27} MTase homolog of the PcG H_3K_{27} MTase proteins to be described (Grossniklaus et al. 1998; Kinoshita et al. 1999; Kiyosue et al. 1999; Makarevich et al. 2006). Later homolog genes like *curly leaf* (*CLF*) (Goodrich et al. 1997) have been described also from *Arabidopsis* and other plants operating in different tissues or developmental stages (Springer et al. 2002).

In conclusion, different PcG complexes have been described, operating in different tissues and/or developmental stages (Levine et al. 2004). Built on WD-40 PcG complexes, they differ in their H_3K_{27} MTase, HDAC, and other components showing different specificities and thus are targeted to different target homeotic genes destined to operate in different developmental stages. It should be stressed again that PcG is a suppressive complex operating to keep silenced genes in the quiescent state during early embryonic development until the activation time. Despite the fact that all PcG genes have been identified from mutations inactivating the coded protein (leading to reactivation of the silenced loci), normally wild-type PcG genes maintain chromatin in their compact, inactive state, leading to transcription inactivation (Schwartz and Pirrotta 2007).

Inactivation, though, with H_3K_{27} methylation, is reversible and not permanent (Matzke et al. 2004). In the case it is accompanied by methylation of H_3K_9 (and of other sites as well), histone deacetylation and DNA methylation, then chromatin is becoming progressively more and more condensed and heterochromatic, leading to more permanent (cemented) inactivation that is almost irreversible during development (Tariq and Pazworski 2004). This characterizes the majority of the heterochromatic genome consisting of REs and TEs.

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Contrary to the view that methylation of different lysines, in cooperation with DNA methylation, leads to more compact, more heterochromatic, thus inactive chromatin, as mentioned already, methylation of the outermost lysine (K_4) of H_3 (H_3K_4) leads to gene activation by chromatin relaxation. This is accomplished by the Trx complex involving H_3K_4 MTase activity. Thus Trx acts antagonistically to PcG. Alvarez-Venegas et al. (2003) have cloned plant homologs of Trx from *Arabidopsis* (ATX-1). ATX-1 found to contain a H_3K_4 MTase, like any other MTase, leading to activation of flower homeotic genes. Other

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different ATP-dependent chromatin-activating complexes of Trx type, identified mostly in yeast (SWI2/SNF2, ISWI, Mi2/CHD, and INO80) with homologous structures found in drosophila and humans, are currently under intense investigation in plants too. Plants have a remarkably high number of ATPases with homology to ATPases participating in chromatin remodeling (Wagner 2003). From 42 putative *Arabidopsis* SNF2-like ATPases (see the Plant Chromatin Database at http://chromdb.org), 4 belong to the canonical SWI2/SNF2 subfamily (Verbsky and Richards 2001). However, only 1 of these, BRAHMA (At2g46020), carries a C-terminal region resembling a chromodomain, a hair mark for binding acetylated lysine residues of histone tails (Hudson 2000; Brzeski and Jerzmanowski 2003). In contrast to the multiplicity of SNF2-like ATPase proteins, *Arabidopsis* has only one gene, *BUSHY (BSH)*, coding for a structural and functional homolog of *SNF5* (Brzeski et al. 1999), but contains four genes encoding *SWI3* homologs. Sarnowski et al. (2005) described also the characterization of knockout mutations in all four *Arabidopsis ATSWI3*. *ATSWI3A* and *ATSWI3B* cause similar blocks of embryo development at the early globular stage. However, unlike *atswi3a,* the *atswiSb* mutations result in aberrant segregation of progeny with arrested ovules, which suggests a possible role for *ATSWISB* in imprinting (see Sarnowski et al. 2005 for detailed analysis of the plant SNF2 complexes).

Dou et al. (2005) made the significant observation that H_3K_4 MTase is physically associated with H_4K_{16} HAT, a known gene activator, since many transcriptional co-activators proved to be HATs (Carrozza et al. 2003). The physical association between H_3K_4 MTase, a known chromatin activator (Hayashi et al. 2005), and H_4K_{17}, another chromatin activator, leads to direct synergy of two chromatin activation mechanisms operating through histones, in reversing silenced chromatin by antagonizing the silencing activity of H_3K_9, H_3K_{27} and H_3K_{79} MTases, which also operate through histones.

Until very recently, the methylation of histones was thought to be an irreversible process, despite the fact that for more than 30 years, a demethylase activity was described (Paik and Kim 1973). The reversibility of histone methylation became apparent a few years ago when antibodies against methylated lysine or methylated arginine residues were used in ChIP assays. These experiments revealed that the methylation of histone residues appeared to be reduced under certain conditions (Martienssen and Colot 2001). This prompted the idea that histone demethylation was a likely possibility, and indeed an amine oxidase specific for removing methyl groups from histones in actively transcripted H_3K_4 chromatin was described, leading in this case to

suppression (see Bannister and Kouzarides 2005 for review of histone demethylation).

Perhaps what is more revealing for the scope of this article is the link between histone methylations–demethylations, and the concomitant effects on DNA methylations are the recent findings that proteins with a Tudor domain are binding to methylated H_3K_{79} or to H_4K_{20}. This is of particular importance because these proteins are involved in binding to damaged DNA and DNA recombination. Many different kinds of stresses lead directly (radiations) or indirectly (other abiotic and biotic stresses) to damages of proteins and DNA by oxygen radicals (see Section V.B). Efficiency of removal of damaged DNA bases, refilling the respective methyl groups on C after this repair, and reestablishment of histone modifications will drastically affect silencing. In this way, epigenetic mechanisms and different kinds of stresses on one hand and these processes with genome plasticity on the other are interconnected (see Section V.B).

C. RNA-based Control Mechanisms

For a long time, RNA has lived in the shadow of DNA and proteins. The shadow has been so obscuring that a whole universe of small RNAs, primarily non-coding for proteins, has been hidden from view until recently. This was due to the methodologies applied in studying mainly the messenger role of large RNAs. The situation changed dramatically in the last few years after the discovery of the significant roles played by small RNA (sRNA). sRNAs were found to interfere (thus the term RNAi for these mechanisms) with many aspects of gene actions and genome organization and, as consequence, to control significant steps in cell-cycle and the development of eukaryotic organisms. As Karp and Ambros (2005) noted, it came as a huge surprise that a major level of gene regulation was completely unknown until the recent discovery of sRNAs.

Like many other significant discoveries, revolutionary discoveries concerning this newly emerging field of RNAi were also made from research groups working with plants. In one case, attempts to boost the expression of an endogenous gene with an extra transgene copy eliminated all expression from both genes: the endogenous gene and the transgene. In this 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 color. 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), which later was shown to occur at the posttranscriptional level (De Carvalho et al. 1992; Van Blokland et al. 1994). PTGS was discov-

ered from another experiment designed to evaluate antisense suppression, which was a promising approach at the time for selectively silencing plant gene expression. In theory, antisense RNA encoded by a transgene should base pair to the complementary mRNA of a plant gene, preventing its translation into protein. Although the control ''sense'' transgene RNAs are unable to base pair to mRNA, and hence should not induce silencing, they often inexplicably did (Smith et al. 1990). A study by Lindbo et al. (1993) showed that plant RNA viruses could be both initiators and targets of PTGS. Plants expressing a transgene encoding a truncated viral-coat protein became resistant to the corresponding virus, a state achieved by mutual degradation of viral RNA and transgene mRNA. In addition to forging a link between RNA virus resistance and PTGS, this study included a remarkably prescient model for PTGS that featured an RNA-dependent RNA polymerase (RDR), dsRNA, and small RNAs, all of which were later found to be important for the RNAi. In a second approach, plants that recovered from one viral infection were resistant against an unrelated virus, provided it carried a small sequence insert from the first virus (Ratcliff et al. 1997). Finally, replicating viroids or viruses that propagated entirely through RNA intermediates left behind an imprint of methylation on homologous DNA. The discovery of silencing-associated sRNAs by Hamilton and Baulcombe (1999) gave this diverse set of homology-dependent events a common specificity determinant and pointed the way to a densely populated sRNA world. Finally, an influential paper by Wassenegger et al. (1994) reported the discovery of RNA-directed DNA methylation in transgenic tobacco plants (see Section II.C). The repression was dependent on partial sequence complementarity between the *lin-4* 21-nt RNA and the 3' (UTR) of *lin-14* mRNA. (For a more detailed history of the discoveries involving sRNAs, see Matzke et al. [2004]); Zamore and Haley [2005]; and the book by Galun [2005].)

Double-strand RNAs could be generated in the cell from many different sources and using different molecular mechanisms: (1) sense together with antisense transcription that has been documented from EST sequencing efforts in a much higher frequency than ever thought; (2) RNA duplication of RNA molecules from different sources, including RNA molecules from RNA viruses and viroids through the action of RNA directed RNA polymerase; (3) transcription of TE and RE with direct repeats in their sequence leading to different intramolecular dsRNA motives; (4) transcription of different primarily intergenic DNA sequences coding for larger precursor RNA molecules like the precursors of mRNAs, which are also cupped and polyadenylated in their 5' and 3' ends, respectively, and are processed down to the

production of sRNAs called miRNAs. dsRNA, the central player in RNA-mediated gene silencing, is chopped into small RNAs by the enzyme Dicer. Farther down sRNAs become associated and guide their own further amplification to many more copies and join an RNA-induced silencing complex (RISC), leading either to mRNA clearage or translational inhibition. In order to guide suppression of homologous targets, the sRNA duplex must become single-stranded. The strand with the 5' phosphate at the less stable end of the helix is incorporated as a guide RNA into an effector complex containing an Argonaute (AGO) protein. Two types of effector complexes have been described. The cytoplasmic complex, RISC, mediates both mRNA cleavage and translational inhibition. Additionally, the RNA-induced transcriptional gene-silencing complex (RITS) operating in the nucleus leads to chromatin modifications and DNA methylation (Matzke et al. 2002).

Since we now have a good understanding of the RNAi pathway, current interest is turning to variations on the basic mechanism. Recent studies involving plants and the nematode *Caenorhabditis elegans* (Pak and Fire 2007; Sijen et al. 2007) deal with the amplification of silencing-related RNA and explain how strong, persistent silencing can be initiated with small amounts of ''initiator'' double-stranded RNA. The amplification process has implications for application of RNA interference to control gene expression in biotechnology and for understanding the effects of silencing RNAs on cell function and organism development (Baulcombe 2007). sRNAs can follow a non-cell autonomous travel throughout the plant organisms using different proteins as carriers (Lucas and Lee 2004) in plants and in some animals; the effects of posttranscriptional RNA silencing can extend beyond its sites of initiation, owing to the movement of signal molecules. Breakthrough grafting experiments indeed demonstrated 100% transmission of the nitrate reductase (Nia) cosuppressed state from silenced rootstocks to nonsilenced transgenic scions (Palauqui et al. 1997). Shortly after this discovery, leaf-infiltration of recombinant *Agrobacterium* cultures (agro-infiltration) into transgenic *Nicotiana benthamiana* expressing the green fluorescent protein (GFP) was found to trigger a systemic, sequence-specific loss of *GFP* expression (Voinnet and Baulcombe 1997). Since then, non-cell autonomous silencing has been documented in several additional plant species, including sunflower, curcubits, *Arabidopsis,* and, to a lesser extent, roots of the legume *Medicago*; it was also recently demonstrated in fern gametophytes (Rutherford et al. 2004). (See Voinnet 2005a for a recent review on non-cell autonomous RNA silencing). With the current increasing interest in using grafted plants in vegetable production (Sapountzakis and Tsaftaris 2002;

Grigoriadis et al. 2005), systemic RNA-silencing scion genes from rootstock RNAs (Lucas and Lee 2004; Athanasiadou et al. 2005) is under intense study as a major determinant of lowering quality characteristics in vegetable fruits.

In conclusion, originating from different resources and using different molecular processes for production, numerous sRNAs are produced that after becoming part of RISC or RITS complexes fulfill divergent jobs. In this framework miRNAs, the most abundant type of endogenous sRNAs in plants and animals, have been implicated in different developmental control mechanisms by acting *in trans* to negatively regulate the expression of target mRNAs from other loci by blocking their translation, by interfering with their splicing, or by destructing them. They regulate others with homology within the restricted miRNA target binding site, but limited homology to the rest of the miRNA precursor. They arise from primary transcripts (pre-miRNAs) transcribed from these endogenous genes by two rounds of endoribonuclease III processing involving first Drosha, producing a hairpin-shaped pre-miRNA, and then Dicer (reviewed by Chen 2005). After Dicer processing, miRNAs emerge as siRNA-dupIex-like structures, but only one strand, the mature miRNA, is then predominantly incorporated into the *RISC* effector complexes. The discarded RNA strand is frequently referred to as miRNA* and is degraded. In plants, miRNAs guide mRNA cleavage, and the highly complementary target sites are readily identified using bioinformatic tools (see Section II.E). In contrast, animal miRNAs preferentially target mRNAs at partially complementary yet evolutionary conserved sites, which are predominantly located within the 3' UTR.

Au: What does the asterisk refer to?

siRNAs are produced by TEs and inverted REs transcription, upon exogenous delivery of dsRNA, transgenic expression of long dsRNA, and RNA viral infections. However, endogenous genes leading to siRNAs production are rare. Thus generally speaking, siRNA usually targets only highly homologs sequences (see the description of tasiRNAs). siRNAs serve as a defense against different ''invading'' nucleic acids such as virus, TEs, REs, transgenes, and injected dsRNA among others (Vance and Vaucheret 2001). Such ''invaders'' trigger silencing by producing long dsRNA that are cleaved into siRNA by Dicer. In this respect, TEs and REs are endogenous sources for siRNA production made from TEs and REs read through transcription, and siRNAs produced this way serve as specific guides for distinguishing specific TEs and REs for inactivation through H_3K_9 and DNA methylation (Lippman et al. 2004). Similarly siRNAs act as guides of RISC in destroying RNA molecules with high homology to the dsRNA trigger. This particular category of siRNAs named rasiRNAs (repeat associate small RNAs)

produced by read-through of sense or antisense transcription of REs and TEs varies in different organisms from 24 to 27 nucleotides. The significance of the variation and the mechanism produced remains to be elucidated, but the main function of this siRNA group is its involvement in establishing and maintaining heterochromatin structure and in controlling transcripts emerging from repeat sequences. Apparently the control on TEs and REs is such that even in case of their activation, the transcript is not only immediately destroyed through PTGS, but sRNA by-products of PTGS used as guides go back to the DNA to silence and put it again under control at the chromatin level. Another category of siRNA consisting of *trans*-acting siRNAs (tasiRNAs) was recently identified. tasiRNAs were initially confounded with miRNAs because the same sequences were cloned multiple times, suggesting a defined register for tasiRNA excision. It was revealed later that specific miRNAs in plants target single-stranded noncoding RNA transcripts for cleavage, which then are used as template for RNA-dependent RNA polymerase (RdRP). Dicer processes the resulting dsRNA in 21-nt increments producing mature tasiRNAs, which then subject mRNAs that have one or a few tasiRNA complementary sites to degradation. tasiRNAs are found to play a critical role in controlling different developmental stages of plants, particularly in phase changing (Willmann and Poethig 2005; Kidner and Martienssen 2005, and Section IV.A).

The description of tasiRNAs indicates that categorizations and different names such as miRNAs and siRNAs may not describe entirely different types of sRNAs. The field is relatively recent with no conclusive knowledge on these very important genome regulators. Their significance grows daily, and their role could be greater than expected (Vaughn and Martienssen 2005). Lu et al. (2005) identified over 1.5 million sRNAs from *Arabidopsis,* representing over 75,000 distinct sequences, using Massively Parallel Signature Sequencing (MPSS) technology. They report that many more genes may be under the control of sRNAs than had been previously imagined (Mattick 2004). For this model plant species, current estimates predict that 2% of genes may be under miRNA control, but the number of genes that might be regulated by siRNAs is not known. It has already been mentioned that siRNAs are known to participate in RNAi-mediated silencing of REs and TEs (Lippman and Martienssen 2004). Thus it is no surprise that the majority of sRNA sequences matched these repeated sequences. Most of the remaining sRNA sequences came from intergenic regions (IGRs), including those derived from miRNAs, which is consistent with previous studies on a much smaller scale (Gustafson et al. 2005). Interestingly, 4,000 protein-coding genes (15% of genes in the genome) and several hundred pseudogenes matched at least one sRNA perfectly,

presumably corresponding to siRNAs (miRNAs usually match imperfectly). However, most of these genes matched only one siRNA, and only a small percent of the total sRNA sequences were from genes. It is possible therefore that many other genes may have matches that went undetected.

Consistent with the idea that siRNA guides silencing as an antiviral mechanism on behalf of the plant, many plant viruses, as a counter-defense mechanism (against the plant's silencing efforts), encode proteins that interfere with processes targeted to their inactivation at one or more steps and thereby act as supressors of their silencing (Roth et al. 2004; Silhavy and Buryuan 2004).

In summary, by identifying small RNAs as agents of gene silencing that act at multiple levels throughout the cell, molecular biologists have created a new paradigm for eukaryotic gene regulation (Matzke et al. 2004). Plant scientists have played an outstanding and leading role in RNA-mediated silencing research. Their successful production of a wide variety of transgenic plants, which displayed an equally rich diversity of gene-silencing phenomena, gave the opportunity to embark in research and analyze these unexpected outcomes. Simultaneously members of the agricultural biotechnology industry avidly pursued transgenic approaches to modulate plant gene expression, to genetically engineer virus resistance, and to find ways to avoid silencing and stabilize transgene expression. The history of gene-silencing research shows once again that plants offer outstanding experimental systems for elucidating general biological principles.

D. The Triangle of Interactions of Epigenetic Mechanisms

By describing DNA methylation, histone modifications, and RNA interference separately, it might appear that they are independent epigenetic mechanisms. On the contrary, the functional relationships between them and their mutual reinforcement (Vire et al. 2006) are unexpectedly complex (Fig. 2.1). DNA methylation mechanisms and histone modifications that lead to chromatin compaction and gene silencing are under intense scrutiny. While results of recent studies indicate intriguing links among the three epigenetic mechanisms, there is still a long way to go before we understand the details of their interaction. For instance, starting with DNA methylation, 5mC binding proteins (5mC BP) not only recognize and bind to methylated DNA but also recruit and form complexes with HDACs and other chromatin remodeling factors (Bird 2002; Fujita et al. 2003; Fuks 2003a; Zemach and Grafi 2007). One such gene coding for a human 5mC binding protein $MeCP_2$ was found to be associated with the human Rett syndrome (Bienvenu and Chelly 2006).

In this way, DNA methylation is linked with modifications of histones. Sarraf and Stanchera (2004) provided results that support a model in which the binding of a 5mC binding protein, MBD1, to 5mC leads to H_3K_9 methylation that is also heritably maintained through DNA replication. In parallel, Tariq et al. (2003), conducting ChIP and immunostaining analysis using antibodies against H_3K_9 in *Arabidopsis met1–3* mutants, clearly demonstrated the CG methylation (but neither CNG or CNN methylation) is a prerequisite for the maintenance of histone methylation (H_3K_9).

To state this another way: Histone modifications are also affecting DNA methylation. Observations made in plants, fungi, and mammals highlight H_3K_9 methylation as a kind of ''beacon'' for DNA methylation (Fuks 2005). H_3K_9, H_3K_{27}, and H_4K_{20} methylation normally leads to inactivation of chromatin, and the relatively small methyl group on histones creates binding sites for regulatory proteins that contain specialized binding domains like HP1 that also attract cytosine MTases leading to DNA methylation (Tamaru and Selker 2001; Jackson et al. 2002; Malagnac et al. 2002; Fuks et al. 2003a). HDACs, histone MTases, and methylcytosine-binding proteins (Nan et al. 1998) lead to the recruitment of DNA MTases (Fuks 2005).

These data support a model in which methylated DNA is part of an epigenetic program that leads to transcriptional silencing (Fuks 2005). Methylation at H_3K_9 creates a foundation for the adaptor molecule HP1, which would, in turn, recruit DNA MTases. The recruited DNA MTases would catalyze CG methylation in the region of the methylated lysine. Methyl-CG-binding domains would then bind to the methylated DNA. The bound methyl-CG-binding domains would attract HDAC complexes, which, in turn, seem to be required to prepare more H_3K_9 methylation. Furthermore, according to the model, methyl-CpG-binding domains would also recruit H_3K_9 MTases. Thus, epigenetic information embodied in methylated residues would flow from histone to DNA and back. Variations of this model are possible to envisage. For example, the well-described DNA MTase-HDAC interaction might provide an alternative route for delivery of the HDAC activity that paves the way for H_3K_9 methylation. Further research is clearly needed to elucidate the precise sequence of events leading to epigenetic silencing. Nonetheless, this model is appealing because it implies that DNA methylation, histone deacetylation, and H_3K_9 methylation act in synergy to generate a self-reinforcing epigenetic cycle that maintains and perpetuates a repressed chromatin state. Such a cycle might be relevant to situations in which genes need to be ''locked'' into a permanently silenced state, as with the case of imprinted genes or of hypermethylated genes in cancer.

The recent discovery of small RNA interference systems showed that sRNA molecules play a role in DNA methylation in addition to their role in RNA processing, translation, and destruction (Wassenerger et al. 1994). RdDM could provide a significant connection between mechanisms that govern the establishment of DNA methylation and the initiation of gene silencing. RdDM facilitates the *de novo* methylation of a target DNA sequence similar to the triggering double-stranded RNA molecule. During the RdDM process, cytosines become methylated regardless of their location (Mette et al. 2000). When dsRNA is directed against promoter regions, this causes their *de novo* methylation and the onset of TGS in plants (Mette et al. 2000; Jones et al. 2001). After removal of the trigger RNA, CG and CNG methylation is maintained but CNN methylation is reduced rapidly (Aufsatz et al. 2002). The maintenance of hypermethylation and TGS triggered by RdDM in the absence of trigger RNA requires MET1 and DDM1 activity (Jones et al. 2001). The DRMs have been shown to be the DNA MTases that are involved in the establishment of methylation by RdDM (Cao et al. 2003). Furthermore, a direct link between RNAi and DRMs could be inferred by results revealing that mutations in components of the RNAi machinery affected DRMs-dependent *de novo* methylation of a FWA transgene in addition to the endogenous FWA locus (Chan et al. 2004). Matzke et al. (2004) and Kanno et al. (2004) found that, in addition, HDACS and a putative chromatin protein are components of RdDM, a fact that points to the involvement of siRNA to chromatin structure (Gendrel and Colot 2005). Mette et al. (2005) reported that transcription of DNA by RNA polymerase II is required for RdDM, pointing to a direct RNA-RNA hybridization model between siRNA and nascent RNA transcripts prior to transcription completion and termination, which leads to DNA methylation and histone modifications like methylations and deacetylations leading to silencing. Thus, the nascent RNA transcript provides the necessary link through RNA-RNA hybridization (and not RNA-DNA hybridization) for a sequence-specific silencing. In parallel, from studies conducted with plants Bao et al. (2004) showed that miRNA could also trigger TGS through RNA-RNA hybridization of miRNA–nascent mRNA hybrids. Thus, miRNA is not restricting itself to PSGS but it can ''mimic'' siRNA in its TGS function, which again makes the functional distinction between siRNA and miRNA more difficult (Ronemus and Martienssen 2005).

E. Methods of Studying Epigenetic Mechanisms

The techniques developed to detect DNA methylation (Lyko 2005) can be divided into three categories according to the information they

Table 2.2. Methods for DNA methylation detection.

Methods Methylation Status	Reference
Immunological method	Sano et al. 1980; Achwal et al. 1984; Vilpo et al. 1986
Nearest-neighbor analysis	Gruenbaum et al. 1983
High-performance liquid chromatography (HPLC)	Gehrke et al. 1984
Nuclear magnetic resonance (NMR)	Wuetrich 1986
Mass spectroscopy (MS)	Annan et al. 1989
Thin layer chromatography (TLC)	Wilson et al. 1986; Gowher et al. 2000; Ramsahoye et al. 2000.
GLOBAL CONTENT	
SssI Methyl-acceptance assay	Wu et al. 1993, Schmitt et al. 1997
Self-primed-in situ labeling (SPRINS)	Andersen et al. 1998
Chloroacetaldehyde assay	Oakeley et al. 1999
High-performance caillary electrophoresis (HPCE)	Fraga et al. 2002
Capillary electrophoresis with laser-induced fluorescence detection (CE-LIF)	Cornelius et al. 2005
Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS)	Song et al. 2006
REGIONAL CONTENT	
Agarose gel electrophoresis upon digest with methylation sensitive enzymes (MSE)	Bird et al. 1979
Chemical sequencing	Church and Gilbert 1984; Saluz and Jost 1986
Cytosine-extension assay	Pogribny et al. 1999
Enzymatic Regional Methylation Analysis (ERMA)	Galm et al. 2002
SPECIFIC (INTENSITY)	
Methylation-sensitive restriction enzyme (MSRE) Southern	Bird et al. 1978
Random amplification coupled with restriction enzyme digestion (CRED-RA)	Cai et al. 1996
Direct bisulfite sequencing	Paul and Clark 1996
Methylation-sensitive arbitrarly primed PCR (MSAP-PCR)	Gonzalgo et al. 1997a
Methylation-sensitive restriction fingerprinting technique (MSRF-PCR)	Huang et al. 1997
Methylatin-sensitive single nucleotide primer extension (MS-SNuPE)	Gonzalgo et al. 1997b
Melting curve combined bisulfite restriction analysis (COBRA)	Xiong and Laird 1997
Hemi-methylation assay	Liang et al. 2002
Bisulphite primer extension—HPLC	Matin et al. 2002

(*continued*)

Table 2.2. (Continued)

Single nucletide polymorphism methylation (SNaPmeth)	Uhlmann et al. 2002
PyroMeth	Uhlmann et al. 2002
Quantitative multiplex-methylation-specific PCR (QM-MSP)	Fackler et al. 2004
Methylation sensitive dot blot assay (MS-DBA)	Clement and Benhattar 2005
Methylation-sensitive multiplex ligation-dependent probe amplification (MS-MLPA)	Nygren et al. 2005
microchip electrophoresis	Zhou et al. 2005
PATTERNS OF ALLELE	
Hydrazine/permaganate sequencing	Ohmori et al. 1978; Fritzsche et al. 1987
Methylation-specific PCR (MS-PCR)	Herman et al. 1996
Methylation sensitive PCR-in situ hybridization (MS PCR-ISH)	Nuovo et al. 1999
Methylation dependent denaturating gradient gell electrophoresis (MB-DGGE)	Uhlmann et al. 1999
Bisulfite-DGGE	Uhlmann et al. 1999, Aggerholm et al. 1999
Methyl Light	Eads et al. 2000
Bisulphite-single strand conformation polymorphism	Burri and Chaubert et al. 1999
Bisulphite-SSCP	Bianco et al. 1999
Methylated DNA binding column—denaturing gradient gel electrophoresis (MBDC-DNGGE)	Masahiko et al. 1999
Methylation sensitive PCR Denaturing HPLC (MSP-DHPLC)	Baumer et al. 2001
In a tube fluorescence melting curve	Worm et al. 2001
Melting curve methylation specific PCR (McMSP)	Akey et al. 2002
CpG methylation and single nucleotide polymorphisms SNPs—DHPLC	Deng et al. 2002
Conversion specific methyLight Con-light MSP	Rand et al. 2002
Denaturing HPLC (DHPLC)	Couvert et al. 2003
Targeted gene methylation (TAGM)	Jessen et al. 2004
Methylation-specific PCR and PCR with confronting two-pair primers (MSP-CTPP)	Sasamoto et al. 2004
Real-time PCR—quantitative analysis of methylated alleles (QAMA)	Zeschnigk et al. 2004
Restriction Landmark Genomic Scanning	Hayashizaki et al. 1993
Methylation-sensitive amplification polymorphism (MASP)	Vos et al. 1995, Reyna-Lopez et al. 1997, Xiong et al. 1999
GENOME PROFILES	
Methylation sensitive representational difference analysis (MS-RDA)	Ushijima et al. 1997

Methylated DNA binding column and denaturing gradient gel electrophoresis	Masahiko et al. 1999
Methylated CpG island amplification	Toyota et al. 1999a, b
Epigenomics microarray	Adorjan et al. 2002
Two-color oligo array	Balog et al. 2002
Identification of CpG Island exhibiting altered methylation patterns (ICEAMP)	Brock et al. 2001
Amplification of inter-methylated sites (AIMS)	Frigola et al. 2002
Methylation- specific oligo array (MSO)	Gitan et al. 2002
Methylation amplification DNA Chip (MAD)	Hatada et al. 2002
Methylation-sensitive digital karyotyping	Hu et al. 2005
NotI Cloning of deleted sequences (NotI CODE)	Li et al. 2002
Demethylation/expression analysis	Suzuki et al. 2002
Different methylation hybridization (DMH)	Shi et al. 2002
Similarly/differentially methylated regions (SMR/DMR) cloning	Strichman-Almashanu et al. 2002
Size fractination profiling	Tompa et al. 2002
Methylated-CpG island recovery assay (MIRA)	Rauch and Pfeifer 2005

provide (Table 2.2). Methods used to measure global methylation level usually detect and quantify different types of modified bases. Some methods from this class are useful in the initial research to determine which species possess certain base modification and to which extent; the other methods from this group are useful for quick rough measures of ongoing hypermethylation in certain tissues. The second category consists of sequence-specific techniques, reliable for methylation analysis of already known genes and sequences (Thomassin et al. 1999). The most precise are based on modification by bisulfite, hydrazine, or permanganate, to accurately measure the level of methylation at given CG sites in target areas of genes. The third category would be the methods that provide information on methylation profiles; they are used for discovery of new methylcytosine hot spots—future biomarkers—without requiring prior knowledge of the DNA sequence.

Recently a computational prediction progmam called HDFINDER was constructed and tested by Das et al. (2006) for predicting the methylation landscape of all 22 autosomal DNA, with 86% accuracy. Detection of chromatin modifications can be accomplished by chromatin immunoprecipitation (ChIP) and the ChIP on chip assay (Orlando 2000; Horak and Snyder 2002; Lippman et al. 2004; Gendrel et al. 2005). The ChIP assay is a powerful tool to identify genome-wide associations between histone proteins and their target genes. The key feature of the assay is that it makes use of an antibody that recognizes the protein of interest, not only free in solution but also when assembled into chromatin. The ChIP assay involves two steps: (1) in vivo crosslinking of whole cells

that fixes protein-protein and protein-DNA interactions; and (2) immunoprecipitation of protein-DNA complexes with specific antibodies. The ChIP assay has been widely used for mapping the location of modified histones in the genome. Using chromatin immunoprecipitation with antibodies against specific histone modifications followed by PCR amplification of specific sequences, it is possible to detect the chromatin states of specific genes. This assay was used by Köhler et al. (2003b) to identify *PHERES 1 (PHE1*) in *Arabidopsis* as the potential target gene of the MEA-FIE Polycomb group protein complex using antibodies against MEA and FIE proteins and PCR amplifications of different regions of the *PHE1* gene after the immunoprecipitations.

A highly promising recent technical advance has been the development of the so-called ChIP on chip assay. In this assay, chromatin immunoprecipitations are performed with antibodies against specific histone modifications, such as dimethyl H_3K_9 or dimethyl H_3K_4, indicators of heterochromatic or euchromatic chromatin regions, respectively. All of the DNA fragments recovered from the precipitate are then amplified. This DNA is enriched for sequences recovered from chromatin immunoprecipitation with antibodies raised against H_3K_9 or H_3K_4 modifications. The amplified DNA is then labeled and annealed to DNA microarray slides containing genomic regions of interest called genomic tiling microarrays (Lippman et al. 2004; Gendrel et al. 2005). In this way it is possible to study entire regions of a genome and map precise locations that are enriched for particular histone modifications. This techinique was used successfully to map H_3K_9 and H_3K_4 histone methylations across a 1.5Mb region in *Arabidopsis* (Lippman et al. 2004; Gendrel et al. 2005).

Finally, RNAi identification of micro-RNAs has been accomplished with biochemical and *in silico* approaches. For the first ''wet'' approach, various biochemical methods were used in order to identify miRNAs in plants; however, only a small number of miRNAs could be isolated. The basic method of identifying miRNA genes has been to isolate total RNA, separate the small-RNA fraction (i.e., RNAs of about20 to 25nt long) on polyacrylamide gels, ligate modified 3'adaptors, reverse transcribe, clone, and sequence these small-molecular-weight RNAs (Elbasir et al. 2001). One drawback of this method is that it is biased for RNAs that are relatively abundant (Jones-Rhoades and Bartel 2004). As an alternative, *in silico* identification of miRNAs has been used widely in the past few years. Because in plants miRNAs have nearly perfect complementarity with their target sequences, it has been easy to develop computational approaches in order to identify miRNAs molecules in plants. Based on the conservation of short sequences between

the genomes of *Arabidopsis* and rice, a search for conserved hairpins and target sequences has been possible. In this way, 91 potential miRNas were detected in *Arabidopsis* and in rice (Bonnet et al. 2004; Jones-Rhoades and Bartel 2004). *Arabidopsis* and rice miRNAs seem to be highly conserved, pointing to the critical role they play in various aspects in plant development. The plant miRNAs that had been identified in the past targeted primarily transcription factors operating at various stages in plant development. Introduction of mutations in miRNA sequences have led to striking developmental abnormalities (Kidner and Martienssen 2005). Currently 117 miRNAs from *Arabidopsis*, 178 from rice, 97 from maize, 16 from *Medicago truncatula*, 22 from soybean, 213 from poplar, 16 from sugarcane, and 72 from sorghum have been reported in the miRNA Registry (Griffiths-Jones 2004). Furthermore, a new computational tool for the identification of novel miRNAs and their targets in plants, named MIRO, was developed recently and will soon be available on the Internet (Ahren et al. unp. results). MIRO is based on evolutionary conservation and internal genome conservation and uses inversion analysis in order to predict hairpins. MiRNA target identification is performed with the MIRANDA algorithm (Enright et al. 2003). Finally a pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes has been reported by Miranda et al. (2006).

III. Plant Biological Phenomena Involving Epigenetic Mechanisms

A. Epigenetic Control of Transposable Elements and Repeats

TEs were discovered and studied as the source of variegated maize phenotypes by Barbara McClintock and Peter Peterson, two illustrious pioneers of twentieth-century genetics. Earlier geneticists had studied variegation, which is obvious in maize seeds and many other plant parts. Alleles were classified as demonstrating somatic variation in contrast to the stable colored and colorless alleles. Parallel detailed analysis by Marcus Rhoades demonstrated the first case of transactivation of gene expression; specifically, the response of the *a1-dt* allele to a dominant *Dotted* factor elsewhere in the genome. McClintock's work established that mobile DNA elements could be responsible for the *in cis* component of mutability, a discovery that surprised everyone (Fedoroff 1999; Walbot 2002). McClintock and Peterson showed that active forms of these elements were required *in trans* to mobilize derivative elements *Ds* and *dSpm,* respectively. More important, they

demonstrated that alleles that acquired a transposon insertion fell under the control of the mobile DNA system. For that reason, McClintock named the transposons controlling elements, despite the fact that mobility was the most surprising property of the transposons mapped and analyzed. McClintock speculated that the controlling elements might be important in molding host gene expression patterns, an insight for which there is growing molecular evidence. It is also equally true that diverse host processes control TEs. TEs and their hosts have co-evolved tolerance: TEs are not completely silenced in the host, and the host is not killed by TEs.

In the 50 years since multiple types of TEs have been discovered, the two basic categories have been identified: retrotransposons and DNA transposons. Retroelements insert through the production of an RNA copy of an existing TE; this RNA transcript is copied by reverse transcriptase into DNA, which inserts into a new chromosomal location. Retrotransposons can proliferate and insert, but they do not excise; as a consequence, retrotransposon-induced mutations have a stable, usually null, phenotype. In contrast, the ''classic'' DNA elements such as *Ac/Ds* and *Spm/dSpm* move as pieces of DNA. These elements are organized into families of autonomous and nonautonomous elements. Walbot (2002) provides data for the different families of TE and their percentages in the maize genome.

Indicative that the methylation patterns of DNA may change during development is the fact that the RNA-silencing patterns can vary temporally during development. For example, work with two maize TEs Suppressor-mutators (Spm) and Robertson's Mutator (Mu) has shown that silencing can increase during shoot development. siRNAs are important for the establishment of transposon silencing by DNA methylation. Plants that have active Spm or Mu transposons at germination display a gradual decrease in transposon activity and an increase in transposon methylation along the primary shoot of the plant. It was further shown that developmentally regulated methylation and silencing are paralleled by a gradual reduction in the polyadenylation of Mu-derived RNA and by an increase in the nuclear retention of these RNAs, resulting in fewer mature transcripts. The developmental regulation of Spm and Mu transposons was demonstrated most clearly in studies with the *pale green* mutant *hcf106* and the *lesion* mutant *Les28*, whose mutant phenotypes are caused by the activity of Mu. Upon inactivation of Mu, the phenotypes of these mutants are suppressed, and sectors of wildtype tissue can be seen. These sectors increase in size and number in subsequent lateral organs as the plant develops. As a result, the juvenile leaves tend to have the greatest transposon activity, the adult

leaves and ears (containing the female gametophyte) an intermediate activity, and the pollen the lowest activity. These changes in epigenetic states, although reversible under the appropriate conditions, are both mitotically and meiotically heritable. Because the pollen has a lower transposon activity than tissues generated during earlier developmental stages, it has a relatively high chance of passing on an inactive transposon state to its progeny.

TEs and REs constitute a large fraction of the DNA in some species of animals, such as mice and humans, or plants, such as maize and wheat. There are three main views about the role of TEs and REs in the genetic material of these organisms. They have been considered as genome parasites or as ''junk DNA'' causing genetic diseases and evolving as retroviruses (Bird 2002). Another view argues that they are maintained in big numbers because of the mechanisms that make large, redundant genomes possible (Fedoroff 2002). Finally since there is evidence in support of each model, most probably synthesis of both is the correct one (Becker and Lonnig 2001; Bestor 2003). TEs were parasitic in nature at their origin, and host genome developed secure control system over their overamplification. Host genome found in those elements an excellent tool to generate variability and maintain its own integrity. For that reason, although TEs can be present in millions in a genome, only a very small fraction of them is normally active. In life-threatening situations, TEs and REs could be activated, increasing the mutation rate and restructuring the genome (Wessler 1996; Takeda et al. 1999). DNA methylation was the first process to be associated with inactivation of TEs and REs. Later evidence has confirmed the necessity of this process (Brutnell and Dellaporta 1994; Fedoroff et al. 1995; Hirochika et al. 2000; Gendrel et al. 2002; Kato et al. 2003).

TEs inserted in or near transcription units can alter gene transcription both quantitatively and qualitatively. Transposon excisions create tremendous allelic diversity by introducing deletions and insertions into genes. Thus, it is true that hosts developed multiple EIS to effectively and stringently control their activity and transposition particularly prior to events leading to meiosis. In plants, somatic cell diversity may itself be a selected feature. Phenotypic variation in the soma can be very beneficial to plants in avoiding herbivory and pathogen attack; one branch that expresses novel chemicals may survive intact while other branches are defoliated (Walbot 1996). Consequently, maintenance of a low level of TE activity in somatic tissues may be selected for based on somatic survival and the fact that flowers arise from somatic tissues. The flowers on the hypothetical branch of novel phenotype may set more seed because photosynthesis locally can support better fruit development. As a

consequence, progeny of these fruit could inherit a novel allele created by TE activity. Somatic selection within plants requires entertaining the idea that the various apical meristems likely to produce flowers are initially identical siblings that diverge as a result of mutation and epigenetic events. According to Walbot (2002), epigenetic regulation of TEs can create epialleles of host genes exhibiting novel quantitative regulation. Maize and other plants are thought to tolerate TE activity because the host can epigenetically silence transposons and also because haploid gametic selection reduces the impact of deleterious mutations (Walbot 1985). The utility of TEs in creating allelic diversity within the plant soma is proposed as an explanation for their retention in the genome. However, it is clear that the plant host is dominant to the TEs since their majority is transcriptionally and transpositionally inactive. There must have been an effective selection for host control on them during evolution. This might also suggest that multiple controlling mechanisms are operating to repress them and explain the high frequency of transgene silencing since transgene incorporation at aberrant sites resembles transposon insertion. Periodic transposon activation involves only a small part of the TEs and a few individuals in a population. Despite the potential beneficial role of TE-mediated diversification of gene expression, it seems that host suppression of all TE activities is the major outcome of the relationship between transposons and their host plant (Walbot 2002; Medstrand et al. 2005; Vitte and Panaud 2005). The importance of studying and understanding the role of TEs and REs as source of new important variants in plant breeding stems from additional recent findings where a TE family, the helitron, cause genomic sequence insertions in maize by an unknown mechanism. These elements can create multiple partial copies of genes or pseudogenes (Morgante et al. 2005). Such helitron actions definitely contribute to the lack of gene colinearity observed in modern maize inbreds (Lal and Hannanah 2005). The study of the helitron family of TEs with members specific to B73 and Mo17 maize inbred lines revealed also an active helitron that, significantly, produces an alternatively spliced and chimeric transcript joining together genic segments of different chromosomal origin contained within the helitron (Lai et al. 2005). This transcript potentially encodes up to four open reading frames. Thus, transcribed helitrons containing multiple gene fragments may occasionally give rise to new genes with novel biochemical functions by a combinatorial assembly of exons. In this way helitrons not only constantly reshape the genomic organization of maize and profoundly affect its genetic diversity, but also may be involved in the evolution of gene function (Brunner et al. 2005). Conclusively, comprehension of genome function could be better achieved

by realizing that repetitive elements are responsible on many occasions for organization and architecture of the genome (Morgante 2006).

B. Epigenetic Mechanisms and Stress

Throughout their life span, plants continually encounter some kind of stress imposed by their environment. Due to their sessile nature, their ability to survive is based on their ability to withstand the consequences of stress and rapidly adapt to such stimuli without relocating themselves to a more favorable environment. For this reason plants evolved sophisticated physiological mechanisms to cope with adversity, either being prepared to face a stressful condition by having accumulated protective metabolites or responding it by deployment of a specific defense. It is well established that the cells contain metabolites, many of which represent preloaded defenses as, for example, antioxidant molecules and antimicrobial compounds. Also, both biotic and abiotic challenges initiate specific signaling cascades, which lead to fast responses for deployment of specific defense mechanisms and/or changing their asic architecture during development in response to such stimuli. How plants integrate endogenous developmental programs with environmental signals and then determine cell fates rapidly enough to ensure survival is not known, but recent experiments have revealed that epigenetic mechanisms play important roles (Costa and Shaw 2006).

Another emerging aspect of stress tolerance and adaptation involves not only the physiological and developmental responses just described, but also genomic responses. Rapid changes in plant genome size have been documented historically without understanding a mechanism that could achieve these rapid size changes (Walbot and Cullis 1985). These genomic changes could be made in response to stress derived from genome changes that have adverse effects on the genome itself (genomic stress), or their cause and origin may be outside of the genome (e.g., environmental). McClintock (1984) introduced the concept of ''genomic stress,'' which can be used to distinguish between stress responses at the physiological level and ''programmed responses to threats that are initiated within the genome itself'' that lead to genomic modifications. These threats may encompass chromosome rearrangements, fragment loss and gain, and transposition of mobile elements, all of which are genetic (since they affect the primary DNA sequence), follow Mendelian inheritance, and are correlated with modifications of the transcriptional activity of many genes. Interestingly, such genomic structural changes may also be induced by environmental factors of either biotic or abiotic nature. There is evidence for transposon changes in barley cultivars

where retrotransposon contribution is different at an examined chromosome location or in the genome as a whole. A study by Kalendar et al. (2000) has correlated the copy number and distribution of the *Bare1* elements of barley with specific environmental conditions, suggesting that dynamic changes in copy number can occur quickly and be stabilized in contiguous populations. Furthermore, brief low doses of UV-B radiation are sufficient to activate quiescent DNA transposons such as Mu elements of maize (Walbot 1999). Perhars more revealing for the role of epigenetic phenomena in the transgeneration memory of stress in plants are the results obtained recently by Molinier et al. (2006). They have shown that *Arabidopsis* plants resulted in high somatic homologous recombination after a UV-C or bacterial flaggelin transgene stress and, more important, these increased levels of somatic homologous recombination persist in the subsequent generation even when the stresses were removed. According to McClintock (1984), genomic ''responses, now known to occur in many organisms, are significant for appreciating how a genome may reorganize itself when faced with a difficulty for which it is unprepared.''

It is evident that these responses to a changing environment have also an epigenetic component (Richards 2006). Alteration of DNA methylation and histone methylation and acetylation may result in response to stress. As mentioned, methylation in plants may be meiotically heritable over many generations and reset the chromatin landscape, causing genome-wide effects. Thus, stress can cause not only physiological but also genomic responses, which in many cases involve or are of epigenetic nature. The paragraphs that follow discuss recent data regarding epigenetic components of stress that are relevant to stress tolerance in plants. The focus is on examples of genomic stress derived from interspecific hybridization and polyploidization, tissue culture, and environmental stress of biotic and abiotic origin. These proved to be major forms of stress able to cause epigenetic genome-wide alterations. The implications of these in the improvement of plant tolerance or defense to various stresses are dealt in Section V.

Polyploidization may encompass duplication of a genome (autopolyploid) or may arise from the fusion of two or more different genomes (allopolyploidy). Polyploidy is an important cause of plant evolution and speciation, and might be more common in plants than in animals, because plants produce powerful spindle inhibitors, such as nicotine and colchicine as a defense against herbivory. Furthermore, plants have few mechanisms to control their temperature; thus, subjection to heat and cold shocks might also promote polyploidy by inhibiting spindle formation (Cronk 2001). Many plants are obvious polyploids, as

judged from chromosome number and chromosome behavior. Although *Arabidopsis*, with its five chromosomes, is not an obvious polyploid, whole-genome sequencing reveals that 60% of its genome is segmentally duplicated, for which a polyploidization event is the most likely cause. Bowers et al. (2003) proposed that *Arabidopsis*, like maize, is ancient tetraploid, and further putative polyploidy events are discernible in its genome. Similar conclusions have been obtained for poplar after the completion of its genome sequence where a polyploidy was revealed. All land plants and many algae will probably have polyploid events in their ancestry. A change of ploidy number is a severe stress for the genome and is associated with changes in the patterns of gene expression and altered phenotypes. In autopolyploids, it is expected that an increase of the number of loci could be related with increase in gene expression. This has been observed, for example in maize, where gene expression for several loci increased as ploidy increased (Guo and Birchler 1994), suggesting that gene expression and copy number are directly related in autopolyploids. However, epigenetic silencing can also occur in autopolyploids, as observed in *Arabidopsis* (Mittelsten Scheid et al. 1996). Later these investigators reported that interaction of active and inactive epialleles of a transgene in tetraploid *Arabidopsis* lines resulted in heritable gene silencing persisting after segregation from the inactivating allele, in a manner resembling paramutation. Further analysis indicated that hypermethylation was associated with silencing of the previously active allele and suggested that a functional chromatin remodeling activity was required for maintaining the silenced state (Mittelsten Scheid et al. 2003; Chen 2007).

In allopolyploids, balanced expression of two or more genomes in one cell requires extensive coordination of gene expression. This is accomplished in early generations of allopolyploid formation, and established allopolyploids display phenotypic and genotypic stability. However, allopolyploids recently produced artificially by scientists lack the stability of their naturally established ancient counterparts. Reestablishment of a balance in gene expression between the parental genomes is achieved by silencing of many genes that sometimes correlates with hypermethylation of the silenced loci (Lee and Chen 2001; Madlung et al. 2002). But in other cases, both up-regulation and down-regulation in gene expression have been observed. Some changes are specific to one of the parental genomes (He et al. 2003), and gene silencing is correlated with increased DNA methylation at the affected loci (Shaked et al. 2001). These changes in DNA methylation and gene expression using synthetic allopolyploids have also been studied in *Arabidopsis* (Madlung et al. 2002), wheat (Kashkush et al. 2002, 2003), and

cotton (Adams et al. 2004) among other plant species. Many expression alterations have been documented including up- and down-regulation of genes that can arise by the onset of polyploidization or in the course of subsequent generations. A subset of these has been found to have epigenetic causes (Rapp and Wendel 2005). All these alterations in gene expression may account for novel aberrant phenotypes that appear when a change in ploidy number is used for generating new genotypes in plant breeding (Wendel et al. 2002; Osborn et al. 2003). Allopolyploid plants can be considered a special type of hybrids where two (or more) homologous genomes are fixed in one nucleus, maintaining their integrity through sexual reproduction. Therefore, the advantage of hybrid vigor and heterosis can be fixed by polyploidization, in contrast to the wide recombination of the parental genomes in segregating progeny of diploid hybrids, which is one of the reasons for inbreeding depression (Fasoula and Fasoula 1997b).

There is evidence that allopolyploidization and hybridization may cause transposon activation (Madlung and Comai 2004). Epigenetic changes in methylation and transcription were reported in rice following alien DNA introgression from *Zizania latifolia*, a related but differentiated species (Liu et al. 2004). The affected sequences included low-copy cellular genes, TEs and REs, suggesting that the alterations likely reflected a general and genome-wide phenomenon. In artificial allopolyploids formed recently between *Arabidopsis thaliana* and *Arabidopsis arenosa,* there was transcriptional activation of a repeat unit with similarity to transposons (Comai et al. 2000). Retrotransposon activation was also observed in rice hybrids (Liu and Wendel 2000) and in polyploid wheat (Kashkush et al. 2003). Some genes adjacent to transposon LTRs in wheat had also altered expression patterns, supporting the hypothesis that hybridization could activate previously silent genes altering heterochromatic regions. Direct evidence for hybridization-induced transposition via demethylation of elements, however, has not been reported so far (Madlung and Comai 2004). By extension, diploid hybrids may also be considered as a special case of allopolyploids, where haploid genomes that may retain significant disparity at various allelic chromosomal locations have to cooperate in a single nucleus and could display the range of atypical gene expression and epigenetic regulation patterns observed in early generations of real allopolyploid plants. Indeed, significant differences have been identified recently between inbred lines of maize with different origins, where large parts of DNA present in one line at a chromosomal position are missing the other and vice versa (Lee et al. 2002; Brunner et al. 2005; Buckler et al. 2006; Messing and Dooner 2006). This might account for

incompatibilities between certain inbred lines and partly affect inbreeding depression observed in segregating populations of F1 hybrids.

There are also data that link tissue culture conditions with stress due to chemical and physical causes, such as wounding, exposure to hormones in the tissue culture medium, and pathogen infection (Madlung and Comai 2004). Somaclonal variation, a frequent result of tissue culture, has been attributed to changes in DNA methylation pattern, activation of transposable elements or retrotransposons, and chromosome remodeling (Kubis et al. 2003). Both hypomethylation and hypermethylation of endogenous single-copy and repeated-gene loci as well as transgene sequences have been reported to occur in calli (Olhoft and Phillips 1999; Jaligot et al. 2000; Kaeppler et al. 2000; Koukalova et al. 2005).

There appears to be mounting evidence for an important role of an epigenetically regulated network of defenses operating in plants during virus infections (see Section III.E). But is there a role of epigenetic mechanisms in infections by other pathogens, such as fungi and bacteria? Data on this issue are scarce, although indirect evidence may provide some clues. Pathogen recognition in plants is almost concomitant with production of reactive oxygen species (ROS) known as oxidative burst, which results from the action of a family of respiratory burst oxidase homolog (Rboh) proteins that encode the key enzymatic subunit of the plant NADPH oxidase (Torres and Dangl 2005). In addition to their well-established signaling roles in plant defense responses, ROS produced during the oxidative burst impose a severe stress termed *oxidative stress* and can damage a variety of biomolecules including DNA itself. This should evoke DNA repair mechanisms that may affect epigenetic patterns and genomic remodeling. Pathogens are not the only elicitors of oxidative stress in plants. Abiotic stress factors can also lead to an increase of ROS and possible epigenetic effects of ROS overload in the cell. Cassells and Curry (2001) reviewed the effects of oxidative damage in eukaryotic cells and cited examples of altered DNA methylation, changes in chromosome number from polyploidy to aneuploidy, chromosome strand breakage, chromosome rearrangements, and DNA base deletions and substitutions. Such changes could explain, at least in part, the range of variability found in plant cells, which is revealed as somaclonal variation in tissue culture.

One of the most common oxygen radical–induced base alterations in DNA is formation of the oxygen radical adduct 8-hydroxyguanosine (8-oxoG) (Cerda and Weitzman 1997). Since there is no known enzymatic mechanism capable of producing 8-oxoG, the only route of its formation in the cell is through oxidative modification of guanine. The oxidation of guanine to 8-oxoG converts the N7 position of guanine from a hydrogen

bond acceptor into a hydrogen bond donor and replaces the 8-hydrogen with an oxygen atom. Replacement of guanine with 8-oxoG alters methylation of adjacent cytosines. The methyl groups of both thymine and 5mC are also susceptible to oxidation. The oxidation of 5mC can generate 5-hydroxymethylcytosine (HmC), 5-formylcytosine, and 5-carboxycytosine. Each modification could potentially interfere with the recognition of the methyl-CpG dinucleotide by methylation binding proteins (see Section II.A). The replacement of a 5mC residue with HmC or the replacement of guanine by 8-oxoG reversed the increase in binding affinity afforded by a 5mC residue (Valinluck et al. 2004). Therefore, oxidation of 5mC to HmC or guanine to 8-oxoG was shown to inhibit MBPs binding, which could affect chromatin structure and result in potentially heritable epigenetic alterations (Zemach and Grafi 2007).

In summary, plant stress responses, although programmed and inducible, may be not enough under certain conditions to ensure survival. These extreme and unpredictable challenges could lead to epigenetic reprogramming of major defense pathways that might provide additional means for their protection and perhaps generation of new variation in their progeny, increasing their chances of survival under the harsh conditions imposed. Plants take advantage of all the three epigenetic mechanisms and combine genetic and epigenetic solutions for enhanced stress tolerance and adaptation.

C. Paramutation in Plants

Paramutation is an allele-dependent transfer of epigenetic information that results in the heritable silencing of one allele by another (Chandler and Stam 2004). In plants, the term *paramutation* was coined by Brink (1956) to describe a heritable change in gene function directed by its allele (Kermicle 1996; Martienssen 1996; Richards 1997). The expression of the one allele, referred to as paramutable, changes following paramutation, thus violating Mendel's first law. The allele that induces the change is called paramutagenic. Once the expression of the paramutable allele has been modified, it is called paramutated, which is designated by an apostrophe after the gene name, for example, *R-r'*. Alleles that do not participate in paramutation are called neutral alleles. When the paramutable/paramutagenic heterozygote is crossed to allow segregation of the two alleles, virtually 100% of the paramutable alleles transmitted display a decrease in expression. This decreased expression level (the paramutant phenotype) persists through many generations. Since there is no change in DNA sequence, but rather there are changes in DNA methylation or chromatin structures, paramutation is classified

as a classical epigenetic phenomenon, and the paramutable and paramutant alleles are called epialleles.

The paramutation phenomenon seems to be associated with a small number of alleles. However, the characteristic traits of paramutant alleles, including high-frequency penetrance and in some cases reversibility of traits, make this phenomenon interesting for breeders. The unknown epigenetic mechanism of interaction, where paramutagenic alleles influence homologous paramutable alleles *in trans*, leads to questions of how one allele influences another, how big the impact of that mechanism is in overall gene regulation, and why such a mechanism would evolve. One answer may be that paramutation is a fast way to adapt easily to a changing environment. The first report on paramutation phenomenon was in *Pisum sativum* (Bateson and Pellew 1915), soon followed by similar observations in other plants like snapdragon (Krebbers et al. 1987) and petunia (Van Houwelingen 1999). The most extensive research has been done in maize (Stam and Mittelsten Scheid 2005). Furthermore, similarity has been observed between paramutation of the *R* locus of maize and the behavior of *A1* allele in certain transgenic petunia plants (Meyer 1996). Other transgenic loci behave like paramutants (Qin and Von Arnim 2002; Mittelsten Scheid et al. 2003), suggesting a link between paramutation and other epigenetic regulation of gene expression. Thereby paramutation can be redefined as *trans*-inactivation between homologous sequences that leads to a high frequency of heritable changes in the gene expression of one of the sequences (Stam and Mittelsten Scheid 2005). The extensive research on this phenomenon has revealed great specificity in behavior of paramutant alleles or great variation between paramutagenic loci in the amount of stability and penetrance (Chandler and Stam 2004). For example, the maize *booster1* (*b1*) gene has been shown to be fully penetrant and highly stable while most other alleles do not show complete penetrance. Maize *b1* gene, which expresses a basic helix-loop-helix transcription factor, is required for activation of anthocyanin pathway in plant. The paramutable form, *B-I*, loses the heritable color phenotype when in a hetrezygotic condition with paramutagenic *B'*, the colorless form of allele. There are alleles neutral to paramutation and alleles that act as stabilizers for paramutable alleles, such as the *b1* gene; but on the contrary, the heterzygosity of another paramutagenic allele, *Pl'*, with a neutral allele leads to loss of paramutagenic strength. Despite the penetrance of the new trait—for example, 100% for the *b1* gene—not all paramutants are so efficient in establishing this new epigenetic state, nor are they so stable. Recombination mapping has defined a 6 kb region located 100 kb upstream of *b1* promoter that included seven tandem

repeats of an 853 bp sequence that is otherwise unique in the genome, whereas neutral alleles only have one copy of this sequence (Stam et al. 2002). DNA sequences are identical in *B-I* and *B'*, indicating that epigenetic mechanisms mediate the stable transcriptional silencing that is associated with *b1* paramutation. The variable transcriptional silencing of the locus is brought about by differentially heterochromatization of the tandem repeats upstream of the locus causing greater DNaseI hypersensitivity in *B'* (silenced) relative to *B-I* (the transcriptionally active allele), where repeats are hypermethylated and have an open chromatin structure. In newly formed *B'* alleles, chromatin structure was intermediate between *B-I* and *B'*.

There are four maize mutants that abolish paramutation: required to maintain repression loci, *rmr1*, *rmr2* and *rmr6* and one named *mediator of paramutation1* (*mop1*) (Hollick et al. 2005).

Recently Alleman et al. (2006) reported the cloning of *mop1,* an RNA-dependent RNA polymerase gene most similar to the RNA-dependent RNA-polymerase (RDRP) described for siRNA production and gene silencing that is again strongly indicative for the critical role played by epigenetic mechanisms in manifestation of paramutations. The same group also reported that transcriptionally silenced transgenes in maize are activated by mutations defective in the paramutation epigenetic mechanism (McGinnis et al. 2006), indicating again that the two phenomena are epigenetically related. There are two major theories proposing two different mechanisms for how homologous sequences can interact in *trans*, bringing about paramutation. One proposed mechanism involves siRNA to mediate chromatin changes. Repeats that would give rise to siRNA are involved in several paramutation phenomena (Walker and Panavas 2001; Stam and Mittelsten Scheid 2005) but not all (Qin and Von Arnim 2002). The second proposed mechanism involves pairing between homologous sequences where paramutagenic locus is proposed to transfer its own transcriptionally inactive state onto the paramutable counterpart via pairing of homologous sequences (Chandler and Stam 2004; Grant-Downton and Dickinson 2004). A most complex model has emerged where the position of the gene and epigenetic memory could direct genes in organizer and transcription factory (Chakalova et al. 2005).

D. Parental Imprinting

Parental imprinting refers to the process whereby only one of parental alleles, either the maternal or the paternal one, is active in an offspring (De Chiara et al. 1991). An epigenetic modification, which must be

mitotically stable, inactivates one of the parental alleles. Therefore, we have the parent-of-origin expression effect where one allele is inherited in a silent state and is not expressed; the other allele is inherited in an active state and consequently is expressed. Gene imprinting has been found both in animals and plants. The first imprinted gene to be reported in mouse was the *insulin–like growth factor-2* (*Igf-2*) by De Chiara et al. (1991). Since then analysis of human and mouse chromosomes has revealed a large number of imprinted genes in these organisms (Kelsey et al. 1999). In mouse, nearly all imprinted genes are associated with differential DNA methylation of maternal and paternal alleles (Brannan and Bartolomei 1999; Feil and Khosla 1999). One of the best-characterized regions of the mouse genome contains a cluster of imprinted genes including *Igf-2*, *H19,* and *Snrpm*. Inspection of this region revealed the presence of differentially methylated regions in or near the imprinted genes. It was recently shown that in mammals, DNA methylation of large (up to 100 kb) specific intergenic regions, named imprinting control centers (ICR), regulate the expression of imprinted genes (Delaval and Feil 2004). For the *Igf2* gene, it was also shown that the *Igf2* paternal allele is silenced by a mechanism involving DNA methylation, an sRNA and the PcG gene *Eed*. Conversely, during female gametogenesis, methylation of the maternal *Igf2* does not occur, and as a consequence the maternal allele is expressed (Delaval and Feil 2004).

In plants, mutations that have either sporophytic or gametophytic parent-of-origin effects have been reported recently in *Arabidopsis*. One class of genes found to be maternally expressed is the *FIS* genes that have been mentioned (see Section II.B), such as *MEA, FIE, FIS2,* which play an essential role in seed development. The study of the MEA/FIE/FIS2 complex has demonstrated that parental imprinting plays a central role in the transcriptional regulation of the *FIS*-class genes (Baroux et al. 2002). It was also mentioned that the target of the *MEA* gene is the gene *PHERES1* (*PHE1*), a MADS box type I gene (Köhler et al. 2003b). *PHE1* is predominately expressed from the paternal allele during seed development whereas the maternal allele is also expressed but maternal transcript levels are much reduced (Köhler et al. 2005). Moreover, it was shown that *PHE1* is only expressed after fertilization. This was the first report of a paternally imprinted gene in plants.

A DNA glycosylase, *DEMETER* (*DME*), is responsible for the activation of the maternal allele *MEA* before fertilization (Choi et al. 2002). Four *met1* (mehtyltransferase1) mutant alleles were isolated in an effort to identify suppressors of *dme* (Xiao et al. 2003). It was suggested that *MET1* is required to maintain cytosine methylation of the *MEA* promoter and that it acts antagonistically to *DME*. Parallel studies with *FIS2*

parental imprinting (Jullien et al. 2006) are also revealing for the essential role of DNA methylation for parental imprinting. This work shows that maintenance of FIS2 imprinting depends on DNA methylation whereas loss of DNA methylation does not affect *MEA* imprinting. Recent reports have shown that *MEA* imprinting is under self-regulation. This is achieved by *DME*-mediated specific demethylation (activation) of the maternal *MEA* allele (Gehring et al. 2006) and by maternal *MEA*-mediated histone methylation (silencing) of the paternal *MEA* allele (Baroux et al. 2006; Jullien et al. 2006). It seems that the other components of the PcG complex are involved in *MEA* autoregulation as *fis2, fie, and msi1* mutants express the paternal *MEA* allele. *MEA* seems to be silenced during the vegetative and male gametogenesis phases of the life cycle of the plant by PcG complexes. In the endosperm, *MEA* expressed from the maternal allele maintains the silencing of the paternal *MEA* allele. In this way *MEA* ensures complete maternal control of *MEA* expression from both parental alleles. Another gene, *FWA*, which encodes a homeodomain transcription factor, has been also reported to be parentally imprinted in *Arabidopsis* (Kinoshita et al. 2004). The maintenance of endosperm-specific and parent-of-origin–specific *FWA* expression depends on *MET1*. The 5' region of the *FWA* contains direct repeat sequences that are hypermethylated in all tissues except the tissues that *FWA* maternal transcript is present (i.e., the female gametophyte and the developing endosperm where the *FWA* promoter is hypomethylated). Furthermore, in the *fwa-1 Arabidopsis* mutant, the 5' region of the *FWA* gene is hypomethylated, and this is associated with ectopic expression of FWA in vegetative tissues (Soppe et al. 2000).

These studies indicate that unlike mammalian imprinting, the imprinting of *MEA* and *FWA* in plants is not established by allele-specific *de novo* methylation but rather by maternal-specific removal of methylation, which is dependent on the activity of the DME DNA glycosylase in the maternal gametophyte. This demethylation leads to specific activation of imprinted genes in the maternal gametophyte. Since the endosperm degenerates during seed maturation or germination, it does not contribute to the genetic or epigenetic information of the next generation. Therefore, the epigenetic state of the endosperm does not need to be reprogrammed, which means that the demethylated and thus activated genes need not be silenced again.

An additional aspect of the regulation of the *FWA* gene imprinting came to light recently. Analysis of the 5' region of the *FWA* gene revealed that it contained retrotransposon-derived tandem repeats that correspond to siRNAs (Lippman et al. 2004). In the same study a genome-wide analysis was performed by ChIP in *Arabidopsis* in order

to define heterochromatic and euchromatic states of chromatin along large regions of the genome. It was shown that heterochromatin is composed of transposons and their related repeats, and they are controlled by the chromatin remodeling factor DDM1. Some of these transposon sequences correspond to siRNAs, suggesting that siRNAs may direct DDM1 to modify chromatin. Considering these results, it is possible that the regulation of *FWA* and other imprinted genes could be governed by transposon-derived sequences and siRNA-dependent DDM1 or MET1 activity.

Many genes with phenotypes and mRNA accumulation patterns similar to MEA are not completely or selectively imprinted in the seed. These include genes that mediate seed phenotypes due to a requirement for expression in the gametophytes (Yadegari et al. 2000; Xiao et al. 2003). Thus, before differential expression can be interpreted as the result of imprinting, differential transcription in the zygote or endosperm must be demonstrated.

Different proposals that are not mutually exclusive in every case of the genes studied have been made to explain imprinting. These proposals include the purifying selection hypothesis already described, which supports the view that any mechanism, imprinting included, leading to haploid phase and allele exposure to selection, could have a purifying selection efficiency, decreasing the mutational load, particularly from TEs and REs activities (Walbot 2002; 2004). The parental conflict proposition also presents an attractive mathematical model proposing that imprinting is driven by conflicts of interest between the mother and father over resource allocation. Imprinting arises when half-sibling embryos from multiple-pollen parents compete with each other on the same mother. Imprinting of growth regulators can serve the interest of polygamous mothers via equal growth of all their progeny, whereas the interests of competing fathers are best served by preferential treatment of progeny carrying their own genes, at the expense of maternal half-sibs (Wilkins and Haig 2003).

The failure of endosperm to cellularize and the runaway cell proliferation in mutants of paternally silent genes, such as *MEA* and the *FIS*-class genes, could result in increased resource demand in these seeds and therefore are consistent with the parental conflict hypothesis. The same could be true for effects of reciprocality on interploidy crosses (see Section V.F). But the conflict model is incompatible with the differential expression data (Wilkins and Haig 2003), which is a widespread phenomenon during seed development (Dilkes and Comai 2004). On the contrary, a differential dosage sensitivity hypothesis in which the dose of a gene product determines the phenotype is

compatible with the wide occurrence of differential expression during seed development (Dilkes and Comai 2004). The dosage hypothesis relies on a dosage sensitivity described by Birchler et al. (2001) according to which it arises at regulatory pathways because the ''stoichiometric relationship of the components of regulatory complexes affects target gene expression.'' In other words, genes causing dosage effects are expected to encode the subunits of macromolecular complexes, and a decrease in one component affects the function and assembly of the whole complex (Vietia 2002). The balance hypothesis applies well to the dosage-sensitivity interpretation of parent-of-origin effects in interploidy crosses (Dilkes and Comai 2004).

E. Viral-Induced Silencing

PTGS can be induced in plants by viral vectors harboring specific genes through the virus-induced gene silencing (VIGS) (Dalmai 2005). Plant viruses have different morphologies, genetic structures, vectors, and host range. Their genome may be single stranded (ss) or double stranded (ds) DNA and ss or dsRNA. However, over 90% of plant viruses have ssRNA genomes that are replicated by a virus-encoded RdRP to produce both sense and antisense (termed plus-strand and minus-strand), and thus accumulate a traceable amount of dsRNA within the plant.

When a plant is infected with unmodified viruses, the VIGS mechanism is specifically targeted against the viral genome. However, with virus vectors carrying a copy of an insert derived from host genes, the process can be additionally targeted against any native or homologous transcripts (Lu et al. 2003). Many groups have used VIGS in order to study the function of a variety of plant genes (Baulcombe 2005). Moreover, VIGS has facilitated the study of resistance gene pathways (see Section V.F). The VIGS technology was applied in tomato for silencing the *constitutive triple response 1* and *2* (*CTR1* and *2*) genes that negatively regulate ethylene responses, by introducing the *Arabidopsis CTR1,2* (Liu Y. et al. 2002). Although there is no experimental evidence so far, it is generally assumed that 85% nucleotide identity would be the lowest limit for triggering the VIGS mechanism (Benedito et al. 2004). Moreover, conserved boxes of gene families can potentially trigger silencing of other members concomitantly (Ratcliff et al. 2001). Overexpression of the *petunia flowering gene* (*PFG*) induces cosuppression of *PFG* itself and of *flowering binding protein 26* (*FBP26*), which shares high overall sequence homology with *PFG* (74%), particularly within the MADS box (88%) (Immink et al. 1999). Consequently, it can be hypothesized that the sequence homologies found in conserved

boxes of gene families are more important for multiple gene silencing than are their overall homologies for VIGS mechanism. Recent findings by Yang and his colleagues (2007) showed that virus-induced gene silencing of NbBTF3 that encodes a *Nicotiana benthamiana* homolog of human BTF3 triggered both leaf yellowing and abnormal leaf morphology but no modification in the development of the plant. In summary, the VIGS system can be used in reverse genetics without requiring plant transformation, thereby allowing the study of multiple genes. Additionally, the procedure is rapid—the VIGS phenotype develops within one or two weeks instead of months or even years when the traditional methods that require transformation procedures are used. Most important, the target mRNA is not silenced until the virus vector infects the plant. It is possible therefore to suppress essential genes for host cell growth and development (Peele et al. 2001). It is important, however, to take into consideration that, until now, this system has been efficiently used in only a few plant species, such as *Nicotiana benthamiana*, tomato, and barley. Nevertheless, the increasingly developing novel vectors will augment the number of species that respond efficiently to VIGS.

In order to protect themselves from degradation, viruses have evolved RNAi inhibitors, which allow them to overcome plant RNAi action and successfully infect the plant. This has been demonstrated with the identification of the potyviral Helper component-Proteinase (HcPro) (Anandalakshmi et al. 1998; Brigneti et al. 1998; Kasschau and Carrington 1998) and the 2b protein of CMV (Brigneti et al. 1998) that suppress PTGS targeting different components in the silencing mechanism. Strong suppressors could prolong and increase virus accumulation, whereas a weak suppressor could not allow a high level of virus accumulation. Lakatos and his colleagues (2004) demonstrated that a viral protein of tombusviruses (p19) inhibits RNA silencing in vivo by strongly binding siRNA in virus-infected cells. When the silencing of the RNAi mechanism was suppressed by p19 in tobacco plants, the expression of various transgenes in transient expression assays was increased 50-fold (Voinnet et al. 2003). Recent findings by Mlotshwa et al. (2005) demonstrate that developmental defects in the viral silencing suppressor P1/Hc-PRO result from the aberrant Dicer activity, suggesting a possible role of Dicers in development, independent of target RNA degradation, and proposing a possible mechanism of surpassing the viral suppression of RNA silencing. New light was shed recently on the mechanism underlying induction and suppression of RNA silencing in antiviral defense by Deleris et al. (2006). These authors showed that DCL4 and DCL2 exhibit specific hierarchical activities in antiviral silencing. DCL4 is the one to exert its antiviral effect first by

producing 21-nt siRNAs, which recruit an antiviral RISC complex. DCL2 also forms 22-nt siRNAs with antiviral activity, but only when DCL4 is inhibited by a viral suppressor. These findings will assist in elucidating the mechanism of the poorly understood antiviral defense process in plants.

F. Transgene Silencing

The spread of techniques that allow introduction and functional expression of foreign genes in plant cells enabled the production of transgenic plants with improved insect and disease resistance, superior quality of seeds and fruits, and increased tolerance to extreme environmental conditions. Vaccines against serious human diseases have also been developed using transgenic plants (Herrera-Estrella et al. 2005). However, the frequent use of gene transfer methods has revealed that many transgenes do not express as expected. Early findings by Matzke et al. (1989) demonstrated that transformation can induce epigenetic phenomena. The copy number of a transgene that integrates into the genome is unpredictable, as is the integration site. Thus, one or multiple copies can integrate at one or multiple loci. Transgene silencing could take place through TGS or at a PTGS. Transgene-induced silencing phenomena in plants could be observed either *in cis*, when it refers to the silencing of transgene expression integrated at a single locus, or *in trans,* when it refers to the silencing effect of one locus on another. Transcriptional *cis*-inactivation can result from the insertion of multiple, rearranged copies of a transgene at a single locus that is located in or next to heterochromatin regions. Therefore, methylation may spread from adjacent sequences into the transgene, leading in this way to silencing (Vaucheret et al. 1998). Moreover, transcriptional *cis*-inactivation may occur when closely linked copies of transgene can attract TGS inspection systems like methylation or heterochromatin-forming proteins although they integrate at a hypomethylated locus. Such RIGS correlate with increased methylation and changes in chromatin configuration (Assaad et al. 1993; Ye and Signer 1996). *Cis*-inactivation can also take place in some instances when single copy of a transgene is inserted at a hypomethylated locus. This phenomenon can be due to the difference in sequence composition between the transgene and the integration region (i.e., when a transgene derived from a monocotyledonous plant was introduced into a dicotyledonous plant), leading to methylation of sequences, which the plant recognizes as foreign (Elomaa et al. 1995). This form of TGS transgene inactivation also involves increased methylation, suggesting again that hypermethylation and chromatin conden-

sation are general characteristics associated with transcriptional silencing. TGS *trans*-inactivation can result from the effect of one transgene on another (i.e., an active copy of a transgene can become repressed by another silenced homologous gene, either linked or not,) and can acquire the capacity of inactivating other copies in subsequent crosses (Meyer et al. 1993). *Trans*-inactivation can affect any transgene that is expressed under the control of the same promoter regardless of the coding region. Sequences as short as 90 bp are sufficient for mediating silencing (Vaucheret 1993). Previous studies proposed that this type of inactivation requires interaction of the sequencing locus with the target sequence by direct DNA-DNA pairing between the loci, resulting in a transfer of the silenced state from one locus to another, or that aberrant promoter transcripts can cause RNA-directed DNA silencing (Mette et al. 1999). Recent findings suggested that siRNAs, produced by a miRNA-directed RdRP, could mediate methylation of genomic DNA (Ronemus and Martienssen 2005). PTGS is occurring when RNA does not accumulate even though transcription occurs. As opposed to TGS, which is meiotically heritable, PTGS is reset after meiosis (Vaucheret 1993; Park et al. 1996). Another difference is that methylation of the promoter region typically results in TGS transgene silencing whereas coding region methylation results in PTGS transgene silencing (Iyer et al. 2000). For many years TGS transgene inactivation was linked to a modification of the epigenetic state of the transgene while the PTGS transgene inactivation was related to RNA degradation. However, this distinction is under consideration after recent findings that suggest the involvement of RNA molecules in the induction of transgene methylation and TGS events such as chromatin modification (Matzke et al. 2004; Depicker et al. 2005). What can be assumed from studies in plants is that the presence of inverted repeats and multiple copies of transgenes is often associated with PTGS. It is believed that ectopic DNA-DNA or DNA-RNA pairing or transcription of inverted repeat structures and simultaneous expression of both sense and antisense transgenes induce the initiation of RNA silencing either by the formation of aberrant RNA transcripts that activate silencing or by the accumulation of normal transcripts that ''exceed'' a threshold level (Lindbo et al. 1993; Muskens et al. 2000; De Buck et al. 2001).

For RNA silencing events, several transgene characteristics can trigger the induction of RNA silencing. The composition of the transgene (promoter selection, promoter strength, transgene stability) determines whether a single-copy transgene will trigger RNA silencing. The possibility of silencing seems higher in homozygous lines for the transgene. Moreover, transformed plants with multiple transgene copies have a

higher possibility of being silenced at a posttranscriptional level, implying that there is a higher likelihood of production of threshold levels of transgene transcripts generating RdRP-mediated primary silencing signals and for inverted-repeat arrangements.

Several studies from various groups on transgene-induced cosuppression in *Nicotiana tabacum* and *Arabidopsis* provided the first evidence that PTGS might play a role in phase change (De Carvalho et al. 1992; Elmayan and Vaucheret 1996; Elmayan et al. 1998). The silencing of transgenes and any endogenous gene by its cognate siRNA, caused by the overexpression of a sense transcript, is known as the phenomenon of cosuppression. Transgenes expressed under the control of the 35S promoter in young seedlings were often downregulated in successive leaves until little or no transcript remained. The level of gene expression determined the degree of suppression: Whereas homozygous transgene transcription was inhibited, hemizygous transgene transcription continued to be active (De Carvalho et al. 1992; Elmayan and Vaucheret 1996; Elmayan et al. 1998). Similar to the inheritance of Pl-Blotched, silencing seems to be reset at every generation, as judged by the fact that the progeny and parents shared the same developmentally regulated transgene-silencing pattern. Finally environmental effects may cause transgene silencing; several reports demonstrated that transgenes were silenced when they were grown in the field but not in the greenhouse (Brandle et al. 1995).

RNA silencing in plants can produce a systemic signal that triggers degradation of homologous RNA at long distances through the plant. The factors involved in such amplification and transmission of the signal are under study via grafting experiments that aim to unravel the mechanisms (Baulcombe 2005; Voinnet 2005b). All these data clearly suggest that transgene silencing still remains a problem to be solved. The phenomenon seems more persistent in plants, probably due to the capacity of plants to fight against invasive DNA and put up with genome duplication.

IV. Epigenetic Mechanisms and Plant Development

A. Plant Development

Despite the over a billion-year dichotomy between plants and animals, there are many common elements in their developmental strategies. The complete genome sequences of animals and plants indicated that, grossly speaking, similar numbers of genes are required for building these multicellular higher organisms. The two groups share the main

developmental strategies, such as cell-cycle, cell-cell signaling, polarity, modularity, as well as basic genetic and epigenetic mechanism of gene and genome control such as DNA methylation, PcG, ATX complexes, and RNAi(Meyerowitz 2002). But there are also fundamental differences in the life cycles of plants and animals.

All plants exhibit at least one form of apical meristem consisting of one or more cells that are functionally analogous to metazoan stem cells because they are histogenetic (i.e., able to generate specialized tissues). Plants, however, differ from animals in that the plant apical meristem has the additional capability of generating organs like leaves and stem, inflorenscences or flowers throughout the life of the plant, whereas the number and form of metazoan organs are embryonically determined. Development continues throughout the life of the plant and proceeds by the often indeterminate, repeated iteration of modules (leaves, roots, and stems). By contrast, animals have a single developmental trajectory, which is completed at maturity and ends with a fixed number of organs. The iteration of these modules is controlled by the signals that plants exchange with the environment. This continuous feedback between development and the external environment is not seen in animals, which, in general, have the power of changing their environment thanks to their capacity to move and their behavior.

The presence of a thick cell wall is also a distinguishing feature of plants. The elaborate cell wall of plants not only makes plants sessile, but also prevents cell movement in plant development. Animal cells can slide past each other to take up programmed developmental positions. By contrast, fate information during development in plants is probably controlled more by cell-cell signaling than by developmental history (cell lineage). As a result, there is no distinction between germ line and soma in plants: the development of germ cells is determined by cell position and not cell lineage (Cronk 2001; Gilbert 2003a).

Au: correct word?

Plants are also characterized by the alternation of generations, a life cycle in which haploid and diploid generations alternate with each other. Very different developmental trajectories, which are linked to ploidy level, are thus contained in the same genome. The specific developmental paths undertaken by haploid spores and diploid zygotes indicate that radically different gene-expression patterns are possible—a phenomenon that might be linked to changes in DNA methylation and epigenetic changes in general. Over evolutionary time from bryophytes, to ferns and angiosperms, there has been a steady reduction in diversity of structures during the haploid phase (Gilbert 2003b). During the course of land plant evolution, heterochronic control mechanisms perhaps shifted the starting point of the diploid phase earlier and earlier in

development at the expense of the earlier haploid organism (Cronk 2001). Gametophytes in flowering plants, for example, are nonphotosynthetic and contain few cell types. Finally, the double fertilization event during sexual reproduction described previously for the production of both embryo and the nutritive tissue endosperm are unique features of flowering plants (Walbot and Evans 2003).

Land plants originated monophyletically from freshwater green algae belonging most probably in the charophycean group. Thus some characteristics of land plants, such as cellulosic cell wall, multicellular body, cytokinetic phragmoplast, plasmodesmata, donated to plants from algae. The origin of a well-defined sporophytic apical stem cell and a system for its proliferation correlated with capacity for organ production and branching occurred sometime between the divergence of modern bryophytes and vascular plant lineages. Roots and their meristem and a multilayered tunica-corpus shoot apical meristem arose later (Graham et al. 2000). It is important to understand these fundamentally unique aspects of plant development, the mechanisms involved, and their consequences. For example, as it was stressed most of DNA transposon-based diversity is generated in somatic tissues; this means that most of the potentially deleterious new mutations and chromosome breaks occur in somatic cells. It is interesting to consider that somatic diversity may itself be a selected feature. Phenotypic variation in the soma can be very beneficial to plants in avoiding herbivory and pathogen attack; one branch that expresses novel chemicals may survive intact while other branches are defoliated (Walbot 1996). Consequently, maintenance of a low level of transposon activity in somatic tissues may be selected for based on somatic survival and the fact that flowers arise from somatic tissues. The flowers on the hypothetical branch of novel phenotype may set more seed because photosynthesis locally can support better fruit development. As a consequence, progeny of these fruit could inherit a novel allele created by transposon activity as well as by the active transposable element system. Somatic selection within plants requires entertaining the idea that the various apical meristems likely to produce flowers are initially identical clones that may diverge as a result of genetic and epigenetic events. Stringent regulation of events preceding meiosis and in the resulting haploid cells could readily be selected based on survivorship in the progeny. In plants, according to Walbot and Evans (2003) and Gu et al. (2003), the gametophytic stage presents an opportunity for natural selection on the haploid genome—as in other haploid organisms, recessive deleterious alleles that would be masked in diploid organisms are removed from the population. In fact, many of the parent-of-origin effects enumerated by

Dilkes and Comai (2004), such as differential expression of genes in the female and male gametophyte, the gametes' delayed expression of genes from one of the other parent in the early zygote development, and differential expression of parental genes in the developing seed, could have a purifying role of a haploid selection scheme, among other roles, such as dosage regulation.

Early data for the significant general role played by DNA methylation during plant development came from examining the effects of genome-wide demethylation in *Arabidopsis*. As mentioned in Section II.AII.A, transformation with an antisense construct of *MET1* resulted in plants with abnormal phenotypes. These included reduced stature, abnormal root morphology, altered leaf size and shape, abnormal floral development, and altered flowering time. The same was later proven to be true for mutations of histone-modifying enzyme genes, such as the *Arabidopsis BRAHMA*, whose silencing results in reduced fertility, curly leaves, homeotic transformations during flower development, and photoperiod-independent early flowering (Farrona et al. 2004). Finally, mutations of genes in the sRNA pathways have similar effects, as exemplified by an allelic series of AGO1 mutations that displayed a complete loss of adaxial/abaxial polarity, ectopic ovule formation, and inversion of polarity in the epidermal cells (Kidner and Martienssen 2005).

The aim of this section is not to describe each developmental step and organ formation. The reader can find detailed information of these topics in excellent recent reviews (Cronk 2001; Walbot and Evans 2003), books (Cronk 2002), or textbooks (Gilbert 2003a). In the following paragraphs an analysis will be undertaken of certain critical plant developmental events or formation of specific organs with emphasis on the particular role played by each EIS.

B. Shoot and Leaf Formation and Development

Flowering plants go through two major developmental life stages: an embryonic stage composed of two phases (early embryonic and late embryonic) followed by a postembryonic stage consisting of a juvenile, adult, and reproductive phase. During their vegetative life (juvenile and adult), plants are composed of three basic organs: shoot, leaves, and roots. Inflorescences and flowers with floral organs such as sepals, petals, stamens, and carpels are the structures formed after the plant makes its transition from vegetative to reproductive phase. Therefore, it is essential to understand shoot and leaf development in order to comprehend full aerial plant morphogenesis.

Shoot meristems are groups of indeterminate cells, which act in a coordinated manner to form the aerial organs. The shoot apical meristem (SAM) initiates during embryogenesis and is a collection of stem cells that reside at the tip of each shoot and are maintained throughout the life of the plant. In *Arabidopsis* the SAM is comprised of several hundred cells that are divided into different functional domains: the central zone (CZ) located at the tip of the meristems composed of slowly dividing stem cells; the peripheral zone (PZ) surrounding the CZ, which is composed of more rapidly dividing cells from which leaf and flower primordia will be formed; and the rib meristem (RM) zone located below the CZ from which cells of the stem form. Axillary meristems initiate from the peripheral zone of the SAM at the axil of a subtending leaf (Steeves and Sussex 1989; Meyerowitz 1997; Sussex and Kerk 2001). The fate of an axillary meristem is regulated by developmental and environmental cues. It may develop as a vegetative branch or as an inflorescence resulting in flowers and seeds. During reproductive development, axillary meristems play a crucial role in the establishment of different inflorescence structures that ultimately lead to the formation of flowers. An axillary meristem may also remain dormant throughout the life of the plant. This flexibility as to fate determination of axillary meristems provides plants with the necessary plasticity that allows them to respond to environmental signals and also to distinguish functions within the shoot. The SAM often suppresses development of axillary branches; this phenomenon is termed apical dominance.

The first step in shoot branching is the establishment of axillary meristems. It is regulated by a series of transcription factors belonging in the GRAS-, MYB-, and bHLH- families of transcription factors. The organization of axillary meristems probably involves communication between the presumptive axillary meristem cells and the vascular system, and is governed by the REVULOTA subfamily of HD-ZIP transcription factors, REV, PHB, and PHV. It was recently shown that the second step in shoot branching, lateral bud outgrowth, is under the control of MAX (more axillary growth) and RMS (ramosus) proteins (Schmitz and Theres 2005). These proteins seem to produce, perceive, and transduce a signal of yet unknown identity that inhibits lateral bud outgrowth. Although its relation to MAX genes is not yet clear, the first gene suppressing lateral bud outgrowth to be identified was the *teosinte branched 1 (TB1)* gene in maize (Doebley et al. 1997; Wang et al. 1999; Hubbard et al. 2002). *TB1* homologs were later identified in rice, tomato, and other crop plants. *TB1* is one of the main genes responsible for changes associated with the domestication of maize from its ancestor, teosinte. Teosinte is typically highly tillered with long axillary

branches developing from the majority of its nodes. Conversely, domesticated maize, which arose from overexpression of *TB1*, exhibits increased apical dominance, as demonstrated by the suppression of axillary bud outgrowth (Doebley et al. 1997). *TB1* is a member of the TCP family of transcription factors such as *barren stalk (BA*) or *ramosa (RA)* (lateral inflorescence bud outgrowth) (Gallavotti et al. 2004; Vollbrecht et al. 2005), which were identified in maize and other plants and shown to control lateral bud outgrowth.

The group of R. Martienssen elegantly demonstrated that all of these genes are heterochronic genes, which delay or prolong a developmental stage at the expense of neighboring (previous or following) developmental stages (Vollbrecht et al. 2005). According to Cronk (2002), heterochronic changes are employed by higher organisms more easily than heterotopic changes. This may be due to the fact that a single genetic or epigenetic change in the promoter area of a gene coding for a developmentally important transcription factor could have significant phenotypic changes that could be assessed and selected. Interestingly, all these genes code for important developmental transcription factors, and the changes—for instance, in the case of *TB1* in maize leading to its higher, thus prolonged expression—are located upstream in its promoter (>41 kb upstream of *tb1*), as it was demonstrated by Doebley and coworkers (Clark et al. 2006).

A great deal of knowledge has been obtained concerning the transition of plants from the adult to the reproductive phase (floral transition). However, the transition from juvenile to adult phase has not been studied as intensively. Juvenile and adult vegetative phases are best distinguished in woody plants, such as *Hedera helix*, where they were first described, but they are also obvious in herbaceous plants, such as maize and *Arabidopsis* (Poethig 2003). In *Arabidopsis,* during the juvenile phase leaves develop round with smooth margins, a low blade-to-petiole ratio and no abaxial trichomes. Conversely, leaves of the adult phase are characterized by an ovate shape, serrate margins, relatively short petiole, downward-curling edges, and abaxial trichomes (Willmann and Poethig 2005).

The processes of juvenile-to-adult and adult-to-reproductive transition in various species are controlled by hormones such as gibberellic acid (GA), phytochrome B, and vernalization (Willmann and Poethig 2005). Most likely, these two transitions are coordinated by these and other unkown factors. A number of genes coding for specific miRNAs as well as genes coding for their metabolism are involved in the transition from juvenile to adult phases, indicative of the significant role played by EIS in controlling plant development (Kidner and Martienssen 2005;

Willmann and Poethig 2005). Changes in the timing of function of these miRNAs exert heterochronic control in the timing of developmental phase changes (Willmann and Poethig 2005).

Leaf primordia, similar to shoots, initiate from flanks of the SAM. Leaf development requires a balance between the proliferation of meristem cells and a commitment to the formation of leaf primordia. Expansion of the leaf blade requires the establishment of adaxial-abaxial polarity. *Adaxial-abaxial polarity* refers to the two opposing faces of a leaf blade, which have distinct cell types with different biological functions (Hudson 2000; McConnell et al. 2001). Early leaf primordia removed from the SAM could only form a small radial leaf without adaxial-abaxial differentiation. This suggested that a meristem-derived signal directs adjacent regions of leaf primordia to become adaxial and regions farthest from the meristem to become abaxial (Bowman et al. 2002). Leaf shape and size are regulated by the expression in the leaf primordia of certain genes such as *CLAVATA1, CLAVATA3, WUSCHEL, KNOTTED1,* and *PHANTASTICA*. Most likely these genes regulate the establishment of hormonal gradients and transport. Finally, establishment of leaf form in the later stages of development involves coordination between different processes, such as establishment of hormonal gradients, release of biophysical constraints, cell division, and cell differentiation (Kessler and Sinha 2004).

Leaf development requires the establishment of proximodistal, mediolateral, and adaxial-abaxial polarities. Several genes have been identified in *Arabidopsis* that play a role in the formation of the adaxial-abaxial polarity, and epigenetic regulation of some of these has been demonstrated. Class III homeodomain/leucine zipper genes, *PHABULOSA (PHB), PHAVULOTA (PHV*), and *REVULOTA (REV)* genes are expressed in a polar fashion (adaxially localized) in leaf primordia and are required for adaxial cell fate (McConnell et al. 2001; Emery et al. 2003). In addition, members of the *YABBI* and *KANADI* gene families are required for determining abaxial cell fate (Eshed et al. 2001; Kerstetter et al. 2001). Moreover, it has been shown that the *ASYMMETRIC LEAVES1*genes *(AS1* and *AS2)* are also involved in establishing leaf polarity by determining leaf adaxial identity, and they were found to positively regulate the *PHB* gene (Xu et al. 2002, 2003). Two miRNAs from the *Arabidopsis* miR165 and miR166 families contain complementary sites for the *PHB*, *PHV,* and *REV* transcripts, and they direct their cleavage *in vitro*. The miRNA 165/166 complemetary site is conserved between *Arabidopsis* and maize. Disruption of the miRNA 165/166 complementary sites leads to the presence of mutant transcripts in the abaxial side and formation of adaxialized leaves. In maize,

mutations in the *rolled leaf 1 (rlf1),* a member of the class III homeodomain/leucine zipper genes, leads to an upward curling of the leaf blade caused by adaxialization or partial reversal of leaf polarity. The miRNA166 represses *rolled leaf1* and in this way mediates maize leaf polarity (Juarez et al. 2004) by restricting expression of the homeodomain/leucine zipper genes in the adaxial site. In a study also in *Arabidopsis,* it was shown that the RDR6 gene acting together with the *AS1* and *AS2* genes also regulate leaf development. The *rdr6* single mutant plants displayed minor altered phenotypes, whereas the *rdr6 as1* and *rdr6 as2* double mutants exhibited dramatically altered phenotypes with severe defects in the leaf adaxial-abaxial polarity and normal leaf morphology (Li et al. 2005). The double mutants exhibited ectopic expression of a class I *KNOX* gene *BREVIPEDICELLUS (BP*), which is down-regulated during leaf primordia initiation. They also displayed dramatically elevated levels of the miRNAs 165/166 and decreased transcript levels of the *PHB* and *REV* transcripts. These results indicated that the *Arabidopsis RDR6*-associated epigenetic pathway synergistically acting with the genes *AS1* and *AS2* represses the expression of BP and miRNAs 165/166 and is required for proper leaf development.

Cell division processes also govern proper leaf development. Studies of mutant *Antirrhinum* plants had assigned a role for proper leaf development to the *CINCINNATA* (*CIN*) gene. Plants lacking *CIN* exhibited leaves with a crinkly appearance whereas wild- type plants had flat leaves (Nath et al. 2003). *CIN* codes for a TCP transcription factor protein (Cubas et al. 1999). TCP transcription factors are involved in regulating the growth of plant organs by arresting cell divisions in specific meristematic zones. It was suggested that in *cin* mutants, there is a delay in cell division arrest in the developing leaf, leading to accumulation of excess cells and a crinkly phenotype. Studies in *Arabidopsis* based on a genetic screen identified a mutant named *jaw-D* whose phenotype resembled the *cin* mutant in that the leaves had uneven shape and curvature. By analyzing global expression profiles, it was demonstrated that the expression of four *TCP* genes was reduced in the *jaw-D* mutant (Palatnik et al. 2003). In addition, a new miRNA, miR-JAW, was identified with sequence similarity to a well-conserved region of the four *TCP* genes. By mutagenesis and transformation experiments, this region was demonstrated to be a true target of the miR-JAW microRNA that guided the cleavage of the corresponding *TCP* transcripts. Abnormal expression and distribution of the TCP mRNAs during leaf cell differentiation lead to abnormal leaf development. In summarary, sRNAs associated with EIS have been proven to be major regulators of genes involved in the vegetative phase of plant development.

C. Flowering

The transition from vegetative growth to flowering is controlled epigenetically primarily by the PcG protein complex described, as well as by certain other chromatin remodeling factors. Although the MEA/FIE/FIS2 PcG complex controlling seed development is the best studied in *Arabidopsis*, the first PcG gene characterized was *Curly Leaf* (*CLF*), and it was shown to be involved in flower morphogenesis. The CLF protein is similar to E(Z) and contains the typical SET domain found in methyltransferases. The *clf* mutants have a phenotype that is similar to transgenic plants with constitutive expression of the homeotic MADS-box gene *AGAMOUS* (*AG*) (Goodrich et al. 1997). These mutants have small and curly leaves, flower earlier, and show partial homeotic transformation of sepals and petals into carpels and stamens, respectively. Further research has shown that CLF is responsible for maintaining AGAMOUS in a repression state (Goodrich et al. 1997).

EMF2 is another polycomb gene that was shown to be involved in floral induction (Yoshida et al. 2001). Mutant *emf2* does not form any rosette leaves but creates small inflorescences whose lateral buds produce only flowers but not additional inflorescences. The *emf2* also shows ectopic expression of *AG*. The EMF2 belongs to the same family with FIS2 of *Arabidopsis* and SU(Z)12 of drosophila. It is likely that FIE, CLF, and EMF2 are all part of a complex similar to the PcG complex for FIE, MEDEA, and FIS2 that is formed during seed development (Grossniklaus et al. 1998) (Fig.3). This hypothesis can also be based on the fact that the *emf2* and *clf* mutants show similar phenotypes: early flowering and curly leaves. Furthermore, a partially complemented *fie* mutant starts flowering in the seedling stage, as is the case for *emf2*. Finally, as there are no other genes identical to FIE in the *Arabidopsis* genome, FIE is probably part of various PcG complexes that control different developmental processes in plants. This is also indicated by the fact that only heterozygous *fie* mutants are viable whereas other *fis* mutants can be made homozygous (Spillane et al. 2000).

One of the most interesting aspects of PcG-based epigenetically regulated processes is the involvement of PcG in the regulation of the vernalization response. Vernalization is the process by which exposure to long periods of cold promotes flowering in plants. Vernalization ensures that flowering does not take place in the fall but in the favorable conditions of the spring. The term *vernalization* originates from the Latin word *vernus*, which means ''spring.'' Plants usually achieve a vernalized state only after a long-term exposure to cold winter conditions. Previous studies had shown that the site of cold perception is the meristem, and during

vernalization the meristem becomes competent for flowering (Sung and Amasino 2004a). Once meristems have been exposed to low temperatures for a sufficiently long period, they ''remember'' that they have been vernalized, and this memory is inherited through cell divisions. After vernalization, plants do not necessarily initiate flowering but become competent to do so (Sung and Amasino 2005).

A major determinant in flowering and vernalization is the *FLOWERING LOCUS C* (*FLC*) MADS-box gene. It has been shown that in *Arabidopsis,* the acceleration of flowering by prolonged exposure to cold is associated with the down-regulation of the *FLC* gene, which usually prevents flowering when it is upregulated. FLC is a MADS-box transcription factor that acts as a repressor of flowering by inhibiting the activation of a set of genes required for the transition of the apical meristem from the vegetative phase to the reproductive phase (Michaels and Amasino 1999). Following prolonged exposure to cold, the *FLC* transcript levels are down-regulated, and they remain low during subsequent development. This observation leads to the concept that this cellular ''memory'' for the cold vernalization response must have an epigenetic basis. Indeed, genetic screens in *Arabidopsis* identified mutants in which the vernalization response was impaired. In this way three genes were identified, *VERNILIZATION 2* (*VRN2), VERNALIZATION1* (*VRN1)*, and *VERNALIZATION INSENSITIVE 3 (VIN3)*. *VRN2* encodes a homolog of the drosophila *Suppressor of Zeste 12* (*Su(z)12*), a member of the PcG transcriptional repressor (Gendall et al. 2001). *VRN1* encodes a plant specific DNA-binding protein (Levy et al. 2002). *VRN1* and *VRN2* were shown to be required for the maintenance of *FLC* repression during subsequent development following prolonged cold exposure. *VIN3* encodes a PHD plant homeodomain finger protein (Sung and Amasino 2004b). PHD finger proteins are often associated with protein complexes involved in chromatin remodeling (Aasland et al. 1995; Gozani et al. 2003). To investigate whether histone modifications were involved in the vernalization-dependent regulation of *FLC*, ChIP experiments were performed (Bastow et al. 2004). It was demonstrated that vernalization results in histone methylation in discrete domains within the *FLC* MADS-box gene. In particular, there was increased dimethylation of H_3K_{27}, which is indicative of a heterochromatic repressed chromatin state. Dimethylation of H_3K_{27} was lost only in the *vrn2* mutant whereas dimethylation of H_3K_9 was absent from both *vrn1* and *vrn*2, indicating that *VRN1* functions downstream of *VRN2*. In another study, the acetylation of H_3K_9 and H_3K_{14} was examined in the chromatin environment of *FLC* (Sung and Amasino 2004b). A region of intron I and a region upstream of the transcriptional initiation site

exhibited decreased acetylation during vernalization, an indication of inactivation. This decrease was maintained after transfer to warm growing conditions. In contrast, in the *vin3* mutant, the vernalization-mediated changes in *FLC* acetylation did not occur. In the *vin3* mutant, the vernalization-mediated increase in dimethylation of H_3K_9 does not take place as is the case for the *vrn1* and *vrn2* mutants (Sung and Amasino 2004b). Furthermore, it was shown that *VIN3* is transiently expressed upon cold conditions both in the wild type and in the *vrn1* and *vrn2* mutants. Taken together, these results lead the researchers to propose a model for the epigenetic basis of vernalization also in *Arabidopsis*. Exposure to prolonged cold induces the expression of *VIN3* that results in deacetylation of the *FLC* locus. This leads to histone methylation and the formation of mitotically stable heterochromatin at the FLC locus by a process involving *VRN1* and *VRN2*. In summary, a series of epigenetic chromatin modifications in the *FLC* locus leads to the epigenetic memory of winter cold, known as memory of winter.

After the transition from vegetative to reproductive meristems, the appearance of flowering plants is determined by the forms of flower-bearing branch systems, known as inflorescences, and the overall structure of the plant. Inflorescence architecture comprises the stereotypical number and arrangement of floral branches that characterize each species of flowering plant (Weberling 1989). The presence or absence of long branches, for example, dictates the capacity for flower and seed production and largely crop yield. Thus, inflorescence architecture reflects reproductive meristems activity, arrangements, and numbers. And the duration of activity of this reproductive meristems correlates with branch length (Sussex and Kerk 2001; Vollbrecht et al. 2005). Developmental decisions such as meristem allocation, fate, and timing (Sussex and Kerk 2001) are thus observed as quantitative variation in the number of main axis inflorescence meristems established, the number of elongated axillary inflorescences, or the timing of shoot bolting. Identification of genes that lead to variation in quantitative aspects of inflorescence morphology can thus provide insights into developmental pathways that lead to diversity in plant reproductive shoot architectures. Plant reproductive ecology is determined, in part, by the architecture of the inflorescence shoot (Schoen and Dubec 1990; Fishbein and Venable 1996; Diggle 1999). Inflorescence architectures display a wide range of diversity among plant species (Coen and Nugent 1994) and are critical determinants of interspecies differences in plant morphology and life history. Significant architectural changes in inflorescence took place during domestication and breeding of many crops. In the last few years, geneticists have identified and isolated several genes

that regulate the development of the inflorescence in *Arabidopsis* (Shannon and Meeks-Wagner 1991, 1993; Bowman et al. 1992; Bradley et al. 1997; Koornneef et al. 1998; Levy and Dean 1998; Schmitz and Theres 1999).

In *Arabidopsis*, genes like *TFL1* (Shannon and Meeks-Wagner 1991, 1993) were cloned, characterized, and studied. In grasses, genes like *ra1,* which imposes short branch identity as branch meristems are initiated, have been cloned and characterized (Vollbrecht et al. 2005). The gene *ramosa1* encodes a transcription factor that appears to be absent in rice, is heterochronically expressed in sorghum, and may have played an important role in maize domestication and grass evolution (Bommert et al. 2005). Characterization of the maize gene *ramosa2* has shown that it encodes a protein with the highly conserved domain lateral organ boundary, which is essential for determining the fate of stem cells in branch meristems of maize (Bortiri et al. 2006). An extensive genome-wide study on quantitative trait loci (QTLs) exerting major effects on quantitive differences in *Arabidopsis* inflorescence was conducted by Ungerer et al. (2002).

Flower patterning is also controlled by various epigenetic regulators. The crucial role of DNA methylation in flower patterning has been demonstrated by examining the effects of genome-wide demethylation in *Arabidopsis*. Transformation with an antisense construct of *MET1* resulted in plants with abnormal flower phenotypes (Finnegan and Dennis 1993; Finnegan et al. 1996; Ronemus et al. 1996). Homeotic transformation of floral organs in these plants resembled the phenotypes of *superman* (*sup*) and *superman/agamous* (*sup/ag*) double mutants (Finnegan et al. 1996). In addition, the leaves of the *MET1* antisense plants exhibited ectopic expression of the two floral homeotic genes *APETALA3 (AP3)* and *AGAMOUS* (*AG*). Examination of the methylation state of the *SUPERMAN* and *AGAMOUS* genes in *MET1* antisense plants revealed that the genes were hypermethylated, and this hypermethylation correlated with the absence of *SUPERMAN* and *AGAMOUS* transcripts in floral buds, respectively (Finnegan 2001).

Flower shape has been shown to be determined by the action of two closely related genes that belong to the family of TCP transcription factors, *CYCLOIDEA* (*CYC*) and *DICHOTOMA* (*DICH*). Expression of both genes is required to produce the typical asymmetric flower morphology in *Antirrhinum* and *Linaria.* Null mutations in the *CYC* and *DICH* genes resulted in the classic *peloric* mutant phenotype, originally described by Linnaeus 250 years ago. *Peloric* is characterized by a change from bilateral to radial flower symmetry both in *Antirrhinum* and *Linaria* (Cubas et al. 1999; Luo et al. 1999). Cubas et al. (1999)

demonstrated that in the *Linaria* mutant, the *CYC* gene is extensively methylated and transcriptionally silenced, and this epigenetically controlled phenotype has been stable for more than 250 years.

Another large family of plant-specific transcription factors has been described recently, the NAC family, which takes its name from the first studied members of the family, *NAM*, *ATAF*, and *CUC2*. Both NAC and TCP transcription factors have been implicated in the establishment of organ boundaries. The NAC transcription factors comprise a superfamily of more than 100 members in *Arabidopsis* and nearly as many in rice (Olsen et al. 2005). Mutations in the NAC genes lead to severe perturbations in embryonic, floral, and vegetative development. The first NAC mutant to be characterized was the *nam* (no apical meristem) mutant in petunia (Souer et al. 1996). The *nam* mutant lacks a shoot apical meristem and dies at the seedling stage. It exhibits fusion of cotyledons, and occasionally plants developed from escape shoots display abnormal flowers. Another NAC mutant, the *cuc2* mutant (for cup-shaped cotyledons) was later characterized in *Arabidopsis* (Aida et al. 1997). *Cuc1* and *cuc2* double mutants display severe defects in the separation of cotyledons, sepals, and stamens and in proper shoot apical meristem formation (Aida et al. 1997; Takada et al. 2001).

A critical role has been assigned to miRNA 164 in the regulation of *CUC1* and *CUC2* (Mallory et al. 2004). The miRNA 164 complementary sites were detected in several NAC genes including *CUC1* and *CUC2,* and miRNA-directed cleavage of the mRNA of those genes was validated experimentally. *Arabidopsis* plants transformed with a miRNA164a- and 164b-resistant version of *CUC1* displayed dramatic effects in cotyledon, leaf, and flower formation. Furthermore, miRNA164c has been also shown to be involved in the *early extra petal1* (*eep1*) mutant in *Arabidopsis*, which is characterized by extra petals in early-arising flowers. MiR164c controls petal number by regulating the transcript accumulation of *CUC1* and *CUC2* (Baker et al. 2005). Finally, another miRNA, miR172, has been shown to target the *AP2 (APETALA 2)* transcription factor and is associated with proper flowering timing and floral organ formation (Aukerman and Sakai 2003).

In summary, all three EIS are major determinants of transition to flowering, flower patterning, inflorescence and flower numbers (thus seed numbers), and flower organ formation and growth.

D. Seed Development

Flowering plants and conifers bear seeds. Seeds contain the embryo and the endosperm, which provides nutrients and sustains embryo devel-

opment and germination. The endosperm stores reserves, such as starch, fatty acids, and proteins. The endosperm of cereal crops (rice, maize, wheat, barley) represents 60% of the world's food and feed supply. Consequently, understanding and manipulating endosperm development for increasing seed yield is of outmost interest for agriculture.

In flowering plants, the seed is formed through the process of double fertilization. Fertilization of the egg cell by a sperm cell from the male gametophyte generates the diploid embryo from which the organs, tissues, and meristems of the plant will be generated. Fertilization of the adjacent central cell by a second sperm cell forms a triploid endosperm, in angiosperms, which supports embryo growth and development by producing storage proteins, lipids, and starch (Brown et al. 1999). In dicots, such as *Arabidopsis*, the endosperm is consumed by the embryo during seed maturation, whereas in monocots, such as cereals, the endosperm persists after embryo development is completed and constitutes the major portion of the mature kernel (Gehring et al. 2004; Olsen 2004).

Epigenetic regulation has been shown to play a crucial role in proper seed development. Proper embryo and endosperm development depends on the maternal allele of a group of genes encoding the PcG transcription regulators. The identification of PcG genes such as *MEA, FIE* and *FIS2,* already described, indicated that the endosperm shows defects in proliferation and polarity when these genes are mutated. In addition, embryos in *mea* and *fis2* mutants rarely reach the heart stage, and there is no embryo development in *fie* mutants (Köhler et al. 2002). In *Arabidopsis*, MSI 1 protein is part of the MEA/FIE complex and interacts directly with FIE (Köhler et al. 2003a). The *msi* mutants also show similar phenotypic behavior to that of the other *fis* mutants, which adds more evidence for its contribution to proper seed development. However, the exact role of this complex in gene silencing has not been completely elucidated.

Microarray analysis in *Arabidopsis* detected two potential targets of the MEA/FIE PcG complex, namely the genes *PHERES1 (PHE1)* and *MEIDOS*, as their transcript levels were shown to be increased in *mea* and *fie* mutants (Köhler et al. 2003b). *PHE1* encodes a MADS-box type I transcription factor, and *MEIDOS* has not been cloned yet. ChIP analysis showed that the MEA/FIE complex strongly associates with the promoter of *PHE1,* indicating that *PHE1* is a direct target of the MEA/FIE PcG (Köhler et al. 2003b). *PHE1* is transiently expressed in embryo and endosperm after fertilization, and it remains highly expressed until the embryo aborts in *fis* mutants. Reduced levels of *PHE1* expression

in *mea* mutants result in rescue of *mea* seed abortion. These observations suggested that *PHE1* has an important role in seed development and that *mea* mutant seed abortion is due to deregulation of its expression. *PHE1* is also parentally imprinted, as it is only the paternal allele that is expressed (Köhler et al. 2005). Homologs of the *E(Z)* and *FIE* genes have been recently identified in maize (Springer et al. 2002), but no mutants are available to analyze gene function. Three *E(Z)* genes have been isolated: *MEZ1*, *MEZ2* and *MEZ3.* Phylogenetic analysis showed that these are orthologs of the *EZA1* gene in *Arabidopsis* (for which no phenotype is yet available) and very distantly related to *MEA*. *MEA* homologs have not been detected in the rice EST or genomic sequence databases. This may suggest that monocots have another *E(Z)* homolog that performs the function of *MEA* and may reflect differences in the regulation of seed development between monocots and dicots (Springer et al. 2002).

Asymmetric expressions of genes in the zygote and the endosperm interfere with seed development. This asymmetry in gene expression is associated with structural differences in the nuclear genomes of the gametes. Plant sperm have highly condensed chromatin, as compared with the egg and central cell (Mogensen 1982; Scholten et al. 2002). Thus, there is a potential imbalance of regulatory factors contributed by the two parents to the products of fertilization (Dilkes and Comai 2004). The pollen parent contributes a small compact nucleus, whereas the female parent contributes an active and decondensed genome, much cytoplasm, and many RNAs. It follows that the female parent might also contribute factors necessary for remodeling and unpackaging sperm chromatin, and development might impose a strict timing requirement on this phase. Interestingly, the zygote and endosperm develop at different rates: The zygote develops slowly whereas the endosperm rushes into a series of cell divisions, engaging in four rounds of mitosis before the embryo divides once (Boisnard-Lorig et al. 2001; Brown et al. 2003). Perhaps, as a consequence of this mechanism, widespread suppression of many paternal genes occurs during early seed development (Vielle-Calzada et al. 2000; Weijers et al. 2001; Guo et al. 2003). As development progresses, this suppression are partially relaxed, resulting in maternally skewed biallelic expression in the endosperm (Weijers et al. 2001; Guo et al. 2003). Transgenes follow a pattern similar to that of endogenous genes (Vielle-Calzada et al. 2000; Baroux et al. 2001; Weijers et al. 2001), displaying either absolute imprinting and differential expression or a reduction in expression from alleles contributed from the pollen parent. Differential expression of paternal alleles has also been demonstrated in maize endosperm, although twice

as many mRNAs are preferentially expressed from the maternal allele (Guo et al. 2003). The dosage sensitivity hypothesis described in Section III.D implies that a dosage-sensitive phenotype affected by any of these gene products, imprinted or not, would result in parent-of-origin effects and could also explain the dosage sensitivity of endosperm development observed during interspecific hybridization. Asymmetric parental contributions during sexual reproduction have long been known to cause problems. Dissimilar paternal and maternal types may be created by different copy numbers of heterochromatic elements, which further could create an impediment to hybridization. If, for example, a critical level of repressor is needed to suppress a locus, a maternal deficiency of repressor would result in locus activation, similar to hybrid dysgenesis in animals. The more rapid the developmental pace, the sooner remodeling must be complete and the greater the reliance on preexisting resources rather than the *de novo* synthesis of required factors. That is, early entry of the fertilized central cell into proliferative cell cycles might underlie endosperm sensitivity (Boisnard-Lorig et al. 2001; Dilkes and Comai 2004; Olsen 2004).

This section has examined different critical plant developmental steps, the formation of different organs, and growth parameters in light of the important role played by the three EIS. All these developmental events (e.g., flowering time, early- or late-spring flowering, and early- or late-leaf senescence) or organ formation and growth (e.g., the number of inflorescences, the determined or indetermined nature of inflorescence, the number of flowers or fertile flowers, the number of seeds and their size, which is actually the size of endosperm or embryo or both in different seed types) are major targets of many plant breeding programs. They are also for many cases major determinants and components of yield and yield stability. Thus, it is important to understand them in molecular, genetic, and epigenetic terms in order to comprehend and enhance plant breeding efficiency in both conventional and modern plant breeding.

V. Implications in Plant Breeding

A. Genetic and Epigenetic Variation

Phenotypic variation is traditionally parsed into components that are directed by genetic and environmental variation. The line between these two components is currently blurred by inherited epigenetic variation, which is potentially sensitive to environmental inputs.

A unifying theme in biology is that the characteristics displayed by organisms are controlled—ultimately—by the nucleotide sequence of their genome. Another cornerstone of modern biology is that inherited information that is transmitted on the chromosomes changes only at random, without direction from the environment toward particular phenotypic outcomes. These elements of our current biological thinking are being tested by recent work in the field of epigenetics. The chromatin and DNA methylation-based mechanisms and the involvement of RNAi mediate a semi-independent epigenetic inheritance system at the interface between genetic control and the environment (Richards 2006).

Epigenetic states in plants, once established, can be inherited through the transmission of epigenetic alleles (epialleles) over many generations (Kakutani 2002). These heritable epigenetic alleles can be considered as a new source of polymorphism and may produce novel phenotypes. An example of the stability and heritability of alleles and epigenetic variation is found in the morphological variant of toadflax *Linaria vulgaris* described 250 years ago by Linnaeus. Ironically, this variant, which has played such a significant role in the history of botany, turned out to be neither a new species (as Linnaeus thought) nor a mutation (as de Vries and others thought), but a fairly stable epimutation due to an alteration in DNA methylation of the *Lcyc* gene. It is not clear what caused the methylation change in the first place, but once formed, it seems to have been transmitted, more or less steadily (although there is residual instability), for many generations.

Such epigenetic variants 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 and pleiotropic allelic interactions. In addition to mutations that create the genetic variation underlying phenotypic traits, epimutations produce a new source of variation for selection. Most important, epigenetic alleles can result from a genome response to stressful environments and may enable plants to tolerate stress (Tsaftaris and Polidoros 2000; Finnegan 2001; Sherman and Talbert 2002; Steward et al. 2002). The two mechanisms generating polymorphism (mutations and epimutations) have been compared by Tsaftaris and Polidoros (2000). Epialleles could emerge at high frequency in a single generation, far exceeding the rate of mutational events that give rise to new alleles. Their reversion rate is far higher and 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. Furthermore, DNA methylation, in particular, can give rise to more permanent mutant alleles at a locus by its mutational role. However, mutations 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: (1) the extent of variation in methylation patterns among individuals within the selection population; (2) the degree to which methylation patterns affect phenotypes; and (3) the extent to which methylation variants linked to superior phenotypes are stably inherited. These are challenging tasks, but the technical potential exists now for genome-wide assessment of methylation pattern and chromatin structure differences between individuals (Zhang et al. 2006). 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 and other EIS in plant evolution, domestication, and breeding.

Research on the regulation of activity of transposable elements just described strongly support the involvement of EIS in generating new genetic variation. Extended inactivation of eukaryotic transposable elements is accomplished through chromatin modifications. Reactivation may occur when the epigenetic mark is lost, which may result to transposition and disruption of gene loci causing genetic mutation, even though it has an epigenetic origin. The possible activation of an inactive element depends on several factors, all of which have been shown to affect chromatin structure including methylation levels. Among these factors is position in the plant, parental origin, presence or absence of other active elements, developmental stage, and influence of the environment (Tsaftaris and Polidoros 2000; and Section III). All these represent possible opportunities for generation of new genetic variation that is heritable since TEs may stably integrate in the regulatory area of a specific locus and exert their effect for many generations.

Plant tissue culture that was frequently used as another source of variation (somaclonal variation) in breeding programs is also found to involve EIS as causes of epigenetic variation. Tissue culture has many applications, including micropropagation, elimination of viruses by meristem-tip culture, production of doubled haploids by anther culture, the use of cell cultures for the production of secondary products, and more recently as a source of target cells in *Agrobacterium*-mediated gene transfer. In these applications it is of paramount importance that the plants derived from culture are true to type. However, uncontrolled instability can occur when plant cells are cultured in vitro because single

cells or tissue explants dedifferentiate from the developmental state in the explanted tissue to form callus and then redifferentiate into new tissue types. Tissue culture inflicts a type of severe stress that includes wounding and genome restructuring to promote developmental changes and reprogramming. This reprogramming of the genome causes phenotypic variability known as somaclonal variation. Dedifferentiation of the cultured cells has been accompanied with changes in rRNA gene methylation in some plant systems (Anderson et al. 1990; Vyskot et al. 1993), but not in others (Avivi et al. 2004; Komarova et al. 2004). The initial reduction of CG and CNG methylation in both intergenic and genic regions of the rDNA cistron in fully dedifferentiated callus was followed by the establishment of stable epigenetic patterns that were maintained throughout prolonged culture. However, regenerated plants and their progeny showed partial and complete remethylation of the ribosomal units (Koukalova et al. 2005). Although these data suggest a role of tissue culture–induced stress in the epigenetic patterns of chromatin structure, a functional relation between methylation and gene expression has been documented by Mitsuhara et al. (2002); Fojtova et al. (2003); and Avivi et al. (2004). Madlung and Comai (2004) discussed the effects of tissue culture stress on genome structure and concluded it is likely that tissue culture compromises the epigenetic homeostasis of plant genomes and can result in secondary genomic effects.

Last but not least, an important source of epigenetic variation emerges from the regulation of gene expression by means of RNAi. As it is described, this epigenetic mechanism can inhibit the expression of an otherwise normal allele and make it seen as a null mutation. For example, Todd and Vodkin (1996) have shown that the soybean color phenotypes are likely the outcome of mutations affecting different enzymes of the anthocyanin and proanthocyanidin pathways. The *I* locus corresponds to a 27-kb-long chalcone synthase gene cluster that exhibits a unique tissue-specific gene silencing mechanism in the seed coats mediated by short-interfering RNA (Todd and Vodkin 1996; Tuteja et al. 2004). Parallel studies with the flavonoid pigment pathway in maize also found that the dominant inhibitory chalcone synthase allele *C2-Idf* (inhibitor diffuse) acts via an endogenous silencing mechanism (Della Vedona et al. 2005). *C2-Idf* is a stable dominant mutation of the chalcone synthase gene, *c2*, which encodes the first dedicated enzyme in the flavonoid biosynthetic pathway of maize. Homozygous *C2-Idf* plants with two defective alleles show no pigmentation. This allele also inhibits expression of functional C2 alleles in heterozygotes, producing a less pigmented condition instead of the normal deeply pigmented phenotype. The gene structure of the *C2-Idf* haplotype differs substan-

tially from that of the normal *c2* gene in that three copies are present. Two of these are located in close proximity to each other in a head-to-head orientation, and the third is closely linked. In nuclei of *C2-Idf/C2* heterozygotes *c2* transcription occurs, but mRNA is destructed by a siRNA coded by the *C2-Idf* allele. Perhaps this is the first indicative result of a new mechanism for explaining alleles frequently observed in nature, exhibiting negative dominance.

The first commercially used cultivar involving RNAi was the rice mutant line Low Glutelin Content-1 (LGC-1; Kusaba et al. 2003). LGC-1 rice has low-protein content and is useful for patients whose diet requires low protein intake, such as those who suffer from kidney disease. Glutelin is the major storage protein in rice, and LGC-1 is again a dominant mutation in the *glutelin* gene. The negative mutant allele produces a hairpin RNA from an inverted repeat of the *glutelin* gene sequence. Via an RNAi mechanism it leads to lower glutelin content in the rice. The LGC mutant was isolated in the 1970s, and the low-glutenin mutant character appears to have been stable for over three generations. This suggests that the suppression of gene expression by natural hairpin RNA-induced RNAi may be inherited in a stable manner. The siRNA example with the rice LCG glutenin gene family, in addition to negative dominance of a defective allele in a normal one, is a revealing example of negative epistasis. The glutenin gene family consists of more than eight members, all of which are suppressed by one allele. These data suggest a new approach as to how the expression of a family of homologous genes could be down-regulated, for breeding purposes, using transgenic technology (see also Section V.G).

B. Improving Plant Stress Tolerance

The abundant evidence demonstrating that epigenetic changes occur as a direct consequence of different stresses has been described in the previous sections. Some of these stresses are internal genomic stresses involving, for example,polyploidization, interspecific hybridization, movement of transposable elements, and so on have already been mentioned. Polyploidization, for example, was identified in Section III.C as a genomic stress factor causing epigenetic changes. Most of the major crops are polyploids, and even if they behave as diploids, many of them have experienced polyploidization events. Allopolyploid plants can be considered as a special type of hybrids where two or more homeologous genomes are fixed in one nucleus maintaining their integrity through sexual reproduction. Therefore, the advantage of hybrid vigor and heterosis can be fixed by polyploidization (Fasoulas 1993). In contrast,

diploid hybrids undergo wide recombination of the parental genomes in segregating progeny. Hindered recombination between the parental genomes in allopolyploids could be unfavorable on an evolutionary time scale. But this disadvantage would be not dramatic in self-fertilized or asexually propagated species, which are genetically uniform. The widespread distribution of allopolyploid hybrids in such species implies that the benefits of permanent fixation of heterosis through polyploidization overwhelm any disadvantage imposed by limiting the recombination of the parental genomes. How a polyploid plant genome ''senses'' the stress of polyploidy and how this leads to epigenetic changes is not understood. Quantitative changes of different transcripts due to gene dosage changes in the pollypdoid could be one possible mechanism inducing the mechanisms of epigenetic changes, but this requires further research.

External environmental conditions imposing biotic and abiotic stresses during plant growth also are proven to induce epigenetic changes in plants. Pathogen attack, for example, is a severely stressful event, and plants have developed an array of defense mechanisms to cope with its effects. These defenses, particularly against viruses, include epigenetic components like RNA silencing, which also leads to genome methylation, as described in detail in Section III.E.

High-temperature stress also leads to epigenetic modifications. Exposure of transgenic *Petunia* plants to high temperature increased the 5mC levels of the 35S CaMV promoter region of a maize *A1* transgene construct (Meyer et al. 1992). This treatment resulted in the change of flower color conferred by decreased activity of the *A1* gene. Other types of stress have also been reported to affect epigenetic states. In *Bryonia dioica*, mechanical stress caused DNA methylation levels to drop from 25% to nearly undetectable in less than an hour, and remained at that level for at least three hours (Galaud et al. 1993). In *Stellaria longipes,* demethylation of ramets coinciding with initiation of rapid stem elongation was observed in plants growing for four days under long-day warm conditions and depended on the relative ratio of Red/Far Red light. The degree of methylation was a crucial factor in controlling the stem elongation response in different ecotypes, since prairie ecotype plants grown in Murashige and Skoog media supplemented with 5-azacytidine (5-AzaC) required greater doses of 5-AzaC, and thus lower methylation levels, than the alpine ecotype plants in order to promote maximal stem elongation (Tatra et al. 2000). Water deficit is another type of stress that has been reported to result in epigenetic modifications. Hypermethylation of tobacco heterochromatin was observed in response to osmotic stress (Kovarik et al. 1997), while hypermethylation

at specific chromatin sites of pea root tips was observed in response to water deficit (Labra et al. 2002).

Environmental stress conditions, such as drought, salinity, high light, or heavy metals, cause a rapid and excessive accumulation of ROS in plant cells. Superoxide dismutatses represent the first line of defense against superoxide accumulation by rapidly converting superoxide to H_2O_2 and molecular oxygen. A fundamental insight into the regulatory role of microRNAs in stress responses of defense genes was provided by the identification of miR389 as a repressor of superoxide dismutases *CSD1* and *CSD2* in *Arabidopsis*. Expression of miR398 was down-regulated by oxidative stress. This down-regulation proved important for the posttranscriptional induction of *CSD1* and *CSD2* expression under oxidative stress conditions. Furthermore, it was shown that relieving miRNA-directed suppression by overexpression of a miR398-resistant version of *CSD2* leads to great improvement of plant resistance to oxidative stress conditions such as high light, heavy metal, and methyl viologen (Sunkar et al. 2006). It is conceivable that other gene targets of miR389 should also be relieved from suppression under oxidative stress. It is important to note that relieving microRNA-guided suppression of defense gene expression might prove to be an effective new approach to improving plant productivity under stress.

Flax exhibits phenotypic and genomic changes associated with environmental factors. In a stressful environment, flax can bear progeny called genotrophs that exhibit stable phenotypic changes associated with highly specific DNA changes at multiple loci. Although the parents remain phenotypically plastic when grown in different environments, the altered phenotypes of the genotrophs are stable. A site-specific insertion sequence (LS-1) was identified in the genotrophs that is also found in natural populations of flax (Chen et al. 2005). An intact LS-1 is not present in the genome of the progenitor flax line. Flax genotrophs support the idea that beyond genetic, a yet uncharacterized mechanism of inheritance might be operating to shape a dynamic plant genome, providing multiple alternatives for chromosomal changes that may confer better adaptation in response to various challenges.

How plants ''sense'' all the above abiotic stresses and how this leads to the epigenetic changes described is not very well known. The accumulaton of oxygen radicals, discruptive for different macromolecules including DNA, under stress conditions of growth is one mechanism already mentioned. Repair efforts on behalf of the plant genome could lead to genetic and/or epigenetic changes in the damaged area. A recently described example involving developmental changes of plant epigermal pattering under nutritional (Pi and Fe)

stress is also indicative of the role of environmental conditions on plant growth (Guimil and Dunand 2006). Under conditions of limiting iron and phosphate, *Arabidopsis* forms many more and longer root hairs (Lopez-Bucio et al. 2003; Muller and Schmidt 2004). However, this is an adaptive response to increase the absorptive surface area in contact with the rhizosphere in order to maximize water and nutrient uptake. How these environmental signals are translated into changes in epidermal cell fate is not understood. Recent experiments have revealed that epigenetic regulation has an important role in the determination of cell fate in the root epidermis (Costa and Shaw 2006). Furthermore, results obtained after treatment of germinating A*rabidopsis* seedlings with trichostatin A (TSA), an inhibitor of histone deacetylases (HDACs), promoted hair cell formation and growth at non–hair cell positions (Xu et al 2005). Application of TSA resulted in the hyperacetylation of core histones H3 and H4 at the *CAPRICE*, *GLABRA2,* and *WER,* a nuclear-localized R3 loci. And mutants of HDAC family members lead to the same phenotype. All these facts strongly support the role of epigenetic mechanisms as mediators of environmental condition effects on changes in the genome.

Au: Correct word?

Breeding plants for stress tolerance, low input demands, better exploitation of limited resources for growth, and stability of performance in general is a major goal for many breeding programs involving different crops. And, despite the success in a number of cases, very little is known about: (a) the underlying genetic or epigenetic mechanisms responsible for the tolerant genotype obtained, and (b) which are the appropriate conditions during selection for the most efficient breeding for stress-tolerant varieties. A better understanding of these mechanisms will help breeders to fulfill the goal of breeding for stress tolerance in a more efficient way.

C. Epigenetic Mechanisms, Yield, and Heterosis

A lasting mystery in biology, from the days of Charles Darwin, is how hybrids display superior growth and fertility over their parents (Darwin 1876). Heterosis, or hybrid vigor, refers to the phenotypic superiority of a hybrid over its parents with respect to traits such as growth rate and reproductive success and plays significant role in evolution. Hybrid vigor, particularly for yield, was rediscovered in maize breeding almost 100 years ago and has subsequently been found to occur in many crop species (Duvick 2001). The importance of heterosis in plant breeding and in agriculture is evident from the dramatic increases in yield over the past 50 years, following the influx of hybrids to crop production

(Duvick 2001). Maize has provided the genetic tools to study heterosis in plant breeding because parental inbreds have been artificially selected for maximum combining ability, and the creation of structured genetic populations enables more accurate quantitative phenotyping than in natural populations. Indeed, much of our genetic knowledge of heterosis comes from classical genetic studies on maize, mainly examining the participation of dominance versus overdominance and their epistatic interactions in the manifestation of heterosis. Despite decades of research, it is widely assumed that the genetic components of heterosis are still obscure (Lippman and Zamir 2007). Recent efforts on mapping and identification of QTLs controlling different phenotypes, including heterosis, addressed the classical models by breaking down heterosis into Mendelian factors and addressing their modes of inheritance, showed that both dominance and overdominance and epistasis have a varying role in heterosis (see Lippman and Zamir 2007 for a recent review). Working with tomato Semel et al. (2006) created populations of isogenic lines (ILs) that control epistasis (allowing dominance or overdominance effects to be studied) and were able to identify a clear overdominant tomato QTL for yield and fitness. ILs are now available for other crops, such as rice, and similar studies on QTLs and heterosis are expected (Tian et al 2006). In a study comparing maize diploid and triploid hybrids of B73 and Mo17 lines (in order to separate the additive effects), Auger et al. (2005) also identified nonadditive gene expression, in addition to the additive gene dosage involved.

Au: Their refers to what?

In addition to commercially used diploid hybrids, such as maize and rice, many important crops, such as wheat, cotton, and canola, are allopolyploids (Wang et al. 2004). Both autopolyploids and allopolyploids can maintain high levels of heterozygosity through generations (Osborn et al. 2003), and hybrid vigor is positively correlated with polymorphism of the contributing genomes in the formation of autotetraploid potato and alfalfa (Mok and Peloquin 1975; Bingham et al. 1994). Theoretically, hybridization of inbreds in diploid species should not be as stressful as allopolyploidization, since it brings together in one nucleus two homologous haploid genomes. But hybrid breeding proved that combining ability of different inbred lines varies greatly, probably due to incompatibilities of haploid genomes even in the same species, which may result in imbalanced gene expression and genomic stress in the hybrid that, even though less severe, could still trigger similar responses as those reported in allopolyploids. Indeed, allelic variation of gene expression that was not due to parental effects has been observed in maize hybrids (Guo et al. 2004). Moreover, allele-specific responses to abiotic stress were recorded, suggesting a functional

disparity of the parental alleles in the hybrid, which may have an impact in combining ability, stability of performance, and heterosis (Guo et al. 2004).

Genomic imbalance in hybrids may not be due only to allelic variation of gene expression. It could also encompass disrupted colinearity of the haploid genomes. Although karyotypic polymorphisms in plants have been studied for many years (Levin 2002), the possibility of polymorphism in gene content and order within species has been largely neglected. Since local gene order differences can be observed between species, there must have been a point in time when they were polymorphic within species, and it is thus reasonable to suppose that some polymorphism in gene order is present within contemporary species as well (Vision 2005). Indeed, different maize inbred lines are polymorphic for the presence or absence of genic sequences at various allelic chromosomal locations due to gene movements caused by transposons (Lai et al. 2005). This polymorphism has been documented for example for the *bz* genomic regions of two North American maize lines that differ extensively in the organization and content of the intergenic retrotransposon clusters in the region, but also in the content of the genes themselves (Fu and Dooner 2002; Brunner et al. 2005; Buckler et al. 2006; Messing and Dooner 2006). It is currently not known how widespread this structural disturbance might be in other plants or animals. But thanks to the advancements with the human genome, the phenomenon of genic polymorphism even between human individuals has been identified in the human genome, indicating that genic polymorphism is by far more extensive than ever thought (Tuzun et al. 2005). The most convincing evidence for structural genic differences within plant species comes from disease-resistance polymorphisms that are known to result from the presence or absence of particular genes (Tian et al. 2002; Scherrer et al. 2005).

At present, the molecular basis of heterosis remains elusive and efforts are ongoing to understand this important phenomenon in molecular terms. Since heterosis is a genome-wide phenomenon, Tsaftaris (1995) proposed that it involves mechanisms of changing globally gene expression. Biochemical and molecular investigations on heterosis indicate that indeed quantitative variation of gene expression may be important in vigor manifestation (Tsaftaris and Polidoros 2000) and molecular models based on classical genetic hypotheses proposed by Birchler et al. (2003). Variation of gene expression could result by a shift in gene regulation in hybrids and support the significance of regulatory mechanisms involved in the quantitative modulation of gene expression. Alternatively, Fu and Dooner (2002) suggested that in different

maize lines, the presence or absence of individual genes that are members of gene families should cause quantitative (additive) effects rather than qualitative ones. Lines lacking different additive genes would complement one another and show hybrid vigor, while lines lacking mostly the same genes would not complement and, consequently, would not exhibit heterosis. As mentioned, Auger et al. (2005) demonstrated that a substantial number of genes are not expressed at the midparent level in hybrids. In mouse hybrids, complementation of alleles produced spatial and temporal expression patterns beyond the range observed in the parents (Cowles et al. 2002; Guo et al. 2004). Similar results of nonadditive gene expression in a hybrid were found using enhancer trap lines in *Drosophila* (Hammerle and Ferru 2003), analyzing zein expression in maize hybrid endosperms (Song and Messing 2003), and examining protein levels in maize root tips (Romagnoli et al. 1990; Leonardi et al. 1991). Adams et al. (2003) also found unequal contributions from the two genomes in newly synthesized allopolyploids of cotton. A growing body of data therefore indicates that when diverse genomes are brought together in intra- or interspecies hybrids, gene expression patterns will not be predicted by simply averaging the expression of the parental lines, but are a consequence of regulatory interactions producing novel effects on genes under hybrid conditions. Thus, regulatory genes will exhibit some measure of dosage dependence, whereas genes that encode metabolic functions will be less likely to show a dosage effect (Birchler et al. 2001). Most of the studies just mentioned tested relatively small numbers of genes, but with the new microarray-based technology available, global estmations of genome-wide expression patterns have become possible (Auger et al. 2005; Stupar and Springer 2006; Swanson-Wagner et al. 2006) with conflicting results mainly due to inherited technical and analytical difficulties of microarray analysis. As stressed by Lippman and Zamir (2007), besides the technical difficulties with these approaches, a fundamental problem is that they cannot associate novel expression patterns in hybrids with any heterotic phenotypes. One way to address this would be to include inbred parents and hybrids that have increased heterosis, in addition to hybrids with low heterosis. This comparison would reveal whether distinct patterns of gene expression or novel activity among genes belonging to specific functional categories were associated with highly heterotic hybrids. This approach with smaller number of genes was followed by Tsaftaris and his colleagues (Tsaftaris and Kafka 1998; Tsaftaris and Polidoros 2000; Kovacevic et al. 2005; Tani et al. 2005), by comparing a common parental line generating a highly heterotic and a nonheterotic hybrid when crossed to two other parental lines and by

examining this set of three parental inbreds and the two hybrids in different conditions of growth, since it is well known that the conditions of growth affect the manifestation of heterosis (see also below). Au: Where?

Epigenetic mechanisms, such as RNAi, chromatin modifications, and particularly DNA methylation could be considered as genome-wide general regulatory mechanisms that globally might affect the expression of many genes that are important for the manifestation of heterosis (Tsaftaris et al. 1997, 1999, 2005; Tsaftaris and Kafka 1998; Phillips 1999). By examining parental transcript accumulation in maize hybrids using allele-specific RT-PCR analysis, Guo et al. (2004) found that 11 of the 15 genes studied showed significant differences between parental alleles, which can be a result of transcriptional or posttranscriptional regulations, such as mRNA degradation or *cis*-acting sequence polymophisms, which can cause allelic difference in transcriptional regulation. In rice, patterns of 5mC in an elite rice hybrid and its parental lines were detected by a methylation-sensitive amplification polymorphism technique (MSAP) (Xiong et al. 1999). There were three classes of patterns of 5mC according to differences in degree of methylation between the hybrid and the parental lines: (1) no difference in methylation status between the parents and hybrid; (2) an increase of methylation level in the hybrid compared to the parents; and (3) a decrease in hybrid methylation level. In the first case, the banding patterns appeared to follow simple Mendelian inheritance. In both of the latter cases, the banding patterns were not inherited in a Mendelian fashion. As described earlier, epigenetic changes in new polyploids might lead to gene repression or to expression of genes that were repressed in the diploid that is derepression (Osborn et al. 2003). These changes could affect phenotypes directly if they involve genes encoding enzymes or structural proteins. Moreover, epigenetic modifications of homolog genes in polyploids in response to environmental cues and developmental programs could be used as a means for adaptive selection and domestication because the best combination of gene expression patterns may be selected (Wang et al. 2004).

In maize, DNA methylation patterns differ between tissues and developmental stages and are influenced by growth conditions (Banks et al. 1988; Rossi et al. 1997; Cocciolone et al. 2001; Sturaro and Viotti 2001; Steward et al. 2002). Results from several studies in maize hybrids and their parental inbred lines carried out in our lab by measuring global methylation using HPLC and local methylation of random sequences using coupled restriction enzyme digestion–rapid amplification (CRED-RA) indicated that: (1) hybrids are, in general, less methylated than their parental inbreds; (2) heterotic hybrids are less methylated

than related nonheterotic hybrids; (3) old, low-yielding inbreds are more methylated; (4) most modern inbreds, especially those selected for high and stable yield under low-density planting in the absence of competition (Fasoula and Fasoula 1997a, b), have lower percentage of methylation in comparison with old progenitor lines (Tsaftaris et al. 1997, 1999, 2001; Tsaftaris and Polidoros 2000; Tani et al. 2005). These data are in agreement with results from other studies revealing an impact of the environmental conditions on DNA methylation. Temperature changes, for instance, altered the activity and methylation state of the transposon *Tam3* in *Antirrhinum* (Hashida et al. 2003). Moreover, when maize seedlings were exposed to cold stress, a genome-wide demethylation occurred in root tissues, suggesting that DNA methylation functions as a common switch of gene expression and that naturally induced changes in DNA methylation may result in heritable epigenetic modification of gene expression (Rossi et al. 1997). However, other studies revealed methylation increases in the DNA of pea root tips exposed to water deficit, which specifically, for the second cytosine of the CCGG target sequence assayed by the MSAP technique, accounted for about 40% of total sites investigated (Labra et al. 2002). Planting density also affected the methylation state of the *Ac* element in maize (Tsaftaris and Kafka 1998). Results obtained for three consecutive years revealed that demethylation (activation) of a methylated *Ac* element was significantly more frequent in plants grown under spread than dense planting.

In subsequent research using Restriction Landmark Genomic Scanning (RLGS), which is a method capable of screening in a single assay the methylation status of more than 1,000 genes, we determined the polymorphism of methylation patterns in maize inbred lines and hybrids, and examined the effects of high-density growth on genome methylation in a maize hybrid selected for its stable performance (Kovacevic et al. 2005). Methylation levels varied up to 20% between different sib inbred lines. A slight increase of methylation was recorded in a stable hybrid growing under high-density stress (Kovacevic et al. 2005), which is in agreement with our previous reports indicating that hybrids are more resistant than inbreds to site-specific methylation changes under stress. This increase was due to both demethylation and new methylation of DNA fragments in the F_1 hybrid, supporting the hypothesis that methylation can be released or repatterned when inbred lines are crossed to generate hybrids. These results show that part of the methylation inheritance is not Mendelian, which indicates that novel regulatory circuits may be formed in the hybrid to account for the quantitative variation in gene expression observed in many studies

(Romagnoli et al. 1990; Leonardi et al. 1991; Damerval et al. 1994; Tsaftaris and Kafka 1998). It is possible that stability of performance is related with increased resistance to changes in methylation patterns under stress, which could explain the differences observed between inbreds and hybrids. Guo et al. (2004) claim that hybrids in general originated from different breeding programs for specific combining ability can surpass their inbred parents in different level, timing, or duration of gene expression, and in their response to developmental and environmental signals.

Overall, these data point to a possible involvement of methylation in manifestation of hybrid vigor in conventional hybrids and encourage further study of the role of epigenetic inheritance in heterosis. Resistance of hybrids in induced genome methylation alterations under different stresses could be at the core of high and, perhaps more important, stable hybrid yield, especially if critical cytosine residues (e.g., regulatory genes, promoter regions of protein-coding genes) are preferably involved. Evidence has been provided that developmental changes of DNA methylation and chromatin structure at, or close to, the promoter region of a gene are responsible for epigenetic regulation of expression (Hoekenga et al. 2000). Other regions of the genome (e.g., heterochromatic DNA) remain highly methylated permanently, throughout plant growth. This could explain why slight changes of total methylation are concomitant with significant changes in gene expression.

The need for robustness that stability of performance on one side, and developmental plasticity and environmental interaction on the other give, suggests that plants would intensively employ epigenetic regulatory strategies that can give heritable, often reversible, changes in their genetic information without immediate altering of their primary nucleotide sequence. In summary, results from these studies support the hypothesis that hybrids perform better than inbred lines as they resist alterations in methylation under stress. Epigenetic changes like DNA methylation are also factors affecting hybrid vigor.

D. Changes in Plant Development and Architecture

Yield and seed yield in particular is a plant characteristic that is difficult to study and understand at the genetic or molecular level. Genetic or molecular genetic methodologies have advanced our knowledge in many different aspects of plant biology, contributed marginally in the understanding of yield. For example, loss-of-function mutants are very informative for establishing the involvement of a gene in a specific biological process; however, in the case of yield, loss of function would

be manifested as a deficiency in growth or a reduction in fruit and seed production, phenotypes that are obviously shared by many essential genes and not diagnostic of a specific role in yield. Gain of function could be manifested by a yield improvement. Yet the relative yield increase in such a case often will not surpass the resolution limit of standard greenhouse or growth chamber. Far more accurate than currently available phenotyping technologies are required for field evaluation of yield. Plant growth and the contribution of plant growth to harvestable yield are continuous processes, features that make the dissection of these processes quite difficult (Van Camp 2005).

Despite these limitations, the successes enhancing yield, particularly seed yield, that have been achieved in recent years are encouraging. In a number of cases, many and diverse approaches that have been successful in enhancing yield involve genes controlling plant architecture and development. The great potential of developmentally important genes that could largely enhance seed yield is illustrated by the green revolution genes, which are based on the improvement of plant architecture; the introduction of semi-dwarf cultivars doubled the crop yield in wheat and rice due to increases in harvest index and the improved environment (by adding water and fertilizer among main inputs) (Khush 1999). The mutations that are responsible for the short stature in wheat and rice have now been identified (Sakamoto and Matsuoka 2004), and both relate to the plant hormone gibberellin. The role of gibberellin in plant dwarfism and the nature of the green revolution genes has recently been reviewed (Sakamoto et al. 2004). The same review also deals with the recent identification of two genes, *MONOCULM1* (Li et al. 2003) and the rice ortholog of *TEOSINTE BRANCHED1* (Takeda et al. 2003), which control the formation of tillers in rice. In spite of the fact that yield is considered a multifactorial trait, the integration of various developmental and physiological processes provides mounting evidence that yield can be increased by genetic modification of single genes affecting yield components, such as plant height and number of tillers. Similar results were obtained in tomato with genes controlling fruit yield (Fridman et al. 2004; Semel et al. 2006). Many other yield-enhancement genes remain elusive, thus feeding a long-standing debate as to whether such genes actually exist. In the past few years significant progress has been made toward the elucidation of plant genes that, as single variables, are able to improve yield. The debate therefore seems to evolve in favor of the proponents of yield-enhancement genes.

Increasing yield through manipulations of single or few genes using conventional or modern biotechnology methods has been reviewed by

Sakamoto and Matsuoka (2004), Beemster et al. (2005), and Van Camp (2005). Some representative examples that show the involvement of EIS mechanisms in controlling plant development and architecture, which influences yield components, will be discussed.

The different sRNAs, and their described associated changes to chromatin structure controlling leaf shape, size, and senescence exert significant role in yield since leaves greatly affect yield (Ku et al. 2001; Lieman-Hurwitz et al. 2003). The stay green gene is perhaps one of the best examples of leaf senescence effect on seed yield (Ying et al. 2000; Valentinuz and Tollenaar 2004). Single genes such as *TB1*, *RA* and others that change dramatically the shoot architecture making maize look so different from its wild progenitor teosinte also have a significant affect on yield. The same is true for genes controlling inflorescence and flower formation since they control the sink capacity, another major component of yield (Giroux et al. 1996;Regierer et al. 2002; Smidansky et al. 2002, 2003). *AP2*, best known for its role in the regulation of flower meristem and flower organ identity, also plays an important role in determining seed size, seed weight, and the accumulation of seed oil and protein (Jofuku et al. 2005). It is well established that *AP2* is epigenetically regulated by miRNA (Chen 2004). In addition, the gene *apetala2* was found to regulate the activity of the stem cell niche in *Arabidopsis* shoot meristem (Wrschum et al. 2006). Results from conventional plant breeding underscore the fact that tillering, profligacy, number of florets, and number of seeds per plant are major determinants of yield and stability of yield under different conditions (Fasoula and Fasoula 1997a, b, 2000; Andrade et al. 1999; Vega et al. 2001; Tollenaar and Lee 2002; Fasoula and Tollenaar 2005). Van Camp (2005) introduced in rice a *SYTa* gene involved in chromatin remodeling (Sanz-Mollinero 2004) and enhanced seed size and seed yield, suggesting the possibility of yield enhancement by changes in the architecture of the seed through EIS mechanisms.

Plant development and developmental genetics was left out of plant breeding thinking in the past. But the data presented in this chapter provide ample support for the role of genetic and epigenetic mechanisms operating mainly in critical regulatory areas of the genome to generate adaptive phenotypic change. The examples described and many others, such as the TB1 and VGT1 loci (Clark et al. 2006; Salvi et al. 2002), provide strong evidence for the role of regulatory elements operating during development and providing instructions as to when, where, and for how long different tool genes will be used in the transforming a genotype to its phenotype. It is time to introduce plant development into plant breeding thinking.

E. Manipulating Parental Imprinting

There has been increased interest in recent years to better understand the role of epigenetic mechanisms that control parental imprinting in seed and particularly endosperm development in plants of high economic value. It had been shown from interploidy cross experiments that the parental genome ratio in *Arabidopsis* seeds is responsible for proper seed development, viability, and size (Scott et al. 1998). Altering the relative contribution of the maternal and paternal genome caused changes in the cell cycle and the differentiation of the endosperm. Even more striking was the observation that the size of the mature seed in *Arabidopsis* was dramatically affected when the parental genome dosage was altered (Scott et al. 1998). Seeds from 4x $\times$ 2x (the seed parent is mentioned first) crosses were lighter than seeds produced by diploids, despite containing more genomes, whereas seeds from 2x $\times$ 4x crosses are heavier than seeds produced by tetraploids. In general, seeds where the paternal contribution was doubled had accelerated mitosis, delayed endosperm cellularization, and larger-than-normal size when mature. Conversely, double dose of the maternal genome led to reduced endosperm mitosis and seeds smaller than the normal size. The balance of the two parental genomes is related to parental imprinting and thereby to the differential expression of the maternal and paternal genes. Similar interploidy crosses experiments in maize showed that disturbing the parental genome equilibrium resulted in abnormal endosperm development and seed abortion (Lin 1984). As in *Arabidopsis*, a 2:1 ratio of maternal to paternal genomes in the endosperm was shown to be crucial for normal maize kernel formation. In addition, like *Arabidopsis*, allele-specific expression was reported in maize for the genes *ZmFIE1*, *NRP1* (no apical meristem-related protein), *MEG1* (maternally expressed gene), and *Mez1,* one of the three E(z) maize homologs (Danilevskaya et al. 2003; Guo et al. 2003; Gutierrez-Marcos et al. 2004; Haun et al. 2007). All three genes are maternally active during early seed development. *NRP1* and *MEG1* are not expressed in the female gamete, which suggests that they are regulated by parental imprinting. There are two *ZmFIE* maize genes, which are homologous to *Arabidopsis FIE. ZmFIE1* is maternally expressed at six days postfertilization, and maternal transcription continues throughout seed development in the endosperm. The second maize *FIE* gene (*ZmFIE2),* is expressed before fertilization and biallelically expressed in embryo, endosperm, and vegetative tissues (Danilevskaya et al. 2003). The function of *FIE* genes in maize is currently unknown as no *fie* mutants have been reported in maize so far. It is possible that *FIE* in

maize also plays a role in endosperm development. *Mez1* displays a monoallelic expression pattern of the maternal allele throughout endosperm development, which is probably due to differential methylation of the *Mez1* promoter in the maternal and the paternal alleles (Haun et al., 2007).

In *Arabidopsis* the *FIS*-class genes (*MEA, FIE,* and *FIS2*) are regulated by parental imprinting (see Section III.D). Only the maternal allele is expressed during the early stages of seed development; it plays an important role in regulating endosperm development (Gehring et al. 2004; Grossniklaus 2005). Thus, further research in the function and epigenetic control by parental imprinting of *FIS* genes in crops could lead to a better understanding of seed development and provide a new tool for crop improvement. This is particularly important for cereals whose endosperm is the main part of seed, making seed size or seed yields the major component of crop yield. Manipulation of parental imprinting could eventually lead to the development of larger seeds in cereals that are cultivated for their endosperm.

Better understanding of parental imprinting could also lead to better understanding and exploitation of the much-desired phenomenon of apomixis (Savidan 2000; Koltunow and Grossniklaus 2003; Bicknell and Koltunow 2004; Spillane et al. 2004). Apomixis, defined as asexual reproduction through seeds, is the production of fertile plant progeny without double fertilization and thus identical to the mother plant (Savidan 2000). About 1,000 apomictic plant species are currently known that can reproduce asexually to form seeds that are clones of the seed parent. However, apomixis is not found frequently in crop plants and conventional breeding has not been successful, so far, in transferring apomixis. The phenotype of *fis* mutants resembles that of apomixis in apomictic plants as a large number of apomictic species exhibit autonomous endosperm proliferation (Spielman et al. 2001; Lohe and Chaudhury 2002; Gehring et al. 2004; Groosniklaus 2005). Mutations in *MEA*, *FIE*, or *FIS2* all lead to initiation of endosperm development without fertilization, and in *mea, fie,* and *fis2* mutants, there is no embryo development in the absence of fertilization. In these mutants, embryos develop abnormally after fertilization and are eventually aborted. Therefore, the *FIS*-class genes may play a crucial role in regulating endosperm and embryo development. Studying such genes in apomictic plants may reveal whether they might also be involved in apomixis. One of the major difficulties in making use of apomixis in cereal crops is the strict requirement for a 2:1 ratio of maternal-to-paternal genomes in the endosperm tissue. Deviations from the 2:1 ratio in many plants and most cereal crops leads to seed abortion (Brichler

1993), a phenomenon that is related to parental imprinting and the dosage sensitivity hypothesis described also in seed development. Indicative of this relation are data proving that maize, when used as the seed parent, can make fertile hybrids with the apomictic relative *Tripsacum dactyloides*. The highest fertility results when using diploid maize and tetraploid *Tripsacum* (Kindiger and Beckett 1992). A study of the functions of FIS homologs in apomictic plants could provide new insights into the process of apomixis. Moreover, manipulation of the genomic imprinting mechanisms associated with seed development may solve the current problems of generating apomixis in economically important crop plants (Grossniklaus 2005).

F. Improving Plant Resistance to Viruses and Other Parasites

Suppression of PTGS is a widespread property and probably a necessary adaptation of RNA and DNA viruses of plants (Voinnet et al. 1999). This is perhaps the most convincing argument that PTGS acts as an antiviral mechanism in plants. Transgenic resistance against viruses based on the PTGS mechanism easily fulfills the current high demands for biosafety. Indeed, PTGS-based resistance does not involve the transgenic production of functional viral genes or proteins, nor does it lead to the presence of transgenic RNA (Goldbach et al. 2003). An important drawback of this approach in biotechnology is the high level of sequence specificity required for RNA degradation. Thus, viruses that contain more than 10% nucleotide discrepancy cannot be subjected to RNA degradation. Another drawback until recently was the size of the transgene. It should be more than 300 base pairs, in order to trigger efficient RNA silencing. There is experimental evidence using genetically engineered miRNA that a 21-nucleotide sequence was sufficient to trigger complete silencing in transgenic plants (Voinnet 2002). However, the major technical limitation for technologies based on RNA silencing is that many important plant crop species are difficult or impossible to transform. Moreover, public concerns over the potential ecological impact of virus-resistant transgenic plants have significantly limited their use so far (Fermin et al. 2004). Virus-induced gene silencing (VIGS) is a technology that exploits an RNA-mediated antiviral defense mechanism. Haque and his colleagues (2007) have produced transgenic *Nicotiana benthamiana* plants that expressed the coat protein gene (CP) of sweet potato feathery mottle virus, and they have shown that RNA silencing spreads in the 5'–3' direction, but not in the 3'–5' direction, along the transgene mRNA.

Until now, most of the applications of VIGS have been in the plant virologists' model plant, *N. benthamiana*. Nevertheless, new vectors are used with the ability to support VIGS in *Arabidopsis* (Dalmay et al. 2000) and tomato (Liu Y. et al. 2002). VIGS technology was used very recently for characterization of genes associated with local and systemic resistance in barley and other cereals (Hein et al. 2005). The main concern for the application of VIGS is the use of genetically modified plant pathogens; thus, all experiments must be carried out in strict control.

The development of RNA silencing as an effective and environmentally safer approach is a long-term goal to control plant virus diseases. Although considerable progress has been made in understanding this mechanism, much still remains to be discovered (Tenllado et al. 2004).

G. The Use of RNAi for Crop Improvement

Improving the nutritional value of plants can be achieved either by classical breeding based on selection of the natural or induced genetic variation or by generating transgenic plants that carry the desired agronomic traits. Transgenic technologies have advantages over classical breeding because the genotypic alterations, which will lead to superior phenotypes, can be specifically designed and introduced to the plant in a highly regulated manner as to abundance and spatial expression. Even though traditional gene knockout approaches have been used in the past to suppress expression of certain genes in plants, RNAi technology provides a way to reduce the product of a gene in a much more efficient and regulated fashion. Most important, RNAi is extremely useful when families of numerous copies of gene homologs need to be down-regulated rather than a single locus.

RNAi technology has been widely used in the past as a tool for analyzing gene function in plants. A common way to achieve RNA interference is to use a transgene that produces hairpin RNA (hp RNA) with a ds RNA region (Waterhouse and Helliwell 2003). Double-stranded RNA had been demonstrated in the past to be a good method to trigger gene silencing in plants. However, RNAi that has been induced by hairpin RNA has proven to be much more efficient than conventional gene silencing methods, which relied on introducing antisense RNA into the plant (Chuang and Meyerowitz 2000). In a hairpin RNA-producing vector, the target gene is cloned as an inverted repeat that contains a spacer of unrelated sequence. This is important for the stability of the construct in *E. coli* after cloning. A strong promoter, such as the 35S CaMV promoter for dicots or the maize ubiquitin 1 promoter for monocots, drives the inverted repeat. The efficiency of

silencing increases dramatically (almost 100% of the transformed plants show gene silencing) when the spacer fragment is an intron (Helliwell and Waterhouse 2003). For genome-wide analysis of gene function, where the silencing of large numbers of genes of unknown sequences is desired, a novel method has been developed named SHUTR (Silencing by Heterologous 3'-UTRs) (Brummell et al. 2003), which relies on the construction of a vector containing an inverted repeat of the 3'-untranslated region (3'UTR) of an heterologous gene and a strong promoter. Gene sequences are incorporated 5' upstream of the 3'UTR of the heterologous gene as to provide a dsRNA region at the 3'end of the transcript. This method was tried out using the the 3'-UTR region of the nopaline synthase (nos) gene from *Agrobacterium tumefanciens*, and it was shown to induce highly efficient silencing of a polygalacturonase transgene in tomato and two transcription factor genes in *Arabidopsis* (Brummell et al. 2003).

Constructs containing tissue-specific promoters have also been used successfully in guiding RNA silencing. For example seed-specific genes have been effectively silenced using the *napin* and *lectin* promoters (Smith et al. 2000; Stoutjesdijk et al. 2002). Similarly, very efficient organ-specific silencing has been achieved both in *Arabidopsis* and *Brassica* using MADS-box gene promoters to transform petals into sepaloid structures (Byzova et al. 2004). A long list of genes, ranging from transcription factors to biosynthetic enzyme encoding genes, has been knocked down using hairpin RNA technology in order for their function to be analyzed (www.pi.csiro.au/RNAi/different gene_eg.htm). Examples include the *Bes1* gene, which codes for a transcription factor involved in regulation of brassinosteroid gene expression in *Arabidopsis* (Yin et al. 2005), and the male fertility gene *Ms45* and several other anther-expressed genes whose transcriptional silencing by RNAi resulted in male sterile plants in maize (Cigan et al. 2005).

RNAi is particularly useful for silencing of genes in polyploids or genes that belong to multigene families. Traditional gene knockout and conventional breeding cannot be used for the repression of a multigene family by the accumulation of mutations for each member of the family, particularly when the members are tightly linked. For example, in the naturally occurring low-glutelin RNAi mutant in rice LCG-1, the glutelin family consists of at least eight members, five of which are clustered in a particular chromosomal location. However, reduction of the levels of glutelin was achieved from a single RNAi locus and not from mutations for each of the members of the glutelin family (Kusaba et al. 2003; Kusaba 2004).

RNAi technology has also been used as a tool for crop improvment. The primary proteins of the maize endosperm, the so- called zein pro-

teins, are classified in four subfamilies, the a-b-g-d zeins (Ueda and Messing 1993). The a-zeins account for 70% of the total zein content, and this nutritional protein class is responsible for the overall low content of seed lysine, a significant amino acid in human and animal diet. The a-zeins belong to a multigene family whose members are clustered in several chromosomal sites (Hunter et al. 2002). In the past, the *opaque-2* (*o2*) maize mutant had been isolated, which displays significantly reduced levels of the 22kd a-zein protein in the endosperm (Mertz et al. 1964; Schmidt et al. 1987). Analysis of this mutant showed that the mutation was the result of differential inhibition of the transcription of the 22kd a-zein transcript (Kodrzycki et al. 1989). Unfortunately, the *O2* gene, which encodes a basic leucine zipper transcriptional factor, controls the expression of not only the 22kd a-zein protein, thus the *o2* mutant although high in lysine has undesirable agronomic traits such as low seed quality and yield (Tang and Galili 2004). Segal et al. (2003) described the production of dominant *O2* maize variant in which selective repression of zein protein synthesis occurred independently of the *O2* gene, which was achieved through the use of RNAi constructs derived from the sequence of the 22kd a-zein. In this mutant, the 22kd a-zein proteins were specifically reduced, whereas accumulation of the other zein proteins remained unaffected. Most important, the altered trait was stably inherited and the mutant maize plants produced normal seeds with high levels of lysine-rich proteins.

Other efforts to increase the lysine content in plants focused on the gene that codes for dihydrodipicolinate synthase (DHPS), the first enzyme specifically committed to lysine biosynthesis. Lysine synthesis is strongly regulated by lysine-mediated feedback inhibition of the activity of DHPS but also by genes regulating lysine catabolism, Lys-ketoglutarate reductase (LKR), and saccharopine dehydrogenase (SDH) (Galili 1995; Galili et al. 2001). Studies with an *Arabidopsis AtLKR/SDH* knockout mutant, which was designed to express a bacterial lysine-insensitive DHPS specifically in seeds, showed that the mutant accumulated high levels of lysine in the seeds but the growth of seedlings from these seeds was dramatically decreased (Zhu and Galili 2003). However, in a subsequent study, coexpression of the bacterial DHPS and a *AtLKR/SDH* RNAi construct both under the control of a seed-specific promoter led to increased lysine content in seed and improved seedling growth (Zhu and Galili 2004), which again supports the advantages of RNAi-mediated technology for altering gene expression in plants.

RNAi technology has been successfully applied to reduce caffeine content in *Coffea arabica*. Decaffeinated coffee is of major interest for

the coffee industry because the stimulatory effects of caffeine cause health problems to sensitive individuals (high blood pressure, palpitations, insomnia). Ogita et al. (2003) constructed transgenic coffee plants where the expression of one of the caffeine-synthesizing enzymes was specifically repressed by an RNAi-based technique, and caffeine content was reduced by 50 to 70%.

Another application of RNAi technology has been the modification of the fatty acid composition of cotton oil. Two key enzymes in the metabolism of fatty acids are the stearoyl-acyl-carrier protein Δ9-desaturase and oleoyl-phosphatidylcholine ω6-desaturase (Liu Q. et al. 2002). RNAi mediated by a hairpin RNA was used in cotton in order to reduce the expression of the genes encoding these two enzymes. The expression of the two desaturases was successfully down-regulated, and the resulting cotton plants produced high levels of oleic and stearic cottonseed oils, which are essential fatty acids for maintaining a healthy heart condition.

In another study RNAi technology was utilized to increase the carotenoid and flavonoid content in tomato (Davaluri et al. 2005). Tomato is the principal dietary source of lycopene, β-carotene, and flavonoids, all of which are phytochemicals that benefit human health. Lycopene and flavonoids are powerful antioxidants that have been associated with reduced risk of heart disease and certain types of cancer. β-carotene is the precursor of vitamin A. Past attempts to regulate genes in the biosynthetic pathways of carotenoids and flavonoids resulted in the increase of the content of either one phytochemical but never of both. The authors demonstrated that suppression of a photomorphogenesis regulatory gene, *DET-1*, using RNAi techniques resulted in tomato fruits containing higher levels of both carotenoids and flavonoids while other fruit quality indicators remained unaltered.

RNAi silencing operates as an ancient self-defense mechanism against foreign invaders, such as viruses and transposons, present in a broad range of eukaryotic organisms. In plants, RNAi serves as an antiviral system. Successful viral infection requires suppression by the virus of gene silencing that was induced by the host plant. During viral invasion, most plant RNA viruses form a dsRNA intermediate, which is cleaved to generate siRNAs that target viral RNA. In order to protect itself from degradation, the virus has evolved RNAi inhibitors, which allow the virus to overcome plant RNAi action and infect the plant successfully. Recently it was demonstrated that a viral protein of tombusviruses (p19) inhibits RNA silencing in vivo by strongly binding siRNA in virus-infected cells (Lakatos et al. 2004). When RNAi silencing was suppressed by p19 in tobacco plants, the expression of various

transgenes in transient expression assay was increased 50-fold (Voinnet et al. 2003). Thus, RNAi silencing suppression could be potentially used as an effective tool for the overproduction of desired proteins in plants.

Krützfeldt et al. (2005) have achieved the chemical engineering of oligonucleotides, termed antagomirs, which can efficiently silence miRNAs in vivo. Antagomirs are single-stranded RNA analogs complementary to miRNAs, which have been chemically modified for stability and cholesterol-conjugated for delivery. Intravenous administration of antagomirs against certain miRNAs resulted in a significant decrease of the respective miRNAs in mice. Because miRNAs play a crucial role in the regulation of gene expression in human disease, this could prove to be a therapeutic technology with enormous potential. Such technology might be applied in plants. For example, it may be possible to deliver such antagomir constructs in the plant liquid-stage endosperm (a multinucleate structure that lacks a cell membrane) in order to manipulate seed development.

H. Securing Stability of Transgenes

Although it is not yet possible to completely rule out transgene silencing, it can be avoided by following several strategies. First, it is advisable to screen transgenic lines for the insertion of single-copy transgenes into hypomethylated genomic regions. It is wise therefore to exclude transgenic lines that have transgene rearrangements and are candidates for silencing (Depicker et al. 2005). This can be accomplished by analyzing the genomic regions adjacent to the transgene integration site and discarding plants with transgenes that are inserted into hypermethylated genomic regions (Meyers 1998). Moreover, because plant genomes are mosaics of isochores, the transgene should match the isochore composition of the host organism. In order to minimize the impact of the sequence composition of the transgene integration site, matrix attachment regions (MARs), which are DNA elements that bind specifically to the nuclear matrix in vitro (Allen et al. 2000), can be positioned on either site of a transgene and may help to reduce variance in expression levels (Vain et al. 1999; Halweg et al. 2005). Another approach is the integration of the transgene into a fixed chromosomal site-by-site–specific integration (Albert et al. 1995). It is important to exclude any plasmid or phage vector in the transgene constructs as they might be recognized as foreign and serve as targets for TGS or PTGS (Iyer et al. 2000). Other factors that should be taken into account are the transformation vectors and the choice of transformation

Au: site or side?

technique. Multiple uses of the same promoters should be avoided, as they can be subjected to TGS (Vaucheret 1993). Multiple uses of identical 3' regions for the generation of a transgenic plant also should be avoided as they can also be targets for TGS or PTGS (De Neve et al. 1999). Silencing has probably evolved as a defense mechanism against parasites and viruses and not as a mechanism to inactivate transgene expression. Therefore, it is possible that a methodical study of these mechanisms will help us to overcome silencing. Plant viruses have evolved their own counterdefenses by virus-encoded silencing suppressors, showing that silence can be broken (Qu and Morris 2005).

VI. Outlook

Frequently, when unexpected phenomena are observed, they first are ignored, then timidly explored, and only later published and debated with firm conviction in a more coherent framework. The third phase of research associated with epigenetic mechanisms operating in plant development and evolution provides new insights into such an important matter. The discovery of sRNAs has introduced a new paradigm of gene function and regulation and uncovered the once-hidden role of function and regulation of non–protein-coding areas of genomic DNA. In addition, these small RNA species provided a link to interconnect the three EIS (DNA methylation, histone modifications, and RNAi) into an interrelated triangle of interactive mechanisms.

Perhaps the described stability of the peloria epiallele for more than 250 years is indicative of the similarities in the stability of some epigenetic and genetic changes (Cubas et al. 1999). The example of low-glutelin variant in rice due to RNA silencing of multiple members of the glutelin gene family and its stability in this case for 25 years (Kusaba et al. 2003) is also indicative for the role of EIS and siRNA in particular in creating useful variation for selection. The genetic alternative for simultaneous knocking out a gene family would require the rather unlikely concurrent mutation of all the individual members of the family (Kusaba 2004). The same holds true for genes with multiple copies in polyploid crops (Lawrence and Pikaard 2003). The low-glutelin rice variant and its study indicates the opportunities offered by the existence of such epigenotypes, which have been selected in these breeding efforts, as a valuable source of material for molecular biologists studing EIS.

Epigenetic mechanisms are currently proven as major mediators of a genomic effect of the environment. What is emerging, with strong

experimental support, is that environmental changes in addition to the induction of the known physiological response on behalf of the sessile plant are playing a major restructuring role on plant genomes. These data are revolutionary with implications not only in plant breeding but also in plant biology and evolution in general.

Developmental genetic and epigenetic variation could be of particular importance for plants. Most structures of a mature plant are formed after embryogenesis by the reiterative action of meristems. By delaying the bulk of growth and differentiation until after embryogenesis, the sessile plants can fashion their form to the environment, allowing development to substitute for behavior to some extent. The structures produced by the apical meristems change through developmental time. The varying epigenetic stages of TEs in somatic cells, for example, is indicative of the extent of this somatic cell variation, of the power of EIS in generating variation, and of the possibilities that emerge. The pattern of plant development allows substantive opportunities for embryogenic cells to vary, and of course such variant cells can subsequently give rise to varying gametes. Epigenetic and genetic systems are involved in the development of individual plants; thus they could be a source of significant and useful variation and could play a vital role in plant breeding.

During a large part of the twentieth century, plant breeding was basically considered an artificial selection scheme applied to a genetically segregating material. Darwin's theory of natural selection, introduced in 1859, proposed a mechanism of evolution at the organism level. As Darwin had no acceptable theory of genetics, natural selection foundered and was bitterly opposed in the early twentieth century (Cronk 2001). However, the rediscovery of Mendelian rules of inheritance by Correns, De Vries, and von Tschermak allowed natural selection to shift to the gene level. R.A. Fisher brilliantly reestablished natural selection as the dominant evolutionary mechanism by showing mathematically its potency in acting on changing allele frequencies in a population. The paradigm of natural selection acting at the gene level led to the ''modern synthesis'' and has lasted to the present day. The shift from Darwin's organism and species selection, to the frequencies of alleles in populations in the neo-Darwinian theory, transferred the emphasis from individuals to populations in both evolution by natural selection and plant breeding by artificial selection. Thus development and developmental genetics were left out of this thinking (Cronk 2001; Robert 2004). However, the recent explosion of the genomic revolution and the availability and comparison of sequences have shifted the emphasis from the neo-Darwinian alleles to single nucleotide differ-

ences and other changes emphasizing the significance of the effect of nucleotide changes in the *cis*-regulatory elements of transcription factors and other regulatory genes. Such changes, leading to altered specificity of DNA-protein binding chromatin modification, to production or new targets of sRNAs, may in turn lead to heterotopic and heterochronic changes in the expression domains of key regulators. Data presented in this review provide ample support for the role of genetic and epigenetic mechanisms operating in these critical areas of the genome driving adaptive changes in the phenotype. As the coding sequences of developmental genes are highly conserved, they are unlikely to be sources of organismal diversity. Studies in maize (Doebley et al. 1997; Wang et al. 2005), rice (Spielmeyer et al. 2002; Li et al. 2003; Li et al. 2006), and tomato (Cong et al. 2002; Liu J. et al. 2002; Van der Knaap et al. 2004) indicate that genetic and epigenetic polymorphisms in the controlling areas operating during development provide instructions as to when, where, and how the ''tool'' genes will be used, which should be the stimulus not only for further work on similar developmental and genetic studies in other crops but also for the making of individual plant development the target of artificial selection breeding (Fasoula and Fasoula 2002). The newly established field of Evo-Devo (Carroll 2005) that brought development to evolutionary natural selection should now be extended to studies of plant domestication (Domest-Devo) and plant breeding (Bred-Devo).

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