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DNA METHYLATION PROFILES DIFFER BETWEEN JUVENILE AND ADULT PHASE LEAVES OF CRAB APPLE (*MALUS MICROMALUS*) SEEDLING TREE

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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. In the present study methylation-sensitive amplification polymorphism (MSAP) associated with the transition from juvenile to adult phase in *Malus micromalus* was explored using a pair of restriction endo-nucleases EcoR II, Bst0.The extracted genomic DNA from juvenile and adult phase leaves of the seedling tree was digested with EcoR II and Bst0I, and amplified using eight primers. In total 77 bands were amplified. Post amplification digestion of these bands with EcoR II or Bst0 I revealed 32 bands containing CC(A/T)GG. Six bands were absent in amplified profiles from juvenile phase digested DNA, appeared in amplified products from digested adult phase DNA, indicating *de novo* methylation at CC(A/T)GG site. Five bands disappeared in Adult phase while these bands were present in Juvenile phase DNA amplified profiles, revealing presence of restriction site without methylation in adult phase. These results are suggestive that demethylation may have occurred in adult phase. Seventeen motifs of DNA methylation at CC(A/T)GG 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

DNA methylation is a strong candidate in modulation of gene expression (Goodrich & Tweedie, 2002, Hsieh & Fischer, 2005). Finnegan (2001) reported that genome-wide demethylation has a pleiotropic effect on the regulation of developmental processes that take place in specific tissues or stages of development in plants. DNA methylation can inhibit transcription directly by blocking the binding of transcription factors by modifying the target sites. However, DNA methylation alone is often not sufficient to block transcription, but it is likely the form of chromatin on a methylated template that renders it transcriptionally inactive (Ng & Bird, 1999). Moreover proteins that bind methylcytocine, such as methylcytocine proteins (MeCp2), interact with a corepressor complex that include histone deacetylase activity and result in transcriptional repression (Finnegan, 2001).

It has been observed that the pattern of DNA methylation changes throughout the life cycle in *Petunia hybrida* (Anderson *et al.*, 1990). DNA methylation changes with age and is regulated by phytohormones (Vanyushin, 2005). It is involved in gene regulation and controls many developmental pathways (Hafiz *et al.*, 2001). In some species like pea, apple, *Acacia* spp., and rose, the level of DNA methylation has been related to different growth stages and environmental conditions (Watson *et al.*, 1987; Li *et al.*, 2002; Baurens *et al.*, 2004; Xu *et al.*, 2004; Hafiz *et al.*, 2006). Methylation also ensures

inheritance of the appropriate developmental state through both mitosis and meiosis. In *Arabidopsis* DNA methylation plays an important role in regulating gene expression of many developmental pathways (Finnegan *et al.*, 2000). 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. This work was, therefore, aimed at studying the DNA methylation patterns, following transition from juvenile to adult phase in a woody perennial fruit tree, *Malus micromalus.* Information obtained through this investigation would contribute towards understanding the biological role of cytosine methylation during phase transition in plants. DNAs of higher eukaryotes are frequently methylated at CpG or CpNG. This methylation interferes with cleavage by certain restriction endonucleases. Endonucleases sensitive to ^{m}5CpG or ^{m}5CpNG 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.

Materials and Methods

Mature seedling tree of apple, *Malus micromalus*, was used as study material. Young leaves with juvenile characters from basal shoots 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 followed by drying on blotting paper.

DNA extraction: Genomic DNA was extracted according to the method described by Chen *et al*., (1997) and 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 Juvenile (J) and adult (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 (5 units each) in 50 µl reaction mixture and incubated overnight at 37°C for EcoR II and at 60°C for Bst0 I.

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 40ºC for 60 seconds, and extension at 72ºC for 120 seconds], and final extension at 72ºC for 5 minute. PCR products were stored at 4^{o}C until used for electrophoresis. Amplified fragments were analyzed along side a standard λ/Hind III+EcoR I molecular weight markers in 1.7% agarose gels containing ethidium bromide in 0.5x TBE (Sambrook *et al.*, 1989).

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
EcoR II	'5 CC(A/T)GG 3'	Yes	Nelson & Mc Clelland 1989
	'3GG(T/A)CC 5'		
	m5	Yes	Mc Clelland *et al*., (1994)
	CC(A/T)GG		
	m5	No	
	C C(A/T)GG		
	m4	No	Mc Clelland *et al*., (1994)
	C C(A/T)GG		
	m4	No	Mc Clelland *et al*., (1994)
	C C(A/T)GG		
	m6	No	Mc Clelland *et al*., (1994)
	CC A GG		
Bst 0 1	5CC (A/T)GG 3'	Yes	Nelson & Mc Clelland (1991)
	'3GG(T/A) CC 5'		
	m5	Yes	Mc Clelland *et al*., (1994)
	CC(A/T)GG		
	m5 m5	Yes	Mc Clelland *et al*., (1994)
	C C(A/T)GG		
	Overlaping metylation	No	Mc Clelland *et al*., (1994)

POST-amplification digestion: 13 μl of PCR product was restricted by 10 units of respective restriction enzyme (Bst0 I or EcoR II) for 30 minutes at appropriate temperature and was run on agarose gel for comparison of the banding pattern.

Results

EcoR II and Bst0 1 recognize the sequence CC (A/T)GG. EcoR II is sensitive to internal cytosine methylation but not to external C methylation and will not cut at the site when internal C is methylated. Bst01 is insensitive to methylation at both C residues and can cut to internal, external or both C methylations. It is only sensitive to over lapping C methylation. 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 Bst01 or EcoR II. The bands absent after restriction of template Bst01 but present after digestion with EcoRII, disappeared when their amplified product was digested with EcoR II or Bst0 I, thus confirming presence of CC(A/T)GG site which may have been modified by methylation in the original template either in the manner $C^{5m}C(A/T)GG$ or $^{5m}C^{5m}C(A/T)GG$ and or overlapping manner.

Genomic DNA from juvenile and adult phase leaves of *M. micromalus* seedling tree that was undigested (control) and digested with EcoRII or Bst0I was amplified using primer S 234. Nine bands were amplified from undigested DNA template of J and A phase (control). When amplified product was digested either by EcoR II or Bst0 1, all the 9 bands disappeared, indicating the presence of CC(A/T)GG site. When template DNA either from J or A phase was digested with EcoRII prior to PCR amplification the 8 bands

persisted and only one band lost. Presence of these bands however indicated methylation of at internal C or on both Cs (internal and external) of the CC(A/T)GG sites present in these fragments. On the other hand, when same template was digested with Bst0I, the 6 bands disappeared in PCR reaction from J phase, while 7 bands persisted in A phase. This pattern indicated methylation only at internal C or at both Cs in J phase, and in overlapping fashion in A phase indicating *de novo* methylation in 4 bands during transition and occurrence of CC(A/T)GG triplets in a cluster form (Table 2).

In RAPD patterns obtained by amplification from both phases using primer S 259, only 3 bands out of 12 contained CC(A/T)GG sites (Fig. 1). One of these bands showed methylation both in the J and A phase. Two bands of 1375 bps, having methylation at internal or both Cs received methylation and it also became methylated in overlapping pattern at cytosines existing near by in adult phase (Table 2).

In S-209 amplified DNA profiles from both phases (J and A), 9 bands were amplified of these 4 containing CC(A/T)GG restriction site as these were lost in post amplification digestion. EcoR II digestion indicated one de-methylated site or only at external C-methylation in A phase while presence of these bands in J phase amplified DNA showed events of methyl group on both cytosines in the restriction site. On the other hand, Bst 0I digestion in A phase exposed de-methylation in one band.

Use of primer S-262 lead to the amplification of 5 fragments containing CC(A/T)GG site. EcoR II digestion of template revealed methylation at both cytosines in all these targetting sites. Bst0I digestion however indicated that these sites lost methylation in two fragments during transition from J to A phase.

Thus out of 21 bands amplified with S-234 and S-259, only 12 contained CC(A/T)GG site. Among 12 CC(A/T)GG containing sites, one remained un-methylated or only at external C. Out of 11 methylated sites in J phase DNA, 6 motifs contained both Cs methylated in the restriction sites while transition to A phase resulted in additional methylation at external /internal Cs in nearby sequences attaining methylation in consecutive/overlapping manner indicating *de novo* methylation.

The primer S-209 and S-262 revealed polymorphism owing to demethylation. Amplification using this set of primers resulted in 14 bands, out of which 9 could be digested post PCR by Bst01. In template DNAs digested by EcoR II, no band lost in J phase indicating that all the CC(A/T)GG sites are methylated at either internal C or both Cs in J phase. Moreover in EcoR II and Bst01 digested templates from A phase, three bands did not appear showing lack of methylation in the amplified profile with S-209. Similarly, 4 fragments disappeared in Bst01 digested A phase DNA template, amplified by primer 262 while only 2 fragments lost in J phase leading to the conclusion that demethylation has occurred at 5 loci presumably during phase transition.

Another set of primers (S-256, S-282, S-295, and S-297) amplified 42 bands, out of which 12 contained CC(A/T)GG sites. All these sites were methylated except two having methylation only at external 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, higher eukaryotes have evolved an array of complex gene regulation strategies to fit in the pattern of development and interaction with the environment. 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).

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

Primer	Sequence of the Primers	Total number of bands in control	Number of bands lost in digestion prior to PCR				Number of bands lost in digestion post PCR	Remarks
			EcoR II		Bst0 I			
			J	A	J	A		
S 234	AGATCCCGCC	9	1	1	6	2	9	*de novo* methylation in adult phase 6 loci and methylation motifs in genomic DNA at 4 loci
S 259	GTCAGTGCGG	12			3	1	3	
S 209	CACCCCTGAG	9		2	2	3	4	Demethylation in adult phase at 5 loci and methylation motifs in genomic DNA at 4 loci
S 262	ACCCCGCCAA	5			2	4	5	
S 256	CTGCGCTGGA	14	2	2	3	3	4	
S 282	CATCGCCGCA	5	-	-	1	1	2	
S 295	AGTCGCCCTT	12	-	-	4	4	3*	DNA methylation motifs at 9 loci in the genomic DNA
S 297	GACGTGGTCA	11	-	-	2	2	2	
Total		**77**	**3**	**5**	**23**	**20**	**32**	

*There was disappearance of four bands in the amplified profile from the template DNA of Juvenile and adult phase digested with Bst01 but only three bands lost in the post PCR digestion. The reason is unknown and further investigation is needed in this regard.

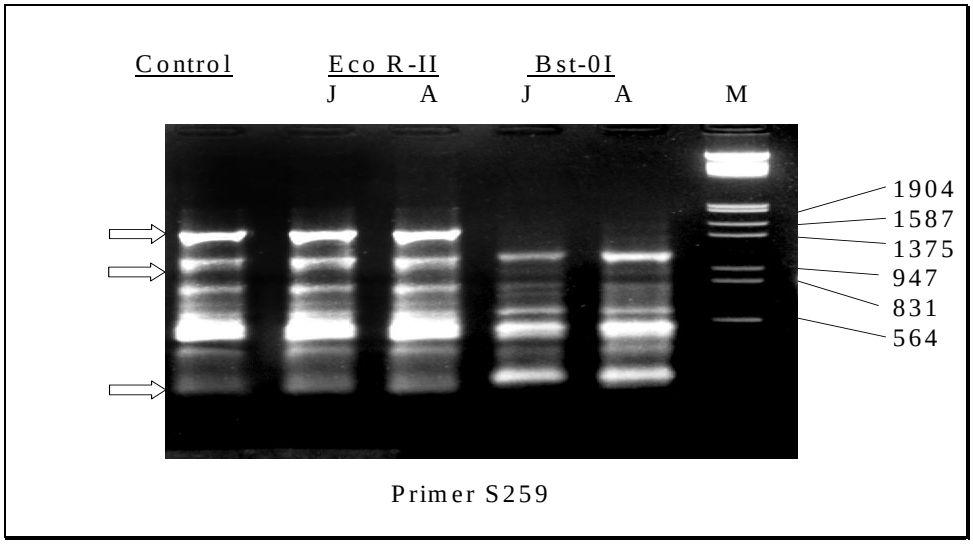

Fig. 1. Pattern of amplified fragments from Juvenile and Adult phase using Primer S259 showing polymorphism

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; Bartee *et al.,* 2001). Presence or absence of methylation results in generation of polymorphism which may be determined by digestion of genomic DNA with a pair of methylation responsive isoschizomeric

restriction enzymes EcoRII and Bst0I. The intact and restricted DNAs from both phases were used as template for PCR reactions.

Out of 22 methylated sites in J phase DNA, 6 contained internal only or on both Cs methylation while in A phase additional methylation at nearby Cs has been established in manner of overlapping. This indicates *de novo* 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). Genomic DNA methylation-demethylation during aging and reinvigoration of *Pinus radiata* has been detected (Fraga *et al.,* 2002). Demethylation of some key genes may result in activation of sub sets of several genes related to the adult phase characteristics. The DNA methylation of many gene-regulatory regions inversely correlates with gene expression (Roloff & Nuber, 2005). In this study 6-*de novo* methylation events have been observed in *M. micromalus* adult phase DNA verses demethylation 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 been 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. 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 region of a gene has been established to impose a more pronounced inhibition (Hohm *et al.,* 1996) and the profiles generated in this study are useful for investigating alterations in gene expression and isolation of genes related phase-specific expression (Hafiz *et al.,* 2001).

As five bands were absent in the patterns of amplified products from EcoRII and Bst0I 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. It was observed that 5 loci undergone demethylation of cytosine residues in the target site during transition from Juvenile phase to physiologically different adult phase (Kaeppler *et al.,*1998). The changes observed are most probably not caused by the general methylation/demethylation but rather occurs due to specific loci. 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 (Hafiz *et al.,* 2006).

The results, 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 (Richards, 1997). 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 through alteration in methylated sequences. The same resulted in polymorphism due to variation in methylation on partially methylated elements during phase change of crab-apple seedling in the present work (Zluvova, 2001; Hafiz *et al*., 2006).

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