doi: 10.17221/55/2016-CJGPB

A Comparative Analysis of DNA Methylation in Diploid and Tetraploid Apple (*Malus* × *domestica* Borkh.)

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Abstract

He P., Cheng L., Li H., Wang H., Li L. (2017): A comparative analysis of DNA methylation in diploid and tetraploid apple (*Malus* × *domestica* Borkh.). Czech J. Genet. Plant Breed., 53: 63–68.

DNA methylation is one of the major epigenetic modifications. It is very important to the regulation of gene expression. Methylation-sensitive amplification polymorphism (MSAP) profiling was applied to a diploid apple cultivar and its derived autotetraploid in order to characterize the level and pattern of DNA methylation at the two different ploidies. The frequency of methylated restriction sites was very similar between the two types (28.0% vs 27.3%), implying that polyploidization had a low effect on the global level of DNA methylation. However, with respect to the pattern of methylation, the frequency of hemi-methylated sites was higher in the tetraploid. When the transcription level of three genes encoding DNA methyltransferase was investigated in various tissues, it was established that *MET1* transcript abundance was the lowest of the three genes throughout the plant, while that of *DRM2* was high in the leaf, flower and fruit, as was that of *CTM3* in the fruit. Polyploidization had no discernible effect on the transcription level of any of the three genes.

Keywords: apple; autopolyploid; DNA methylation; MSAP; methyltransferase

It has been estimated that most angiosperm species have experienced at least one round of whole genome duplication during their evolution (Soltis 2005). Increasing a plant's chromosome number in this way can have profound effects on morphology, physiology and biochemistry, in some cases resulting in a major increase in productivity (Einspahr *et al*. 1963). Much of the regulation of the multiple gene copies present as a result of polyploidization relies on epigenetic modifications to one or more of the orthologs (Blanc *et al*. 2003). In particular, DNA methylation has been shown to be responsible for some of the phenotypic effects of polyploidization (Adams & Wendel 2005). Among the four nucleotides, cytosine (C) is the most susceptible to methylation. In plant genomes, methylated C occurs mainly when the C is present as either a CG dinucleotide or a CHG (where H can represent either A, C or T) trinucleotide; less frequently, CHH trinucleotides can be methylated. Three classes of DNA methyltransferase (MET, CMT and DRM) control C methylation (Finnegan & Kovac 2000): METs target mainly CG dinucleotides, while CMTs prefer CHG trinucleotides and DRMs CHH trinucleotides (Cao *et al*. 2000; Finnegan & Kovac 2000; Cao & Jacobsen 2002). The activity of these enzymes is thought to regulate many of the genes which control critical developmental switches, including seed development (Cao *et al*. 2000; Finnegan & Kovac 2000; Jullien *et al*. 2012).

The pattern of DNA methylation can alter during plant growth and development (Donini *et al*. 1997; Gehring & Henikoff 2007). In the sweet chestnut (*Castanea sativa*), the level of methylation is much higher in the dormant shoot than in the growing shoots of a juvenile tree (Hasbún *et al*. 2005). In both apple (*Malus domestica*) and pear (*Pyrus* spp.), the methylation of a transcription factor is believed to be important for the regulation of pigment accumulation in the fruit (Telias *et al*. 2011; Wang *et al*. 2013).

Here, the methylation-sensitive amplification polymorphism (MSAP) profiling technique was applied

doi: 10.17221/55/2016-CJGPB

to compare levels of C methylation between the diploid and tetraploid forms of an apple cultivar. Although originally developed to detect methylation in the CCGG tetranucleotide (Reyna-Lopez *et al*. 1997), MSAP has been found to be informative for characterizing the extent and pattern of CG methylation more generally (Xiong *et al*. 1999), for the analysis of the relationship between DNA methylation and heterosis (Li *et al*. 2013), and for correlating DNA methylation to pathogen resistance (Sha *et al*. 2005). The expression levels of DNA methyltransferase genes in various tissues were also examined and compared. The aim of the present study is to enhance the fundamental understanding of DNA methylation polymorphism in apples with different ploidy, and to provide a basis for further studies on the potential role of DNA methylation in apple polyploidy genetic breeding.

MATERIAL AND METHODS

Plant material and the extraction of genomic DNA and total RNA. Both the diploid and autotetraploid forms of Hanfu (HF) apple were raised on a rootstock of the M26 variety for 4 years. The trees are maintained at the Shandong Institute of Pomology (Taian, China). Total DNA was extracted from young leaves using a CTAB-based method (Cheng *et al*. 2003), while RNA was extracted from apical rolled young leaves, the big bud stage flowers and young fruits (50 days after full bloom), following Cao *et al*. (2012).

MSAP analysis. The MSAP profiling involved the use of a single pre-selective primer combination, along with combinations based on seven *Eco* and seven (*Hpa*II/*Msp*I-derived) primers (HM), each carrying three selective bases (Table 1). The template represented genomic DNA restricted with either *Eco*RI and *Hpa*II or *Eco*RI and *Msp*I (enzymes obtained from New England Biolabsinc., Beverly, USA). The methods used for restriction digestion, adapter ligation, pre-amplification and selective amplification followed those given by Li *et al*. (2015). The amplicons were resolved by electrophoresis through a 6% denaturing polyacrylamide gel and were visualized by silver staining (Dong *et al*. 2006). Only clear and reproducible fragments recovered from a replicated experiment based on independent DNA extractions were retained for fragment analysis.

Quantitative reverse transcription PCR (qRT-PCR). The cDNA first strand was synthesized from a 1.5 μg aliquot of total RNA using a RevertAid first-strand cDNA synthesis kit (Fermentas, Bourlington, Canada). The qRT-PCR primer pairs (Table 1) were designed using Primer Express software (Applied Biosystems, Foster City, USA) based on gene sequences present in *Arabidopsis thaliana* and apple, involving the genes *MET1* (AAA32829.1, EU273287.1), *DRM2* (AED92056.1, XM008392223.1) and *CMT3* (AAK69756.1, NC024251.1). Each 20 μl PCR contained 10 μl SYBR Green PCR mix, 1 μl cDNA, 0.4 μM of each primer and sterile ddH_2O added in a final volume of 20 μl. The reactions were initially denatured (95°C/60 s), then subjected to 40 cycles of 95°C/15 s, 60°C/60 s. Relative transcript abundances were calculated using the $2^{-\Delta\Delta Ct}$ method, following normalization against the abundance of 18S (NC_018554.1) rRNA transcript. Three biological replications with

Table 1. Adapter and primer sequences

Primers/adapters	Sequences (5'-3')
EcoRI adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
HpaII-MspI adapter	GATCATGAGTCCTGCT CGAGCAGGACTCATGA
Eco + A	GACTGCGTACCAATTCA
HM + T	ATCATGAGTCCTGCTCGG
Eco + ATA	GACTGCGTACCAATTCATA
Eco + AAT	GACTGCGTACCAATTCAAT
Eco + AAG	GACTGCGTACCAATTCAAG
Eco + ACT	GACTGCGTACCAATTCACT
Eco + ACA	GACTGCGTACCAATTCACA
Eco + ATG	GACTGCGTACCAATTCATG
Eco + ATC	GACTGCGTACCAATTCATC
HM + TGA	ATCATGAGTCCTGCTCGGTGA
HM + TTT	ATCATGAGTCCTGCTCGGTTT
HM + TTG	ATCATGAGTCCTGCTCGGTTG
HM + TTA	ATCATGAGTCCTGCTCGGTTA
HM + TAT	ATCATGAGTCCTGCTCGGTAT
HM + TCT	ATCATGAGTCCTGCTCGGTCT
HM + TCA	ATCATGAGTCCTGCTCGGTCA
DRM2-F	TGACTGGCGGCCTAGCGTAT
DRM2-R	ATCCCATTCCAATCAAATCTGATCT
18S-F	GAAAGACTCGGCATTCAGGC
18S-R	GCACGCACCTTTTTGTTTGT
CMT3-F	TTCCAGCCAAAGACGACACC
CMT3-R	GGCGGAGGGTTTGTTGATCT
MET1-F	AAGAGCCTGCTGGTGATGC
MET1-R	GTCCTCCATTGATGAAATCCACT

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Table 2. DNA methylation levels in diploid and tetraploid apple based on methylation-sensitive amplification polymorphism (MSAP) analysis

Ploidy	Total sites	None-methylated CCGG sites (%)	methylated CCGG sites (%)		
			fully methylated sites	hemi-methylated sites	total
Diploid	503	362 (72.0)	94 (18.7)	47 (9.3)	141 (28.0)
Tetraploid	421	306 (72.7)	59 (14.0)	56 (13.3)	115 (27.3)

three technical replications for each sample were used for real-time analyses.

Data analysis. To characterize the DNA methylation status of CCGG tetranucleotides, comparisons were made between the MSAP profiles derived from *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I digests. *Hpa*II and *Msp*I are isoschizomers which both recognize CCGG; however, while *Msp*I cleaves either a non-methylated site, a hemi-methylated (mC in one DNA strand only) site or a fully methylated CmCGG site (but neither hemi-methylated nor fully methylated mCCGG nor mCmCGG site), in contrast, *Hpa*II cleaves only non-methylated (CCGG) and hemi-methylated (mCCGG) sites (Lu *et al*. 2008; Li *et al*. 2015). The resulting fragments therefore fall into four types (I through IV): type I fragments, which are shared by both digests, are formed when the CCGG is non-methylated; type II fragments, which are present in the *Eco*RI/*Hpa*II but not in the *Eco*RI/*Msp*I digest, are difficult to interpret in plant genomes, where methylation can occur at both CCG and CG motifs; type III fragments, which are present in the EcoRI/MspI but not in the *Eco*RI/*Hpa*II digest, are produced when a CmCGG site is present; finally type IV fragments (not present in either digest) are detected where the CCGG site varies between genetically distinct samples (here the diploid and tetraploid cultivar) (Fulneček & Kovařík 2014). An estimation of the global DNA methylation level was calculated from the ratio between the number of methylated fragments and the total number of fragments. The combined number of type III and IV fragments represented the number of fully methylated ones, and the combined number of type II through IV the number of methylated fragments.

RESULTS

DNA methylation level and patterns. Ten chosen selective primer (generating the higher number of amplified bands) combinations generated 924 fragments, with the number of fragments generated per combination varying from 22 to 53. Of these, 256 were not consistent between the two digests: 153 represented fully methylated sites and 103 hemi-methylated ones. In the diploid template, 141 fragments were methylated (94 fully methylated, 47 hemi-methylated), while in the tetraploid template, the respective numbers were 115, 59 and 56. Overall, the level of DNA methylation within analysed CCGG loci was 28.0% and 27.3% for diploid and tetraploid genotypes, respectively (Table 2).

MSAP fragments obtained from PCRs primed with 23 primer combinations (higher percentage polymorphism or generating the higher number of amplified bands) were used to compare the CCGG methylation profiles of the diploid and tetraploid apple genotypes (Figure 1). The MSAP bands that revealed different methylation patterns could be divided into four types (Table 3): A (single-stranded locus, the same as methylation status in the locus,

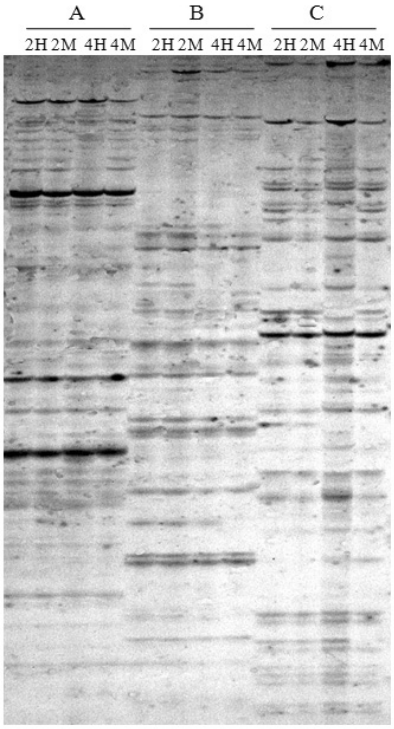

Figure 1. Part of an electrophoresis map of methylation-sensitive amplification polymorphism (MSAP)
2 – diploid; 4 – tetraploid; H – *Eco*RI + *Hpa*II double digestion; M – *Eco*RI + *MSP*I double digestion; A – *E*ATA + *HM*TGA; B – *E*AAT + *HM*TTT; C – *E*AAG + *HM*TAT

doi: 10.17221/55/2016-CJGPB

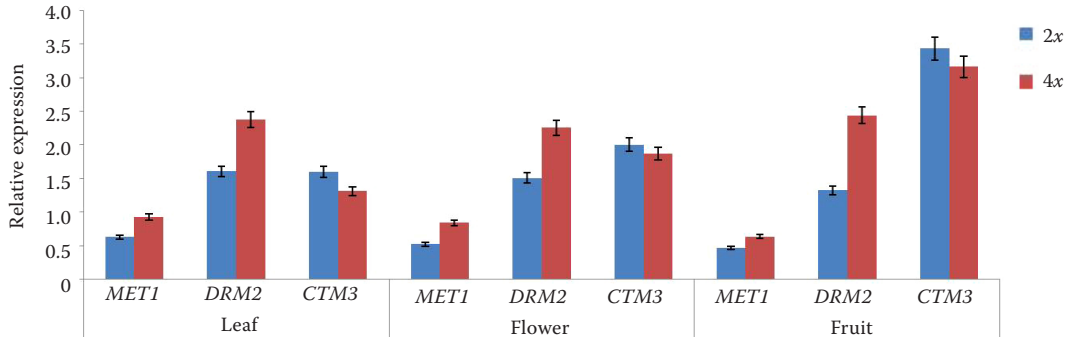

Figure 2. qRT-PCR derived relative transcript abundances of *MET1*, *DRM2* and *CTM3* in the leaf, flower and fruit of the diploid and tetraploid forms of Hanfu apple
The error bars represent the standard error (n = 3)

type I), B (demethylation, type II), C (hypermethylation, type III) and D (uncertain type methylation, type IV), including 15 subclasses. In all, 749 fragments were polymorphic, distributed as 329 fragments of A type, 254 fragments of B type, 153 fragments of C type, and 13 fragments of D type. A comparison of diploid and tetraploid DNA methylation patterns showed that hypermethylation accounted for 33.9%, decreased types for 20.5% (Table 3). The results showed that DNA methylation patterns undergo significant changes after tetraploidization.

Table 3. Comparison of DNA methylation patterns between the diploids and tetraploids of Hanfu apple

Pattern	4H	4M	2H	2M	No. of polylmorphic bands	Ratio (%)
A					329	43.9
A1	1	1	1	1	296	
A2	1	0	1	0	10	
A3	0	1	0	1	23	
B					254	33.9
B1	1	0	1	1	59	
B2	0	1	1	1	42	
B3	0	0	1	1	69	
B4	0	0	1	0	45	
B5	0	0	0	1	39	
C					153	20.5
C1	1	1	1	0	16	
C2	1	1	0	1	42	
C3	1	1	0	0	68	
C4	1	0	0	0	20	
C5	0	1	0		7	
D					13	1.7
D1	0	1	1	0	5	
D2	1	0	0	1	8	
Total					749	

The transcription of genes involved in DNA methylation. *MET1*, *DRM2* and *CMT3* transcription profiles were obtained from the leaf, flower and fruit of both the diploid and tetraploid genotypes using qRT-PCR (Figure 2). All three genes were transcribed in each of the three organs of both ploidy types. Of the three genes, *MET1* has the weakest transcription. The abundance of its transcript was highest in the leaf and lowest in the fruit, and was higher in the tetraploid than in the diploid. *DRM2* was most strongly transcribed throughout the plant in the diploid, but noticeably less so in the tetraploid, especially in the fruit. *CTM3* was transcribed most strongly in the fruit, and there was no perceptible difference in transcript abundance between the two ploidy types.

DISCUSSION

Polyploidy, the acquisition of chromosome doubling, has been an important factor in plant evolution (Masterson 1994). After chromosome doubling, plants exhibit obvious predominance in morphology, physiology, biochemistry, and other aspects to adapt themselves to the environment quickly (Chen & Ni 2006). However, extensive epigenetic modifications may be associated with the expression of genes involved in various regulatory networks in new polyploidy cells (Wang *et al.* 2004). Differential epigenetic modifications of homoeologous genes in polyploids may play an important yet unrecognized evolutionary role. A mechanism for rapid and stochastic establishment of genome-specific gene expression may control the expression of duplicate genes in polyploids, leading to natural variation and evolutionary opportunities for adaptive selection and domestication (Wendel 2000). One of the epigenetic variations is DNA methyla-

doi: 10.17221/55/2016-CJGPB

tion, which is important to genome activity. Given the importance of DNA methylation to gene expression, changes in cytosine methylation resulting from genome merger and/or polyploidy formation could clearly have genome-wide epigenetic consequences of relevance to polyploidy evolution (Geiman & Robertson 2002). In the present research, the application of MSAP profiling has exposed only a slight difference in the global DNA methylation level between the diploid and tetraploid versions of HF apple (28.0% vs 27.3%). Nevertheless, the pattern of CCGG methylation was quite divergent between the two ploidy types, with adjusted fragments accounting for over half of the MSAP fragments. Of these, the demethylated form was the most frequent, followed by the supermethylated form. The general tendency was that the DNA methylation degree increased. This meant that no or uncertain type DNA methylation loci in diploids were changed to hypermethylation state in tetraploids, while hypermethylation could lead to transcription shutdown or inhibition to maintain tetraploid genome stability. Similar conclusions have been drawn from comparisons between ploidy variants of species in the genera *Solanum* (Stupar *et al*. 2007), *Brassica* (Albertin *et al*. 2005) and *Arabidopsis* (Wang *et al*. 2006).

Plant polyploidization is normally accompanied by various phenotypic changes that are partially induced by new methylation changes during the interaction of different genomes. Extensive DNA methylation differences have been reported in synthesized *Brassica* (Xu *et al*. 2009; Lukens *et al*. 2006). DNA methylation is affected in plants by methyltransferases and demethylases. The transcription of genes encoding DNA methyltransferases varies during plant development (Yamauchi *et al*. 2008). *MET1* gene is expressed in vegetative organs or in reproductive organs, but decreases with the maturity of the organ (Fujimoto *et al*. 2006; Teyssier *et al.* 2008). *DRM* gene can be expressed in roots, leaves, stems, flowers, and its expression intensity has no direct relationship with the maturity of tissues and organs (Wada *et al.* 2003). *CMT3* genes are unique to plants, isolated from maize, oil palm, tomatoes and so on (Papa *et al.* 2001; Rival *et al.* 2008; Teyssier *et al.* 2008). The present qRT-PCR analysis was able to show that *MET1* was most strongly transcribed in the leaf, while *CTM3* transcript abundance was highest in the fruit. Nevertheless, *MET1*, *DRM2* and *CMT3* were all strongly transcribed throughout the plant (Figure 1). Since there was a very small difference in the global DNA methylation level between the diploid and tetraploid versions of HF, the implication is that the systems of methylation and demethylation together determine the global pattern of DNA methylation.

Acknowledgments. This research was supported by funds ear-marked for the Fruit Germplasm Resources Collection, and by grants from the New Germplasm Innovation Program (Lu No. 2014-96), the National Science and Technology Plans Program (2013BAD02B01) and the China Agriculture Research System (CARS-28).

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doi: 10.17221/55/2016-CJGPB

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Received for publication April 27, 2016
Accepted after corrections December 27, 2016
Published online April 5, 2017