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THE METHYLATION-SENSITIVE AMPLIFICATION POLYMORPHISM IN JUVENILE AND ADULT PHASE CRAB APPLE (*MALUS MICROMALUS*)

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Abstract

Cytosine methylation has been implicated in regulation of gene expression, genomic imprinting and chromatin remodeling, resulting both in temporal and developmental regulation. Keeping in view its importance in development, this study was carried out to explore the methylation-sensitive amplification polymorphism (MSAP) associated with the transition from juvenile to adult phase in *Malus micromalus.* For detection of methylation in the genome of *M. micromalus*, a pair of restriction endo-nucleases HpaII-MspI was used. Genomic DNA from juvenile and adult phase leaves of the seedling tree, that was undigested (control) and digested with each enzyme HpaII and MspI, was amplified using selected primers. In total 72 bands were amplified with the help of 12 primers. Post amplification digestion of these bands with MspI or HpaII revealed 35 bands containing CCGG. Ten bands absent in amplified profiles from J phase digested DNA, appeared in amplified products from digested A phase DNA, indicating *de novo* methylation at CCGG. Five bands disappeared in A phase while these bands were present in J phase DNA amplified profiles, revealing presence of restriction site without methylation in A phase. These results are suggestive that demethylation may have occurred in A phase. Seventeen motifs of DNA methylation at CCGG remaining similar in both phases seem to have been maintained from basal to crown part of the seedling tree. Amplified profiles produced from restricted DNA from both phases showed polymorphism due to differential methylation.

Introduction

Phase change is an important phenomenon in plant life. It is a complex mechanism, which is under control of several key genes that govern subsets of many genes. The coordinated expression of gene(s) managed by/results in a series of intricate biochemical events, hitherto, incompletely understood, are nevertheless being actively investigated. One of the significant hypotheses being developed recently relies on DNA methylation as a strong candidate in modulation of gene expression**.** Finnegan and co-authors (1998) have reviewed the process of epigenetic control of gene expression and concluded that methylation is the most widely studied mechanism involved in this process. The event is carried out by transfer of methyl group from S-adinosyl methionine by specific DNA methyl transferases. In most mammalian and plant DNA, methyl transferases target CpG or CpNG sequences as their substrate (McClelland *et al*., 1994*)*. Cytosine methylation occurs predominantly in the CG and CNG sites (Dai *et al*., 2005). Over 70% CpGs are methylated in somatic cells (Richard *et al*., 2005) DNA binding proteins, including transcriptional activators can be sensitive to the presence of methylated cytosines in DNA (Inamdar *et al*., 1991). The transcriptional activity has been shown to be inversely correlated with methylation of cytosine residues within the promoter region of a gene

(Zhou *et al*., 1996, Pikaard, 1999). Methylation of CpG islands has a strong correlation with transcriptional suppression, and lack of methylation is required for expression of the associated gene (Richardson, 2003).

It has been observed that the pattern of DNA methylation changes throughout the life cycle in *Petunia hybrida* (Anderson *et al*., 1990). In plants DNA methylation changes with age and is regulated by phytohormons (Vanyushin, 2005). In some species like pea and maize the level of DNA methylation has been related to different growth stages (Watson *et al*., 1987). Methylation also ensures inheritance of the appropriate developmental state through both mitosis and meiosis (Barry *et al*., 1993). In *Arabidopsis* DNA methylation plays an important role in regulating many developmental pathways (Finnegan *et al*., 1996). These and many other reports suggest that local epigenetic differences, which might occur between two tissues, or two distinct growth stages in a plant, might alter phenotypes. In the light of the available literature, it is evident that studying methylation pattern in the genome of a plant and how it is altered would be important. This study was, therefore, aimed at studying the DNA methylation patterns, following transition from juvenile to adult phase in a woody perennial fruit tree, the *Malus micromalus.* Information obtained through this investigation would contribute towards understanding the biological role of cytosine methylation during phase transition in plants.

Materials and Methods

DNAs of higher eukaryotes are frequently methylated at CpG or CpNG. This methylation interferes with cleavage by certain restriction endonucleases. Endonucleases sensitive to m5CpG or m5CpNG methylation, as well as isoschizomer that recognize identical sequences but differ in their sensitivity to methylation are useful for studying the level and distribution of methylation in eukaryotic DNA. In this study we have used HpaII and MspI for the study.

Mature seedling tree of apple, *Malus micromalus*, was used as study material. Young leaves with juvenile characters from basal shoots < 50-cm height from ground level and young leaves with mature phase characters were collected from crown shoots of the same tree. Samples were washed thoroughly with tap water and rinsed with distilled water to remove insect eggs or any residual pesticide, dried with blotting paper.

DNA extraction: Genomic DNA was extracted according to the method described by Chen *et al*., (1997). Two-gram plant material was ground to a fine powder in liquid nitrogen with the help of porcelain pestle and mortar and transferred to 50 ml polyvinyl tube followed by addition of 10 ml of homogenization buffer (0.4 mol/L glucose, 3% soluble PVP, 2.3% DDTC, 10 mM β-mercaptoethanol, 20 mM EDTA pH 8). The contents were centrifuged at 10,000 rpm for 10 minutes at 4^{o}C and supernatant was discarded. To the pellet containing crude nuclear fraction, an equal volume pre-warmed nuclear lysis buffer (100 mM Tris HCl pH 8, 20 mM EDTA, 0.5 M NaCl and 1.5% SDS) was added, mixed and incubated at 65^{o}C with occasional gentle mixing. After 1 hour an equal volume chloroform:ethanol:isopentanol (80:16:4) was added and after thorough mixing contents were centrifuged at 10,000 rpm for 10 minutes. Aqueous phase containing DNA was precipitated by addition of an equal volume of isopropanol. Precipitated DNA was dissolved in TE buffer (pH 8) and subsequently purified twice

with phenol:chloroform (1:1) and re-precipitated with 100% ethanol. Hooked out with glass rod, washed with 70% ethanol, air dried and dissolved in TE buffer (pH 8). DNA concentration was determined both by A_{260}/A_{280}, and in ethidium bromide stained agarose gel by comparison with λ/Hind III digested DNA markers. The DNA, was further purified to remove any residual organic solvents and proteins according to Vogelstein & Gillespie (1979).

DNA Methylation detection in juvenile and mature phase of crab apple in this experiment was performed following the Couple Restriction Endonucleases Digestion-Polymerase Chain Reaction (CRED-PCR) by Cai *et al*., (1996)

Genomic DNA digestion: Two restriction endonucleases were used separately to digest genomic DNA from J and A phase leaves either before or after Polymerase Chain Reaction (PCR) as mentioned in Table 1. To achieve complete digestion, 1 µg genomic DNA was digested with excess of restriction enzyme (10 units each) in 50 µl reaction mixture and incubated overnight at 37°C.

Table 1. Cleavage site sequences and methylation sensitivities of the restriction endonucleases used in this study.

Restriction Enzyme	Restriction site & methylated nucleotides	Restrictability of the site	References
Hpa-II	5C ↓ CGG 3 3 GGC ↑ C 5	Yes	Nelson & McClelland 1989
	$C^{5m}CGG$	No	McClelland *et al*., (1994)
	$^{5m}CCGG$	No	McClelland *et al*., (1994)
	$^{5m}C^{5m}CGG$	No	McClelland *et al*., (1994)
Msp-I	5C ↓ CGG 3 3 GGC ↑ C 5	Yes	Nelson & McClelland (1991)
	$C^{5m}CGG$	Yes	McClelland *et al*., (1994)
	$^{5m}CCGG$	No	McClelland *et al*., (1994)
	$^{5m}C^{5m}CGG$	No	McClelland *et al*., (1994)

RAPD amplification: The template DNA (15 ng) was amplified in a total volume of 36 µl reaction mixture, containing 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 2 µM of the primer, 1.25 u *Taq* DNA polymerase. PCR program included pre-amplification denaturation at 94ºC for 4 minutes, 45x [denaturation at 93ºC for 15 seconds, annealing at 37ºC for 60 seconds, and extension at 72ºC for 120 seconds], and final extension at 72ºC for 5 minute. PCR products were stored at 4ºC until used for electrophoresis. Amplified fragments were analyzed along side a standard λ/Hind III+EcoRI molecular weight markers in 1.7% agarose gels containing ethidium bromide in 0.5x TBE (Sambrook *et al*., 1989).

POST-Amplification digestion: 13 µl of PCR product was restricted by 10 units of respective restriction enzyme (MspI or HpaII) for 30 minutes at appropriate temperature.

Results

HpaII and MspI both recognize and cut sites with a CCGG sequence, however HpaII is sensitive to methylation at both or at either of the cytosine of the cytosine residue.

Therefore, HpaII is unable to cut at sites where either of the Cs is methylated (Mc Clelland *et al*., 1994). On the other hand MspI is sensitive to outer C methylation either alone or in combination with internal C methylation, however, it is not inhibited by methylation at internal C. Digestion of DNA template with the REs prior to PCR resulted in non-appearance of some bands from PCR amplified DNA profiles, which were seen in profiles obtained from undigested templates indicating probability of modification in the restriction site of the enzyme.

For further verification, PCR amplified products from the digested templates were restricted with MspI or HpaII. The bands absent after restriction of template with MspI but present after digestion with HpaII, disappeared when their amplified product was digested with HpaII, thus confirming presence of CCGG site which may have been modified by methylation in the original template either in the manner ^{5m}CCGG or ^{5m}C^{5m}CGG.

Genomic DNA from juvenile and adult phase leaves of *M. micromalus* seedling tree that was undigested (control) and digested with HpaII or MspI was amplified using primer S 209. Two bands were amplified from undigested DNA template of J and A phase (control). When amplified product was digested either by HpaII or MspI, one band disappeared, indicating the presence of CCGG site. When template DNA either from J or A phase was digested with HpaII prior to PCR amplification the same bands persisted. Presence of this band however indicated methylation of CCGG site present in it. On the other hand when same templates were digested with MspI, the band disappeared in PCR reaction from J phase, while persisted in A phase. This pattern indicated methylation only at internal C in J phase, and either at internal C or both Cs in A phase (Table 2).

In RAPD patterns obtained by amplification from both phases by using primer S 217, only four bands out of eight contained CCGG site. Two of these bands showed methylation both in the J and A phase. One band having methylation at internal C received/shuffled methylation and its outer C also became methylated in adult phase (Table 2). One band disappeared in both phases after restriction only with MspI showing methylated restriction site at internal C (C^{5m}CGG).

In S 249 amplified DNA profiles from both phases (J and A), four bands were amplified, all containing CCGG restriction site. HpaII digestion indicated one un-methylated site, both in J and A phase. On the other hand MspI digestion in J phase exposed internal methylation in three bands while none remained undigested. Thus restriction by HpaII and MspI revealed internal methylation on three of these sequences in J phase, while during transition from J to A phase these sequences either received an additional methyl group at external C or shuffled existing methyl group to the external C (Fig. 1).

Amplification profile obtained with S259 primer showed 11 bands, out of which only two could be digested by HpaII. When HpaII digested DNA was used as template, one of these bands (~300bp) was absent from amplified profile of J phase and both form A phase. MspI profile of J phase, on the other hand, showed an additional band of ~300bp, while both were present in A phase, indicating *de novo* methylation during phase transition.

Use of primer S295 leads to the amplification of 7 fragments including four containing CCGG site. HpaII digestion of template revealed methylation in all these sites. MspI digestion however indicated that these sites gained internal methylation during transition from J to A phase.

Table 2. Total number of bands amplified from J and A phase DNA in undigested control and number of bands lost in digestion prior to PCR.

Primer	Sequence of the primers	Total no. of bands in control	No. of bands lost in digestion prior to PCR				No. of bands lost in digestion post PCR	Remarks
			HPaII		MspI			
			J	A	J	A		
S 209	CACCCCTGAG	2			1		1	*de novo* methylation in adult phase at 10 loci and methylation motifs in genomic DNA at 3 loci
S 217	CCAACGTCGT	8	2	2	3	2	4	
S 249	CCACATCGGT	4	1	1	4	1	4	
S 259	GTCAGTGCGG	11	1		1	1	2	
S 295	AGTCGCCCTT	7			4		5	
S 256	CTGCGCTGGA	5			2		2	Demethylation in adult phase at 5 loci and methylation motifs in genomic DNA at 2 loci
S 267	CTGGACGTCA	6	1		1	1	2	
S 290	CAAACGTGGG	5	1		2		3	
S 223	CTCCCTGCAA	7					4	methylation motifs in genomic DNA at 12 loci
S 257	ACCTGGGGAG	3			1	1	2	
S 293	GGGTCTCGGT	11					4	
S 294	GGTCGATCTG	3			1	1	2	
TOTAL		**72**	**4**	**5**	**16**	**11**	**35**	

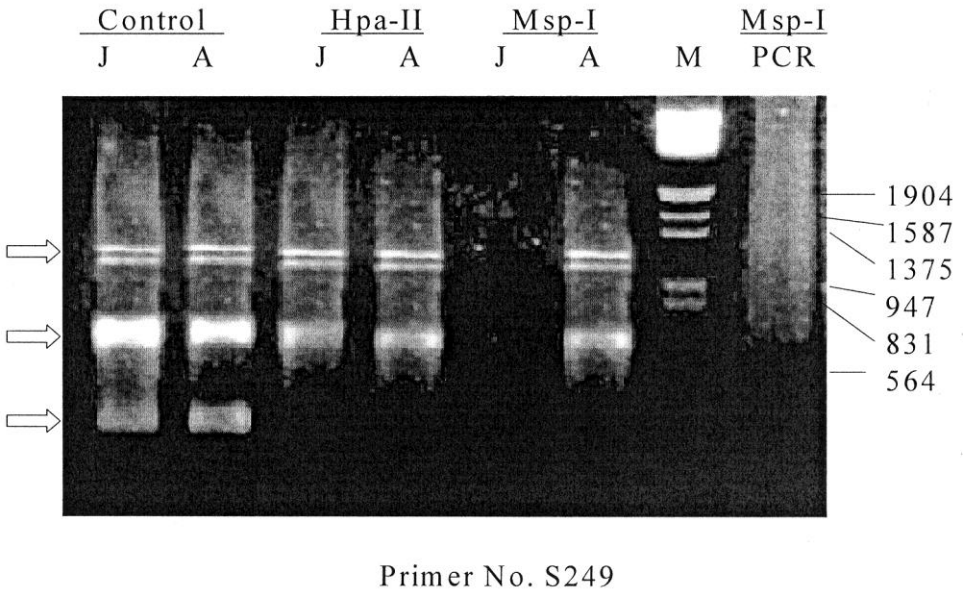

Fig. 1. J and A phase DNA amplified profiles by using Primer S 249, showing polymorphism due to variation in methylation following phase change.

Thus out of 32 bands amplified with S209, S217, S249, S259 and S295, only 16 contained CCGG site. Among 16 CCGG containing sites, four remained un-methylated in J phase DNA while three in A phase DNA. Out of 12 methylated sites in J phase DNA, 10 contained internal methylation, while transition to A phase resulted either in additional methylation at external C or shuffling of methyl group from internal to external C of the CCGG site.

The primer S256, S267, and S290 revealed polymorphism owing to *de novo* de-mthylation. Amplification using this set of primers resulted in 16 bands, out of which 7 could be digested HpaII. In template DNAs digested by HpaII no band appeared in J phase indicating that all the CCGG sites are methylated at either or both Cs in J phase. Moreover in HpaII digested templates from A phase, two bands did appear showing lack of methylation, leading to the conclusion that de-methylation has occurred presumably during phase transition.

Out of 7 bands containing CCGG site, MspI digestion of J phase genome could verify only one methylation event on internal C, and 5 on either external or both Cs. This evidence is suggestive of *de novo* demethylation at 5 loci.

Another set of primers (S223, S257, S293, and S294) amplified 24 bands, out of which 12 contained CCGG sites. All these sites were methylated, except two having methylation only at internal C both in J and A phase DNA. These results suggest lack of any change in methylation status during phase transition.

Discussion

During evolution from simpler to complex forms of life, the expression of genes seems to have become more important rather than mere their number. Higher eukaryotes have, therefore, evolved an array of complex gene regulation strategies to fit in the pattern of development and interaction with the environment. On the road to genomics, having surpassed the difficulties in sequencing, probably the next most demanding task is to unravel the mechanism of control of gene action. Cytosine methylation plays a critical role in directing patterns of heterochromatin formation in genome of plants with effect on both gene expression and genome stability (Bird, 2002). It plays a fundamental role in plant development, differentiation and physiology (Richards, 1997; Buryanov & Shevchuk, 2005). The role in plant development has been demonstrated by at least three different kinds of evidence: parent-specific expression of some genes in developing seeds, control of flowering time and floral morphogenesis, and correlation with silencing of mobile genetic elements and transgenes (Zluvova *et al*.**,** 2001)

In this study the DNA methylation pattern in genomic DNA of the seedling tree of *Malus micromalus* during transition from J to A phase has been investigated. Methylation generally occurs at cytosine residues in CpG doublets, and CpNG triplets of plant genomes (McClelland *et al.,* 1994). Presence or absence of methylation results in generation of polymorphism which may be determined by digestion of genomic DNA with a pairs of methylation responsive isoschizomeric restriction enzymes HpaII and MspI. The intact and restricted DNAs from both phases were used as template for PCR reactions.

Out of 12 methylated sites in J phase DNA, 10 contained internal methylaion, while in A phase additional methylation at external C has been established. This indicates *de nove* methylation activity, which has previously been demonstrated in mature phase DNAs of some plant species.

Watson *et al*., (1987) reported that methylation is generally low in young seedlings of pea, but DNA obtained from the apical buds of mature pea seedling is highly methylated. Similarly genomic DNA of immature tissues and protoplasts have been reported to possess significantly lower levels of cytosine methylation than that of mature tissues in tomato (Messeguer *et al*., 1991). In this study 10-de *novo* methylation even have been observed in *M. micromalus* adult phase DNA versis demthylation at 5 loci (Table 2). This shows an overall increase in methylation activity in adult phase, however, a general conclusion may be restrained owing to limited size of the data being reported. Although the level of increasing epigenetic modification has correlated with DNA methylation (Bestor *et al*., 1992) it is not possible to demonstrate any change in gene expression in the absence of the genetic characterization of the loci under investigation. The DNA methylation of many gene-regulatory regions inversely correlates with gene expression (Roloff & Nuber, 2005). Nevertheless association of increased levels of methylation with loss of gene expression has been reported by many investigators (Rochi *et al*., 1995, Janousek *et al*., 1996). Whereas presence of ^{m}CpG / ^{m}CpNGs exclusively in the coding region is reported to reduce expression, methylation in a promoter sequence has been established to impose a more pronounced inhibition (Dieguez *et al*., 1997; Hohm *et al*., 1996).

As six bands were absent in the patterns of amplified products from HpaII-MspI restricted A phase DNA, it seems that demethylation has taken place at target sites in these fragments. Methylation state of DNA can influence the binding affinity of proteins, including transcription factors (Schmulling & Rohrig 1995). Janousek *et al*., (1996) observed that 5-aza C-hypomethylation of CpG and CpNG in the *Melandrium album* genome caused activation of X-chromosome. In the present investigation we have found 5 loci to undergo demethylation of cytosine residues in the target site during transition from Juvenile phase. The changes observed are most probably not caused by the general methylation/demethylation but rather specific loci are involved. In the light of these and other similar studies and in the absence of specific information regarding the elements controlling the length of juvenile period and the time of transition to adult phase, one might hypothesize involvement of a demethylation dependent epigenetic switch. Hence further investigations in this area may be very interesting and revealing.

The result in addition to demonstrating *de novo* methylation/demethylation, have also indicated lack of change of methylation status of certain loci (Table 2). This also indicates the specific nature of the process, yet the specific function of methylation present in these DNA motifs remains unknown. Presences of such CpG/CpNG methylated islands are not unique to *M. micromalus*, but also exist in several plant species including maize, tobacco and wheat (Anteguera & Bird 1989). The methylated moieties detected in apple genome maintained persistently from basal to crown part of the tree can, therefore, be included in fully methylated sequences.

As the samples were collected from young leaves of both phases (J and A) from the same apple seedling tree, the differences in methylation levels, detected may be due to the state of development. The study being reported, consequently reveals that the methylation-specific amplified polymorphism provides strong clues to the possible involvement of the methylation in phase transition. In addition to validating the finding of Fedoroff *et al*., (1989) that according to methylation states, the genome of a plant can be divided into three categories; fully methylated elements which are genetically and transcriptionally silent (cryptic), hypo-methylated elements which are active, and partially methylated elements designated programmable which may exhibit a variety of developmental expression programs.

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