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Review

DNA methylation - an essential mechanism in plant molecular biology

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Abstract

DNA methylation is a common phenomenon in plants. In plant genomes, its level is comparatively lower than that of animal genomes. It is involved in gene regulation and controls many development pathways. Methylation status of particular DNA sequence controls the potential for transition from vegetative to reproductive growth. It is believed that fully methylated elements are genetically and transcriptionally silent, however, some methylated genes may also be expressed. While hypomethylated elements are active and partially methylated elements, designated programmable, exhibit a variety of development expression programmes during plant development. DNA methylation plays an important role in the evolution of plant species through alloploidy or polyploidy. The methylation pattern in parental plants is highly heritable which is of great interest for plant breeders. DNA methylation also plays an important role in genome defense system by inactivating and methylating the invasive DNA sequences. A methylated sequence may suppress gene expression in other sequences. The generation and breeding of transgenic plants becomes complex due to inactivation of transgenes and instability of their expression. The pattern of methylation is maintained by methyltransferase through DNA replication. Several methods are in use to detect methylated nucleotides motifs that may help in identification of some essential genes.

Introduction

Genomic DNA methylation

The genome of an adult vertebrate cell has 60-90 % of the cytosines in CpG dinucleotides methylated by DNA methyltransferase (Riggs and Porter 1996). The genomes of fishes and amphibians are, on average, about twice as methylated as those of mammals, birds and reptiles (Jabbari *et al.* 1997). In plant genomes, the level of methylcytosines is comparatively low (Shimizu *et al.* 1997). This modification is up to 30 % and is not restricted to CpG doublets and CpNpG triplets (Gruenbaum *et al.* 1981), but also occurs in non-symmetrical DNA sequences (Meyer *et al.* 1994; Pelissier *et al.* 1999). The G + C content of nuclear DNA recorded in tobacco is 40.3 % (Wagner and Capesius 1981) and in tomato 40.7 % (Messeguer *et al.* 1991). However, the content of 5-methylcytosine in plant DNA is comparatively higher than animal DNA (Messeguer *et al.* 1991). In plant genomes, a large amount of CpG sequence was found in upstream regions of start codon and comparatively less in coding regions (Shimizu *et al.* 1997). The lowest CpG content was observed in non-coding regions (Messe-

guer *et al.* 1991). Genomic DNA methylation plays an important role in the evolution of plant species, plant development and physiology through involvement in gene expression mechanism.

Evolution of plant species and DNA methylation

The occurrence of heritable C-methylation patterns in evolutionarily divergent species suggests not only an ancient origin of the mechanisms that generate and maintain these patterns but also indicates an important role for methylation in species where it occurs (Bird 1986, Cedar 1988, Smulders and Rus-Kortekaas 1995). Polyploidy has played a major evolutionary role in the formation of many plant species (Soltis and Soltis 1995). The production of new polyploid species is often accompanied by extensive genomic modifications in a short period of time (Walbot and Cullis 1985; Riesenberg *et al.* 1996). The mechanism by which several genomes coexist in the same nucleus is largely unknown. This mechanism may involve adjustments in DNA sequences (Feldman *et al.* 1997). The duplicated sequences escape silencing if they are embedded in non-intersecting chromosomal environments or if they contain a significant degree of sequence divergence (Meyer and Saedler 1996). Many plant genomes carry a large proportion of duplicated loci (Whitkus *et al.* 1992). The methylation induced by repeated sequences counter-balances DNA amplification processes, generating heterogeneous epigenetic patterns in repeated sequences that can be further modulated by environmental factors during evolution (Meyer and Saedler 1996). Considerable portion of the genomic DNA is fully methylated and remains esoteric (Fedoroff *et al.* 1989). Methylation polymorphism also represents an additional source of variation. Allele-specific methylation is apparently inherited and is a dynamic process as new alleles with heritable methylation patterns are likely to occur with a relatively high frequency in plant populations. Allele-specific methylation polymorphism is not only common in plants but also represents an important form of genetic variation. In tomato, this has implications not only for creating new genetic variation of potential evolutionary significance but it also has practical implications (Messeguer *et al.* 1991). The occurrence of alleles which are not only differentially methylated, but also display tissue-specific patterns of methylation, may help reconcile the apparent paradox of stable inheritance of methylation patterns, from parent to offspring, with developmental variation in methylation within an individual (Silva and White 1988, Messeguer *et al.* 1991).

Plant physiology and DNA methylation

A large part of literature on the subject has provided much circumstantial evidence in support of the hypothesis that DNA methylation is involved in gene regulation. DNA binding proteins, including transcription factors can be sensitive to the presence of methylated cytosine in DNA and the transcription activity has been inversely correlated with methylation level of cytosine residues within the promoter region of a gene (Jones and Buckley 1990, Hadifield *et al.* 1993, Zhou *et al.* 1996, Virmani *et al.* 2000).

An increase in DNA methylation can lead to the formation of a condensed chromatin structure (Selker 1990, Lewis and Bird 1991, van Blokland *et al.* 1997). The possible mechanism of this process involves the interaction of a methylated sequence in a CpG-rich region with a methylated DNA-binding protein to form a secondary structure that is insensitive to endonucleases. This structure not only inhibits RNA transcription from the region but also alters DNA replication time (Jablonka *et al.* 1985, Selig *et al.* 1988, Pikaard 1999). Recently Kass *et al.* (1997) have proposed that the assembly of specialized nucleosomal structures on methylated DNA helps to explain the capacity of methylated DNA segments to silence transcription more effectively than conventional chromatin.

The production of transgenic plants is very quickly becoming a routine for a number of crops to generate plants that are resistant to insect-pests, diseases and adapted to specific soil or ecological conditions. The level of transgenes expression in plants is usually unpredictable (see review Finnegan and McElroy 1994). Inactivation of transgene in plants due to methylation has been reported in tobacco (Matzke *et al.* 1989) and *Petunia hybrida* (Meyer *et al.* 1993). However, there are also numerous evidences of positive effects of plant transformation (Cerutti *et al.* 1997). A more disturbing problem in transgenic agricultural plants is instability of trans-

gene expression between generations (Meyer *et al.* 1992) and non-Mendelian inheritance of the transgenic phenotypes (Scheid *et al.* 1991, Park *et al.* 1996). Instability rates have been determined with higher numbers of insertion loci and in crosses between independent transgenic plants, in successive generations of tobacco (Cherdshewasart *et al.* 1993, Schmulling and Rohrig 1995). In the history of plant genetics, there are several examples of non-Mendelian phenomena demonstrating that identical DNA sequences can adopt alternative information states which may be inherited through many generations of organisms (Stokes and Richards 2000). Perhaps the most familiar examples of such epigenetic modifications include paramutation of *b* and *pl* alleles in maize (Depicker and Montagu 1997) and transgene silencing in *Chlamydomonas* (Cerutti *et al.* 1997). In paramutation, one allele alters the activity of its allelic partner on the homologous chromosome through a chromatin level change that leaves the underlying DNA sequence unaltered (Hollick *et al.* 1997). In transformants of *Chlamydomonas*, expression of a eubacterial gene aadA is transcriptionally suppressed by reversible epigenetic mechanism. Gene silencing does not correlate with methylation of the integrated DNA and does not involve large alterations in its chromatin structure (Cerutti *et al.* 1997). Epigenetic phenomena are a consequence of chromatin level regulation directed at transposonable elements to region in their potentially damaging effects (Martienssen 1996, Steimer *et al.* 2000).

The inactivation mechanism is unclear. It has been suggested that plant genomes are mosaics of compositionally homogenous DNA segments, with defined GC content termed "Isochores" (Matassi *et al.* 1989). Disruption of the normal make up of an isochore by insertion of a foreign DNA fragment that differs in GC content may mark this region for inactivation and methylation (Finnegan and McElroy 1994). For example, a single copy of maize AI transgene in petunia, which became hypermethylated, even though flanking plant DNA sequences remained hypomethylated (Meyer and Heidman 1994). At the time of DNA-DNA pairing duplicated sequences are readily *de novo* methylated and transcriptionally inactivated (Selker and Garrett 1988, Assaad *et al.* 1993, Rossignol and Faugeron 1994). RNA induced methylation of genomic DNA could provide a means to control over expressed nuclear genes (Wassenegger *et al.* 1994, Baulcombe 1999).

A methylated sequence may have epigenetic effects, suppressing gene expression in other sequences. This has been observed in studies with transgenic plants, where activity inhibition of some methylated genes appears to depend upon DNA methylation in other sequences (Matzke *et al.* 1989, Linn *et al.* 1990). Methylation plays an important role in the genome defense system that inactivates and methylates invasive DNA sequences, such as viral DNA, transposable elements, and multiple copies of transgenes (see review Matzke *et al.* 1995). For instance, methylation inhibited propagation of tomato golden mosaic virus DNA in transfected protoplasts (Brough *et al.* 1992). Recent research in RNA-mediated virus resistance and co-suppression has provided insight into the interactions between plant viruses and their hosts, and spawned several models to explain the phenomenon (Waterhouse *et al.* 1999). Several studies have depicted an association between post-transcriptional gene silencing and coding region methylation in plants (English *et al.* 1996, van Houdt *et al.* 1997). A non-metabolic, transgene-specific, diffusable messenger mediates the propagation of *de novo* post-transcriptional silencing through plants. Transgene-specific post-transcriptional silencing is transmitted with 100 % efficiency from silenced stock to non-silenced scions expressing the corresponding transgene in tobacco (Palauqui *et al.* 1997). Post-transcriptional gene silencing is a homology-dependent process that reduces cytoplasmic RNA levels and is associated with methylation of DNA. To study the RNA-DNA interactions and *de novo* methylation, Jones *et al.* (1999) investigated post-transcriptional gene silencing of a transgene and a endogenous gene in *Nicotiana benthamiana* and proposed an epigenetic model of post-transcriptional gene silencing in which transgene methylation is associated with an RNA-DNA interaction that ensures that post-transcriptional gene silencing is maintained (also see review Stokes and Richards 2000).

Plant development and DNA methylation

According to methylation states genome of a plant can be divided in to three categories; fully methylated elements are genetically and transcriptionally esoteric, while hypo-methylated elements are agile and partially methylated elements designated contrivable, exhibit a variety of developmental expression programs (Fedoroff *et al.* 1989, Monk 1995). However, methylated genes may also be expressed. Amedeo *et al.* (2000) have isolated an Arabidopsis gene, MOM, whose disruption releases transcriptional silencing of methylated genes. According to them, MOM is the first known molecular component that is essential for transcriptional gene silencing and does not affect methylation pattern. In rice, silencing of a ß-glucuronidase gene is independent of DNA methylation and is correlated with repetitive transgene structure (Wang and Waterhouse 2000). The partial methylation, which shows differential methylation during development has been reprogrammed and reset to make tissue specific gene expression and phase change (Furner *et al.* 1998). Many authors have considered methylation involvement in the mechanism for cell-and organ-specific gene expression (Riggs and Chrispeels 1990, Boyes and Bird 1991, Monk 1995).

In *Arabidopsis*, it has been observed that DNA methylation plays an important role in regulating many developmental pathways (Yanofsky 1995, Finnegan *et al.* 1996). Burn *et al.* (1993) suggested that the methylation status of particular DNA sequences controls the potential for transition from vegetative to reproductive development. Distinctive DNA methylation patterns have been observed in the apical meristem of J and A phase in olive (Mazzuca *et al.* 1995) and apple tree (Hafiz 1998). Mazzuca *et al.* (1995) have also proposed that the transition seems to be related to a change in gene expression. In some species of fungi, the level of DNA methylation has been related to different growth stages (Juppe *et al.* 1986, Russell *et al.* 1987, 1989, Reyna-Leopez *et al.* 1997). It ensures inheritance of the appropriate developmental state through both mitosis and meiosis (Holliday 1987, Vyskot *et al.* 1995). The pattern of DNA methylation changes throughout the life cycle of plants, *e.g.* in wheat (Brown 1989, Brown *et al.* 1989), pea (Watson *et al.* 1987), *Petunia hybrida* (Anderson *et al.* 1990) and in crab apple (Hafiz 1998). It is interesting to note that in plant cell nucleus, specific factors are present which are responsible for activity, substrate and site specificity of methylation during the two phases of genome modification (Kass *et al.* 1997, Finnegan *et al.* 1998). In the cells of immature seeds, minor modulations of genome methylation during its replication may cause heritable changes in genome expression (Kirnose *et al.* 1995). Single dose treatment of 5-azacytidine to rice seeds caused heritable changes in genome methylation and gene expression (Sano *et al.* 1990). Similar observations have been reported in broccoli (King 1995), flax (Fields and Durrant 1994) and tobacco (Palmgren *et al.* 1993).

Messeguer *et al.* (1991) have suggested that lower levels of methylation in immature/juvenile tissues may be, at least in part, to the fact that these tissues are likely experiencing higher levels of cell division and thus contain higher proportions of hemimethylated DNA. While there is still some contention on this issue, it is generally accepted that there is little or no lag time between DNA replication and methylation (Razin *et al.* 1984). If this holds true for apple, it seems unlikely that higher levels of hemimethylated DNA in immature/juvenile tissues can account for their over all lower levels of methylation (Hafiz 1998). It is interesting to note that in *Petunia hybrida* plant, ribosomal RNA genes in adventitious roots formed from stem cuttings, were hypomethylated (Anderson *et al.* 1990). Higher rooting ability of juvenile tissues of trees can be attributed to low levels of methylation of the genomes. The lower levels of methylation might be indispensable for higher growth rate of juvenile phase meristem (Watson *et al.* 1987).

DNA methylation maintenance

The specificity of CG and CG/CNG methylation in animals and plants, respectively is attributed to the properties of the MTase (methyltransferase) that create and maintain the pattern of methylation throughout DNA replication. The purified vertebrate MTases show a strong preference *in vitro* for methylation of CG dinucleotides, as compared with CA and CT (Adams *et al.* 1993). In plants, two distinct DNA MTases have been purified from *Pisum*

sativum. The presence of two DNA methyltransferases raises the possibility that they serve different functions, one for CG dinucleotides, the other for the trinucleotides CAG and CTG (Yesufu *et al.* 1991, Pradhan and Adams 1995, Pradhan *et al.* 1999). *In vitro*, most MTases act preferentially on hemimethylated DNA, which is the intermediate during replication of a fully methylated template. Together with the symmetry of CG and CNG sequences, this could explain the maintenance of a given methylation pattern. The genes encoding for the EcoRII DNA methyltransferase (M.EcoRII MTase) modifies a cytosine in the DNA sequence CCWGG, which contains a CNG methylation motif, characteristic of plant DNA. The DNA methyltransferase maintains the methyl-CpG content of both daughter DNA duplexes following replication (Holliday 1987). Methyltransferase localizes to the chromosomal replication complex and maintenance methylation takes place less than one minute following replication (Razin *et al.* 1984). It is of great interest for plant breeders that methylation pattern present in parental plants is highly heritable and is passed on to the offspring's in a normal Mendelian fashion (Messeguer *et al.* 1991). The fully methylated elements in plant genome are genetically and transcriptionally occult and are maintained with high fidelity, while partially methylated elements are scheduled, exhibit a variety of development expression programmes (Hafiz 1998, Fedoroff *et al.* 1989). The mechanism of *de novo* methylation and demethylation has been explained by Kass *et al.* (1997).

The *de novo* methylation of CpG dinucleotides is a regulated process. There is co-existence of transcriptional activator and repressors, if repressor operates more effectively than transcriptional machinery, the methyltransferase enzyme performs *de novo* methylation. If components of regulatory complexes can bind to DNA immediately after replication with reasonable efficiency and before DNA methyltransferase begins to modify the template, then they prevent DNA methylation around their binding sites. The sequences then might become progressively demethylated (Kass *et al.* 1997).

DNA methylation detection

Several methods are applicable to detect cytosine or adenine methylation in nuclear DNA and cell organelles, such as plastid chloroplasts and mitochondria. Procedures such as the hydrolysis of DNA, followed by comparison of Tm and bouyant density (Hake and Walbot 1980, Razin *et al.* 1984), and HPLC determination of nucleoside composition (Messeguer *et al.* 1991, Cai and Chinnappa 1999) can determine methylcytosine content, but can not provide any information about the location in the genome.

The techniques helpful to identify methylation of the residues specific to genes, or particular regions of DNA include southern blot analysis of DNA that has been digested with a pair of isoschizomers restriction enzymes (REs) having different sensitivities to methylation in restriction sites (Messeguer *et al.* 1991, Poulsen *et al.* 1993) and bisulphite base conversion method followed by PCR and sequencing (Frommer *et al.* 1992). Another method consisting of reaction of monoclonal antibodies with methylated nucleotides is also under use (Oakeley *et al.* 1997). Xiong *et al.* (1999) used the technique of methylation-sensitive amplified polymorphism (MSAP), which is a modification of the amplified fragment length polymorphism (AFLP) to assess the extent and pattern of cytosine methylation. This method makes use of differential sensitivity of a pair of isochizomers. However, the procedures are somewhat laborious.

A novel technique based upon the couple restriction enzyme digestion and random amplification (CRED—RA) of genomic DNA developed by Cai *et al.* (1996) allows detecting loci in DNA having methylation. The procedure is quite simple and applicable in many situations (Cai and Chinnappa 1999). However, combination of one of the two methods may result in a high degree of precision and success. The detection of methylated nucleotide motifs may be helpful in identification of some essential genes.

Conclusions

1. DNA methylation is a common phenomenon in plant genomes.
2. It plays an important role in evolution of plant species.
3. It remains dynamic through out plant life and contributes to plant development and organ differentiation.
4. It should be considered an important factor in expression of transgenes and breeding task.
5. Several methods are applicable in detection of methylated DNA moieties. The combination of two or more methods may result in more precision.
6. The phenomenon can help in identification and probing of some essential genes.

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