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Mini Review

Epi-Alleles in Plants: Inheritance of Epigenetic Information over Generations

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Epigenetic modification of plant gene and transposon activity, which correlates with their methylation, is often heritable over many generations. Such heritable properties allow conventional genetic linkage analysis to identify the sequences affected in epigenetic variants. Machinery controlling the establishment of the epigenetic state and role of the epigenetic controls in plant development are also discussed.

Keywords: DNA methylation — Gene silencing — RNA-directed DNA methylation — RNA interference — Transposon.

Abbreviations: *Ac*, *Activator*; *BAL*, *BALL*; *CMT*, *CHROMOMETHYLASE*; *DDM*, *DECREASE IN DNA METHYLATION*; *DRM*, *DOMAIN REARRANGED METHYLTRANSFERASE*; *En*/*Spm*, *Enhancer*/*Suppressor-mutator*; *MET*, *METHYLTRANSFERASE*; *MOP*, *MODIFIER OF PARAMUTATION*; *PAI*, *PHOSPHORIBOSYLANTHRANILATE ISOMERASE*; PTGS, post-transcriptional gene silencing; RdDM, RNA-directed DNA methylation; RNAi, RNA interference; *SUP*, *SUPERMAN*; TGS, transcriptional gene silencing; TSI, transcriptionally silent information.

Nucleotide sequence is not the only heritable information on the chromosome. Epigenetic information, which is based on DNA methylation or chromatin states, is also heritable during cell propagation. In both plants and mammals, DNA methylation correlates with epigenetic suppression of transcription. Mammalian epigenetic phenomena, such as parental imprinting and X-chromosome inactivation, are developmentally regulated, and re-programming of the epigenetic states occur in each generation. Similarly, the methylation pattern in the mammalian genome undergoes reorganization by extensive demethylation and "de novo" methylation during gametogenesis and early development (Monk et al. 1987). In contrast, the epigenetic states of plant genes are often inherited over generations. I review here the epigenetic phenomena in plants, with special emphasis on the epigenetic inheritance over generations. Such heritable "epigenetic alleles" may be involved in diverse phenomena ranging from development to genome evolution and defense against transposons.

Epigenetic inheritance of transposon activity

The recent accumulation of genome sequence information revealed that transposable elements and their derivatives are a major constituent of the genome of vertebrates and higher plants (SanMiguel et al. 1996). Considering the abundance of transposons in their genome, it is surprising that only a low proportion of spontaneous mutations is caused by them. Mechanisms may exist that suppress uncontrolled transposition of these elements. Eukaryotic genomes with a high proportion of such repeated "junk" sequences are generally associated with a high methylated cytosine content. Most of the methylated cytosine is found in transposons and repeats in the mammalian genome. Some eukaryotic species with less genomic DNA methylation, such as *Drosophila*, suffer from a high frequency of transposon-mediated mutations compared with vertebrates and higher plants. These findings led Yoder et al. (1997) and colleagues to propose that primary function of eukaryotic DNA methylation may be defense of their genome from deleterious effects of endogenous transposons.

Historically, epigenetic regulation of transposons has been extensively studied in plants since McClintock's early findings. Maize transposons such as *Activator* (*Ac*) and *Enhancer*/*Suppressor-mutator* (*En*/*Spm*) sometimes change from active to inactive states and again to active state (McClintock 1951, McClintock 1958). The changes affect all detectable activities of the transposons (which McClintock called "controlling elements"), such as transposition, chromosome break, and modification of gene expression. The active or inactive states are often heritable over generations. The correlation between the reversible transposon activities and DNA methylation has been found on *Robertson's Mutator* (Chandler and Walbot 1986), *Ac* (Schwartz and Dennis 1986) and *En*/*Spm* (Banks et al. 1988). An interesting feature of these systems is that modification of the activity in the transposons or their derivatives affect the activity of the nearby host genes (reviewed by Martienssen 1996a, Fedoroff 1996). Again, methylation correlates with the epigenetic state of these systems. Paramutation, another type of interesting epigenetic silencing of endogenous genes in maize, may also be derived from control of transposons (Martienssen 1996b). Interestingly, *modifier of paramutation 1* (*mop1*) mutation of maize, which prevents paramutation at *b1*, *r1* and *pl1* loci, also reverts methylation and silencing of the *Mutator* transposon (Lisch et al. 2002).

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Arabidopsis mutants with reduced DNA methylation provide powerful systems to directly investigate the role of DNA methylation. Reduced methylation in repeat sequences results from the mutation in *Arabidopsis* gene *DDM1* (*DECREASE IN DNA METHYLATION 1*), which encodes a protein similar to the chromatin remodeling factor SWI2/SNF2 (Vongs et al. 1993, Jeddeloh et al. 1999). In the hypomethylation background induced by the *ddm1* mutant, several silent repeated sequences are reactivated transcriptionally (Jeddeloh et al. 1998, Hirochika et al. 2000, Steimer et al. 2000). For example, a sequence called TSI (or transcriptionally silent information), which is a part of the repeated transposon-like sequences Athila, is silent in wild-type plants, but transcribed in the *ddm1* mutant (Steimer et al. 2000). DNA methylation seems to be necessary for silencing this element, because mutations in DNA methyltransferase genes, such as *MET1* (*METHYLTRASFERASE 1*) or *CMT3* (*CHROMONOMETHYLASE 3*), result in transcriptional TSI reactivation (Steimer et al. 2000, Lindroth et al. 2001). In addition to the transcriptional activation, the *ddm1* mutation induces high frequency transposition of at least two classes of the endogenous *Arabidopsis* elements, MULE (Mutator-like, which is similar to maize Mutator elements; Singer et al. 2001) and CACTA, which is similar to the maize *Enhancer/Suppressor-Mutator* (*En/Spm*) transposons (Miura et al. 2001). Both of these elements are not mobile in wild-type Columbia background. These observations suggest that DNA methylation effectively suppresses transposon activity.

Independent of these studies on epigenetic control of transposons, epigenetic control of gene expression has been extensively studied using transgenic plant systems (reviewed by Matzke and Matzke 1995, Vaucheret et al. 1998, Waterhouse et al. 2001). In short, transgene-silencing in many systems has been categorized into two types: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). TGS affects transcriptional initiation, whereas PTGS results from targeted degradation of RNA. TGS is heritable over generations, whereas PTGS is reset after meiosis and recurs in every generation at some stage of plant development. Much evidence suggests that PTGS is effective in defending plants against RNA viruses. In contrast, TGS and DNA methylation may be important for defense against transposable elements as stated above. The meiotically heritable property of TGS would be advantageous for defense of the genome against transposon, as the silenced state is maintained throughout development.

Meiotically heritable epigenetic alleles affecting plant development

Some of meiotically heritable epigenetic changes affect plant development. Jacobsen and Meyerowitz (1997) found the first of such examples through characterizing unstable alleles of the *SUPERMAN* (*SUP*) gene. Mutations in the *SUP* gene alter floral pattern formation by affecting the floral whorl boundary (Sakai et al. 1995). Unlike previously characterized *sup* mutations, the new alleles, called *clark kent* (*clk*), revert to the normal allele at about 3% per generation (Jacobsen and Meyerowitz 1997). Although results of fine mapping and complementation by *SUP* transgene indicate that *clk*s are allelic to other *sup* mutations, they do not have any change in the nucleotide sequence of *SUP* gene (Jacobsen and Meyerowitz 1997). Instead, in the *clk* plants, the *SUP* gene was heavily methylated and transcriptionally silenced (Jacobsen and Meyerowitz 1997). Thus *clk*s are epigenetically suppressed alleles of the *SUP* gene. The epigenetic state is heritable over generations and behaves like a real mutation, except that they revert to the wild-type allele at low frequency. The factors causing initial epigenetic change of the *SUP* gene in these alleles are unknown. Counter-intuitively, similar epigenetic silencing of the *SUP* gene was observed in *met1* mutants and *MET1* antisense lines. In these lines, overall DNA methylation level is reduced but the *SUP* gene is hyper-methylated (Jacobsen and Meyerowitz 1997, Jacobsen et al. 2000).

In *Arabidopsis*, more examples of such epigenetic alleles affecting plant development have been identified using the DNA hypomethylation mutants and the linkage analysis. Both *ddm1* mutants and antisense *MET1* lines exhibit a variety of developmental abnormalities (Finnegan et al. 1996, Ronemus et al. 1996, Kakutani et al. 1996, Kakutani 1997). Insertion mutation induced by activation of endogenous transposon under the *ddm1* mutant background is one of the mechanisms for the developmental abnormalities (Miura et al. 2001, Singer et al. 2001). However, it does not explain the high frequency occurrence of similar phenotypes in independent *ddm1* lines and *met1* lines (Finnegan et al. 1996, Ronemus et al. 1996, Kakutani et al. 1996, Kakutani 1997). For example, late flowering traits are frequently observed in *ddm1* inbred lines and in lines expressing *MET1* antisense RNA, suggesting the underlying mechanism is non-random and possibly epigenetic (Ronemus et al. 1996, Kakutani 1997). This late flowering phenotype is inherited as a monogenic dominant trait mapped to a chromosomal region containing *FWA* (Kakutani 1997). *FWA* is one of the flowering-time loci previously found by conventional mutagenesis and linkage analysis (Koornneef et al. 1991). *FWA* gene was subsequently cloned by a map-based approach (Soppe et al. 2000). Both *fwa-1* and *fwa-2* mutants are semi-dominant (Koornneef et al. 1991), suggesting that they are gain-of-function mutations. Intragenic revertants with normal flowering time have been recovered from *fwa-1* after mutagenization, again suggesting that the original *fwa-1* mutation is a gain-function mutation (Soppe et al. 2000). All the three revertant alleles of *fwa-1* have putative loss of function mutation in the *FWA* gene, indicating that it is the responsible gene (Soppe et al. 2000). However, both *fwa-1* and *fwa-2* alleles do not show change in the nucleotide sequence compared with the wild-type allele. Instead, *FWA* gene is ectopically expressed in the *fwa-1* and *fwa-2* mutants (Soppe et al. 2000). Thus, they are gain-of-function epigenetic alleles, analogous to the loss-of-function epigenetic alleles of the *SUP* gene. The ectopic expression of the *FWA* gene is associated with

hypomethylation of direct repeat around the transcriptional starting site (Soppe et al. 2000). The ectopic expression and hypomethylation of the repeats were also observed in the late flowering *ddm1* and *ddm2* (*met1*) lines, suggesting that the hypomethylation can generate the meiotically heritable epigenetic mark responsible for the late-flowering phenotype (Soppe et al. 2000, Lindroth et al. 2001, Kakutani unpublished).

Another interesting example of developmental abnormalities induced under the *ddm1* background is a dwarf phenotype called *ball* (*bal*). This phenotype is also inherited as a monogenic Mendelian trait (Kakutani et al. 1996). The *BAL* locus was mapped to the clustered disease resistance (R) genes (Stokes et al. 2002). Over-expression of R gene seems to be responsible for the *bal* phenotype, as constitutive expression of the R gene At4g16890 in transgenics mimics the phenotype (Stokes et al. 2002). Interestingly, the epigenetic overexpression state of the *BAL* locus is heritable but metastable: it reverts at high frequency to the normal silent state after exposure to EMS or irradiation (Stokes et al. 2002). In short, epi-alleles of both *FWA* and *BAL* genes result from overexpression with loss of silencing in the repeated sequences.

Is such inheritance of the epigenetic state over generations unique to plants? Stable chromosomal inheritance of the epigenetic state during mitosis and meiosis has also been found in fission yeast (Grewal and Klar 1996), although genomic DNA methylation has not been found in this organism. In the fission yeast systems, association of heterochromatin protein, rather than DNA modification, may be responsible for the epigenetic inheritance (Nakayama et al. 2000). Moreover, inheritance of the epigenetic state was even been found in mouse: epigenetic modification of some alleles of the *AGOUTI* gene, which is associated with IAP retrotransposon insertion, is heritable over generations (Michaud et al. 1994, Morgan et al. 1999), although the stability is much lower than those in the plant epi-alleles discussed above. The stable inheritance of the epigenetically suppressed state over generations might be an evolutionary prototype of epigenetic control. If so, mammals may modify it to control development through control of the DNA methylation pattern. Consistent with the idea that developmental control of DNA methylation evolved relatively late in evolution, a change in the DNA methylation pattern has not been found in zebra fish (Macleod et al. 1999).

Control of DNA methylation

The inheritance of DNA methylation states over generations is not confined to the sequences affecting development, such as *FWA*, *BAL* and *SUP*. Methylation in the majority of *Arabidopsis* genomic sequences seems to be controlled in a similar way. Centromeric 180-base repeats, rDNA clusters and CACTA family transposons become hypomethylated by *ddm1* mutation. All these sequences remained hypomethylated even after out-crossing to the *DDM1* wild-type background (Kakutani et al. 1999, Kato and Kakutani unpublished). The inheritance of the hypomethylation was also observed by global genomic level measured by TLC and HPLC (Vongs et al. 1993, Kakutani et al. 1999).

The stable inheritance of the DNA methylation pattern over generations raises the question how methylation patterns are initially established. In other words, why are some sequences (sites) methylated while others not? Very intriguingly, Mette et al. (2000) found that when RNA with inverted repeat sequence was generated from a transgene, the corresponding sequence in another locus was methylated de novo. A similar phenomenon has previously been found in viroid-infected transgenic plants (Wassenegger et al. 1994). These phenomena, called RNA-directed DNA methylation (RdDM), could be induced in various sequences and generally occurs at all cytosines including non-symmetrical sites (Wassenegger et al. 1994).

Although global de novo methylation comparable to that during mammalian development has not been found in *Arabidopsis*, its genome has copies of genes structurally similar to mammalian de novo DNA methyltransferase DNMT3s (Okano et al. 1998, Okano et al. 1999). As they have a novel arrangement of the motifs required for DNA MTase catalytic activity, they are called "domain rearranged methyltransferase" or *DRM1*, *DRM2* and *DRM3* (Cao et al. 2000, www.chromdb). Loss of function mutation of *DRM1* and *DRM2* genes does not affect pre-existing methylation in the *FWA* or *SUP* loci, but blocked de novo methylation of these sequences triggered by the transgene with inverted repeats, suggesting that the *DRM* genes are de novo methylase (Cao and Jacobsen 2002).

An interesting example of control in the methylation of the endogenous sequence has been found in the *PHOSPHORIBOSYLANTHRANILATE ISOMERASE 2* (*PAI2*) gene. The *PAI2* gene is methylated and silenced in ecotypes with inverted duplication of similar sequences *PAI1-PAI4* at another locus (Bender and Fink 1995, Melquist et al. 1999). In ecotypes without the duplicated *PAI1-PAI4*, however, *PAI2* is unmethylated (Melquist et al. 1999). In addition, after introduction of the inverted repeat copy by transformation or genetic hybridization between ecotypes, the unmethylated copy was methylated de novo (Luff et al. 1999). The de novo methylation of *PAI2* gene may result from direct DNA–DNA interaction or by the RdDM mechanism directed by transcripts from the *PAI1-PAI4* inverted repeat. High-density methylation including non-CpG sites, similar to the RdDM, has also been found in *clk* epi-alleles of the *SUP* gene (Jacobsen and Meyerowitz 1997). In both *SUP* and *PAI2*, the high-density methylation is associated with transcriptional silencing. Screens for *trans*-acting mutations that release silencing of these genes identified several mutant alleles of the *CHROMOMETHYLASE 3* (*CMT3*) gene (Lindroth et al. 2001, Bartee et al. 2001). Chromomethylases are structurally related to other DNA methyltransferases but contain additional chromodomain motifs (Henikoff and Comai 1998, Papa et al. 2001, Lindroth et al. 2001, Bartee et al. 2001). *cmt3* mutations affect methylation in non-CpG sites, especially CpNpG sites, rather than CpG sites (Lindroth et al. 2001,

Bartee et al. 2001). *MET1* seems to be involved in maintenance methylation of CpG sites, whereas *CMT3* seem to be necessary for methylation in non-CpG sites. Biochemically, it is not known whether CMT3 has maintenance or de novo methylation activity. However, the *CMT3* product may be a component of machinery causing de novo methylation at non CpG sites, because transformation of the *cmt3* mutant with the wild-type *CMT3* gene results in re-methylation of the *PAI* sequence (Bartee et al. 2001).

In addition to the *CMT3* gene, Jackson et al. (2002) also identified mutations in another gene from the screening that release silencing of the *clk* allele. This new gene, named *KRYPTONITE* (*KYP*), encodes a protein similar to enzymes methylating the ninth residue of histone H3. Indeed, the KYP protein shows H3 methylase activity in vitro (Jackson et al. 2002). Since the *Arabidopsis* homologue of HETEROCHROMATIN PROTEIN 1 (HP1) physically interacts with both CMT3 and methylated histone, it may function as a link for recruitment of CMT3 to heterochromatic region (Jackson et al. 2002). The methylation of histone H3 is associated with heterochromatin formation in many eukaryotic systems. The involvement of histone methylation on DNA methylation has also been found in *Neurospora*. Working on a *Neurospora* mutant defective in DNA methylation, Tamaru and Selker found that H3 methyltransferase, called DIM5, is necessary for DNA methylation (Tamaru and Selker 2001). The methylation of histone H3 is necessary for stable heterochromatin formation in many eukaryotic systems, and DNA methylation may function to further stabilize the silent state.

A most notable example of involvement of double-strand RNA in the epigenetic process is post-transcriptional gene silencing (PTGS)/RNA interference (RNAi). PTGS has first been described as enigmatic phenomena called "co-suppression" in the late 1980s and has been studied extensively in plants (Jorgensen 1995). In 1998, Fire et al. (1998) reported an analogous phenomena, RNA interference (RNAi), a targeted degradation of mRNA in *C. elegans* induced by double-strand RNA. Since then, genetic and biochemical studies in *C. elegans*, *N. crassa*, *Chlamydomonous*, *Drosophila* and *Arabidopsis* are revealing the conserved mechanism for PTGS/RNAi (reviewed by Waterhouse et al. 2001). RdDM has only been found in the plant kingdom, but it would be very interesting to learn whether some of the components of RNAi and RdDM overlap. As RNAi was observed even in organisms without detectable genomic DNA methylation, such as *C. elegans* or *Drosophila*, cytosine methylation might have been evolved from RNAi as another layer of genome defense mechanism. Interestingly, major targets of methylation by *CMT3* are transposons and RNAi-based process may be involved in target recognition (Tompa et al. 2002).

Although TGS and PTGS/RNAi have originally been regarded as separate phenomena, recent identification of RdDM suggests an important link between them. In mammalian epigenetic systems, establishment of gene silencing is often accompanied by production of non-coding RNA, such as Xist and Air (Avner and Heard 2001, Reik and Walter 2001, Sleutels et al. 2002). It is intriguing to speculate that RNA metabolism related to PTGS/RNAi machinery functions for the establishment of the epigenetic states, while TGS/DNA methylation machinery in plants functions for its maintenance.

Epigenetic control of plant development

PTGS machinery may be involved in plant development. Mutations in the *AGO1* gene, which is necessary for PTGS, also result in various types of developmental defects (Fagard et al. 2000, Bohmert et al. 1998). In addition, mutations in the *CARPEL FACTORY* gene, a candidate homologue of an RNAi component, Dicer, result in unregulated cell division in floral meristems (Jacobsen et al. 1999). Identification of direct targets of PTGS involved in these developmental processes would be an important breakthrough. In *C. elegans*, lin-4 and let-7 are founder members of short (ca. 22nt) microRNA, which control animal development (Grishok et al. 2001). By direct cloning procedures, many additional members of microRNA have been cloned in *C. elegans* and in humans (Lagos-Quintana et al. 2001, Lau et al. 2001, Lee and Ambros 2001). Application of such an approach in plants might lead to identification of targets of PTGS machinery controlling plant development.

Do DNA methylation and TGS machinery also control plant development? Although several endogenous target genes for the DNA methylation and TGS mutant have been identified (*SUP, FWA, BAL* and *PAI,* for example), it is still not known if the DNA methylation and TGS of these genes play a role during normal plant development. TGS in plants is often so stable that it is inherited over generations. In addition, separation of somatic and germ cells is not as clear in plants as it is in animals: the shoot apical meristem of a plant contributes to the generation of many organs as well as gametes. Provided that the DNA methylation pattern is inherited by the next generation, irreversible change of the methylation in the apical meristem may not be a good strategy for controlling development. Instead, control of development by DNA methylation might be possible in terminally differentiated tissues which do not contribute to the next generation; an obvious example is endosperm. This review does not cover another type of transcriptional gene silencing mediated by polycomb proteins. This type of chromatin silencing, which is reset in each generation, is important for many developmental processes including flowering and endosperm formation (reviewed by Preuss 1999, recent advances in Gendall et al. 2001, Kinoshita et al. 2001, Yoshida et al. 2001). The possible connection between silencing mediated by polycomb proteins and DNA methylation is another challenging field (Finnegan et al. 2000, Adams et al. 2000, Vielle-Calzada et al. 1999, Vinkenoog et al. 2000).

Note added in proof

Important papers have recently appeared on plant small RNAs (Llave et al. *Plant Cell* 14: 1605–1619, Reinhart et al. *Genes Dev.* 16: 1616–1626, Rhoades et al. *Cell* 110: 513–520) and interplay between DNA and histone modifications (Johnson et al. *Curr. Biol.* 12: 1360, Gendrel et al. *Science* 297: 1871–1873).

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