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DNA Methylation in Genomes of Several Annual Herbaceous and Woody Perennial Plants of Varying Ploidy as Detected by MSAP

Ai Li · Bao-Quan Hu · Zhen-Yi Xue · Li Chen · Wei-Xing Wang · Wen-Qin Song · Cheng-Bin Chen · Chun-Guo Wang

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Abstract Polyploidization is known to accompany altered DNA methylation in higher plants, which plays an important role in gene expression regulation and maintaining genome stability. While the characteristics of DNA methylation in different polyploid plants are still to be elucidated; here, status of genomic DNA methylation in a series of diploid, triploid, and tetraploid annual herbaceous plants (watermelon and *Salvia*) and woody perennials (pear, *Poplar*, and loquat) were explored by methylation-specific amplified polymorphism analysis. The results indicated that levels of DNA methylation in triploid watermelon and *Salvia* were lower than their diploid parents. In triploid *Poplar* and pear, higher levels of DNA methylation were detected, and no significant difference was observed between triploid and tetraploid in all tested materials. Further data analysis suggested that about half of the total detected sites underwent changes of DNA methylation patterns in triploid watermelons and *Salvia*, as well as an obvious trend towards demethylation. However, the changes of DNA methylation patterns in three triploid woody perennials were only 17.54–33.40%. This implied that the characteristics of DNA methylation are significantly different during the polyploidization of different plant species. Furthermore, the results suggested that the level of DNA methylation was nonlinearly related to the ploidy level, and triploid plants displayed more interesting DNA methylation status. The characteristics and possible functions of DNA methylation in different ploidy series are further discussed.

Keywords DNA methylation · MSAP · Annual herbaceous plants · Woody perennial · Polyploidization

Abbreviations

MSAP	Methylation-sensitive amplification polymorphism
PCR	Polymerase chain reaction

Introduction

The duplication of genomes (polyploidy), either of the same genome (autopolyploidy) or of diverged genomes (allopolyploidy or amphiploidy), is a major force of evolution. In plants, it is estimated that polyploidy occurred in 50–70% of flowering species (Masterson 1994), and most of them have undergone one or more polyploidization events during their evolution (Spring 1997; Wolfe and Shields 1997). Some very important crops, such as maize (Gaut and Doebley 1997), soybean (Shoemaker et al. 1996), and *Brassica* (Lagercrantz and Lydiate 1996) are of polyploid origin. Genome sequencing data provides further evidence for polyploidy, although *Arabidopsis* has a smaller genome, it is also an ancient tetraploid (Blanc et al. 2000). After polyploidization, plants were usually shown to

A. Li · B.-Q. Hu · Z.-Y. Xue · L. Chen · W.-Q. Song · C.-B. Chen (✉) · C.-G. Wang (✉)
College of Life Sciences, Nankai University, Tianjin 300071, People's Republic of China
e-mail: Chencb@nankai.edu.cn

C.-G. Wang
e-mail: wangcg@nankai.edu.cn

W.-X. Wang
College of Horticulture and Landscape, Xi Nan University, Chongqing 400716, People's Republic of China

obviously increase the capabilities of adapting to their environment or possess traits that are desirable for domesticated crops such as increased abiotic and biotic stress tolerance, early flowering time, increased organ size, and biomass (Hilu 1993; Liu and Wendel 2002). Consequently, polyploidization has not only been regarded as one of the most important driving forces for plant genome evolution and speciation, but also makes an enormous contribution to agriculture. Recent investigations have identified that polyploidization events can result in rapid genetic changes in the genome structure (Swapna et al. 2010; Song et al. 1995; Pires et al. 2004; Pontes et al. 2004,) or gene expression profiles (Lu et al. 2006; Adams et al. 2003; Kashkush et al. 2002; Adams et al. 2004). Moreover, beyond variation at the DNA sequence level, DNA methylation, as one method of primary epigenetic regulation, was also identified to play an important role in the formation of polyploids (Levy and Feldman 2004; Lee and Chen 2001; Liu and Wendel 2003; Wang et al. 2004). Characteristics of DNA methylation in the polyploidization of *Arabidopsis* (Madlung et al. 2002), wheat (Shaked et al. 2001; Han et al. 2003), rice (Zhang et al. 2006), cotton (Keyte et al. 2006), and *Cucumis* (Chen and Chen 2008) revealed that the rapid adjustments of DNA methylation levels and patterns occurred. However, most investigations about DNA methylation and polyploidization were focused on the annual herbaceous plants as mentioned polyploid *Arabidopsis*, wheat, cotton, rice, and *Brassica* (Madlung et al. 2002; Zhang et al. 2006; Chen and Chen 2008). In contrast, relatively few attentions have been dedicated to elucidating the relationship between DNA methylation and polyploidization in woody perennials such as *Poplar*, pear, probably due to their large-size genomes, long growth cycles, and complex genetic backgrounds, and further to elucidating the different characteristics of DNA methylation between the annual herbaceous plants and woody perennials. In the present study, three ploidy series of woody perennials, in addition to two ploidy series of annual herbaceous plants were used as the samples. Analysis of the DNA methylation status was carried out. Consequently, the aim of this work was to provide an insight into the characteristics of DNA methylation in the polyploidization of certain annual herbaceous plants and woody perennials, and the relationship between the level of DNA methylation and the ploidy level.

Materials and Methods

Plant Materials

The materials for this study included five different combinations of triploids with their corresponding diploid and/or tetraploid parents. The first cross involved diploid (2×) watermelon (*Citrulluis vulgaris* Schrad.) × tetraploid (4×) watermelon. The second cross involved diploid (2×) *Salvia* (*Salvia miltiorrhiza* Bunge) × tetraploid (4×) *Salvia*. The third cross involved diploid (2×) pear (*Pyrus* × *bretschneideri* Rehd.cv. "Yali") × tetraploid (4×) pear. The forth cross involved diploid (2×) loquat (*Eriobotrya japonica* Lindl.) × tetraploid (4×) loquat. The fifth combination involved diploid (2×) *Poplar* (*Poplar Latin*), and newly synthesized triploid (3×) *Poplar*, which was created by pollen doubling. The tetraploids of watermelon, *Salvia*, and loquat were obtained after colchicine treatment of the diploid plants, while the tetraploid pear was created by bud mutation of diploid. All triploids, except triploid *Poplar*, were generated by crossing diploid with tetraploid as the female recipient of pollen.

DNA Isolation

The ploidy levels of all materials were determined by counting chromosome numbers in root cells using the squash technique as described previously (Urdampilleta et al. 2006). Genomic DNA was isolated from young fresh leaves of different ploidy plants by the cetyltrimethylammonium bromide method (Murray and Thompson 1980) with some modifications. DNA was dissolved in 50-μl TE (pH 8.0) after treatment with RNase. Finally, the concentration and purity of DNA sample was measured by absorbance and the ratio of OD_{260}/OD_{280}, using NanoDrop®ND-1000 (Nanodrop Technologies, USA).

Digestion and Ligation Reactions

DNA samples were separately digested with *Eco*RI (promega, USA) + *Hpa*II (promega, USA) and *Eco*RI + *Msp*I (promega, USA), of which *Hpa*II and *Msp*I are isoschizomers. They can recognize and digest the same site (5′-CCGG-3′), but display differential sensitivity to DNA methylation. *Hpa*II is inactive if either cytosine is fully methylated (methylation of both strands), while it can cleave the hemi-methylated sequence (only one strand methylation). *Msp*I is sensitive only to methylation at the external cytosine. It cuts in the case of inner cytosine methylation (CmCGG), but not in the case of outer cytosine methylation (mCCGG). Therefore, in methylation-sensitive amplification polymorphism (MSAP) analysis, different polymorphic bands from polymerase chain reaction (PCR) amplification can reflect the DNA methylation status. Here, the digestion reaction was performed in a volume of 15 μl containing 400 ng DNA template, 3 U *Eco*RI, 3 U *Hpa*II (or *Msp*I), and 3 μl 5×ligase-digestion buffer (0.05 M Tris-HCl, 0.05 M $MgAc_2$, 0.25 M KAc). The mixture was incubated at 37°C for 24 h. After that, the digested

fragments were ligated to the adapters in a buffer containing 0.8 U T4 DNA ligase (TaKaRa, Japan), 30 pM *Eco*RI adapter (Table 1), 30 pM *Hpa*II or *Msp*I adapter (Table 1), 0.4 mM ATP, and 1 μl 5×ligase digestion buffer in a final volume of 20 μl. The mixture was incubated at 16°C overnight, inactivated at 65°C for 10 min, and stored at −20°C.

Preamplification and Selective Amplification

Two amplification processes were carried out according to amplified fragment length polymorphism procedure (Portis et al. 2004), respectively. Preamplified PCR reactions were performed in a final volume of 25 μl containing 5 μl of diluted ligation products, 0.4 μM of E_{00} and H/M_{00} preamplification primer (Table 1), 0.5 U of *Taq* polymerase (TaKaRa, Japan), 200 μM of dNTPs (TaKaRa, Japan), and 2.5 μl of 10×PCR buffer. The DNA fragments were amplified for 25 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 1 min, as described by Xiong and Xu (1999) with some modifications. Following that the preamplification product was diluted 1:10 (*v*/*v*) with ddH_2O, and 1 μl of these diluted samples was mixed with 2 μl of 10× PCR buffer, 0.3 μM of *Eco*RI and *Hpa*II/*Msp*I selective amplification primers (Table 1), 0.5 U of *Taq* polymerase (TaKaRa, Japan), and 200 μM of dNTPs (TaKaRa, Japan) in a final volume of 20 μl. The PCR conditions were as follows: 13 cycles at 94°C for 30 s, 65°C for 30 s (reduced by 0.7°C each cycle), 72°C for 1 min, 23 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min.

Denaturing Polyacrylamide Gel and Data Analysis

Of the selective amplified products, 2.5 μl, mixed 1:1 (*v*/*v*) with a loading buffer, were heated at 100°C for 10 min and quickly chilled on ice for 2 min. The entire mixture was loaded onto 6% denaturing polyacrylamide gels. Electrophoresis was performed at a constant power of 35 W for 2 h. After silver staining, statistical analysis was carried out. Only clear and reproducible bands that appeared in three independent PCR amplifications (staring from the digestion–ligation step, i.e., the first step of MSAP) were scored and further transformed into a binary character matrix, where "1" indicated the presence and "0" showed the absence of a band. Following that, the formula as described by Zhao et al. (2007) were used to elucidate the differences between triploid and diploid or tetraploid of each ploidy series. In this formula, n_1 represents the total sites of diploid or tetraploid; n_2 represents the total sites of triploid;

$$p = \frac{y_1+y_2}{n_1+n_2}, \quad q = 1-p, \quad \sigma_{p_1-p_2} = \sqrt{pq\left(\frac{1}{n_1}+\frac{1}{n_2}\right)}, \quad U = \frac{p_1-p_2}{\sigma_{p1-p2}}$$

y_1 represents the total DNA methylation sites, hemimethylation sites, or fully methylation sites of diploid or tetraploid; y_2 shows the total DNA methylation sites, hemimethylation sites, or fully methylation sites of triploid; p_1 is the percentage of total methylation sites, hemimethylation sites, or fully methylation sites of diploid or tetraploid; and p_2 is the percentage of total methylation sites, hemimethylation sites, or fully methylation sites of triploid.

Results

Validation of the Ploidy Level by Chromosome Counting

All materials used in the present study were determined by counting chromosome numbers in root cells for their ploidy levels. The results indicated that in each cell, the corresponding chromosome numbers were correct as

Table 1 Sequences of adapters and pre-selective amplification primers used for MSAP analysis

	*Eco*RI (E) 5′-3′	*Hpa*II/*Msp*I (H/M) 5′-3′
Adapter-1	CTCGTAGACTGCGTACC	GACGATGAGTCTAGAA
Adapter-2	AATTGGTACGCAGTC	CGTTCTAGACTCATC
Pre-amplification primers	GACTGCGTACCAATTC (E_{00})	GATGAGTCTAGAACGG (H/M_{00})
		GATGAGTCTAGAACGGCA(H_{15})
	GACTGCGTACCAATTAAC(E_{32})	GATGAGTCTAGAACGGCT(H_{18})
	GACTGCGTACCAATTACA(E_{35})	GATGAGTCTAGAACGGGA(H_{19})
	GACTGCGTACCAATTACG(E_{37})	GATGAGTCTAGAACGGGT(H_{22})
Selective amplification primers	GACTGCGTACCAATTAGC(E_{39})	GATGAGTCTAGAACGGTG(H_{25})
	GACTGCGTACCAATTAGG(E_{41})	GATGAGTCTAGAACGGCAA(H_{47})
	GACTGCGTACCAATTCAA(E_{47})	GATGAGTCTAGAACGGCAT(H_{50})
	GACTGCGTACCAATTCAT(E_{50})	GATGAGTCTAGAACGGCTA(H_{59})

anticipated, implying that all materials were stable with respect to ploidy level (Fig. 1). Further karyotypic analysis indicated that no obvious chromosome deletion events occurred in any materials.

DNA Methylation Level of the Same Plants but with Different Ploidy Level

Fifty-six pairs of selective primers were used to analyze the DNA methylation status of watermelon, *Salvia*, pear, *Poplar*, and loquat polyploidy series, respectively. The data indicated that in the two annual herbaceous plants, the DNA methylation level of diploid, triploid, and tetraploid watermelon was 28.00%, 22.90%, and 27.37%, respectively, and the corresponding value of different ploidy *Salvia* was 43.66%, 38.87%, and 39.79%, respectively (Table 2). In the two woody perennials, pear and loquat, the DNA methylation level of each ploidy level was distinctly lower than the corresponding value of watermelon and *Salvia*, and the DNA methylation level of diploid and triploid *Poplar* was 16.10% and 24.11%, respectively (Table 2, Fig. 2). Further statistical analysis indicated that the total levels of DNA methylation of triploid watermelon and *Salvia* were significantly lower than those of their diploid parents (*U* values ranged from 2.00 to 2.11 and $U_{0.05}$=1.96). Similarly, the value also showed a degressive trend corresponding to the tetraploid parents, although this did not reach a statistically significant level (*U* values ranged from 0.40 to 1.79 and $U_{0.05}$= 1.96). In contrast, the total level of DNA methylation of triploid pear and *Poplar* showed significantly higher values than those of their diploid parents (*U* values ranged from 2.41 to 5.72 and $U_{0.05}$=1.96). While the DNA methylation level of triploid loquat showed no significant difference compared to its diploid parent (*U* value was 1.04 and $U_{0.05}$=1.96).

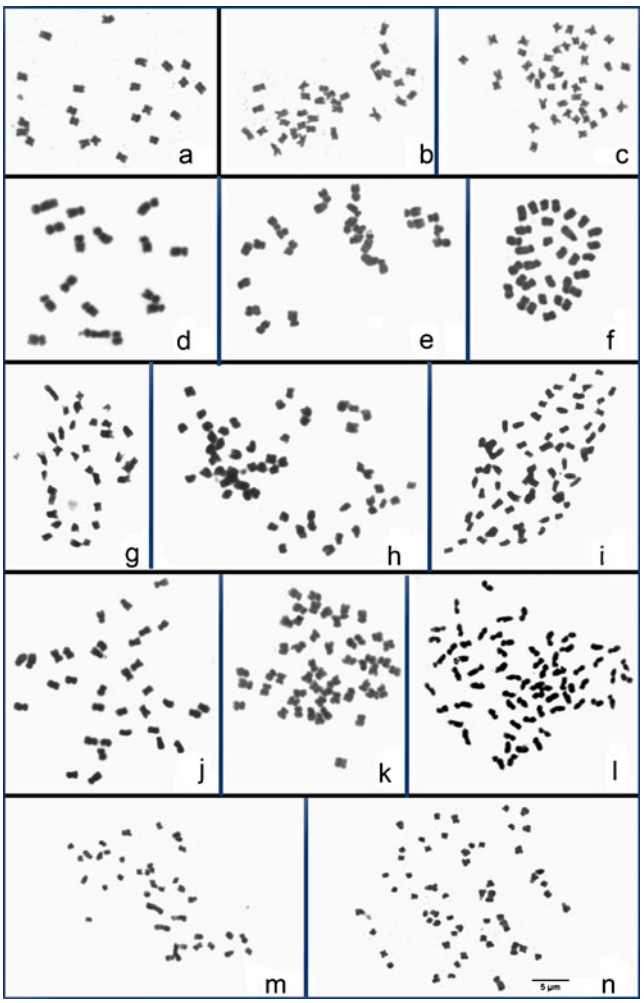

Fig. 1 Chromosome analysis of watermelon, *Salvia miltiorrhiza*, pear, loquat, and *Poplar*. **a** Diploid watermelon (2*n*=2×=22), **b** triploid watermelon (2*n*=3×=33), **c** tetraploid watermelon (2*n*=4×=44), **d** diploid *Salvia* (2*n*=2×=16), **e** triploid *Salvia* (2*n*=3×=24), **f** tetraploid *Salvia* (2*n*=4×=32), **g** diploid pear (2*n*=2×=30), **h** triploid pear (2*n*=3×=45), **i** tetraploid pear (2*n*=4×=60), **j** diploid loquat (2*n*=2×=34), **k** triploid loquat (2*n*=3×=51), **l** tetraploid loquat (2*n*=4×=68), **m** diploid *Poplar* (2*n*=2×=38), and **n** triploid *Poplar* (2*n*=3×=57); *scale bar* 5 μm

DNA Methylation Patterns of the Same Plants but with Different Ploidy Level

Based on the polymorphic bands detected by MSAP, the DNA methylation patterns in each ploidy series of watermelon, *Salvia*, pear, *Poplar*, and loquat all could be categorized into four groups (group A, B, C, and D; Fig. 3), of which type D corresponded to patterns that were undecided by the present method, which accounted for less than 2% of all tested sites in the present data. This was not shown and considered in the subsequent assays. When pair-wise comparisons between triploid plants and their diploid or tetraploid parents were carried out according to the hypermethylation and demethylation ratio of triploid plants, the five ploidy series materials could be categorized into three types. One type including the annual herbaceous plants watermelon and *Salvia* showed an obvious trend of demethylation (*U* values ranged from 1.28 to 11.48 and $U_{0.05}$=1.96). In the second type, including two woody perennials pear and *Poplar*, no significant differences between the hypermethylation and demethylation ratios were detected (*U* values ranged from 0.50 to 1.00 and $U_{0.05}$=1.96). Interestingly, the third type was only associated with loquat, and although this was also regarded as a woody perennial, the character of DNA methylation patterns in triploid loquat was not similar to those of triploid pear and *Poplar*. Moreover, it is also not consistent with those of triploid watermelon or *Salvia* (Table 3).

Table 2 DNA methylation levels of the same plant with different ploidy level

Species	Type	Total bands	Total methylated bands CCGG GĈGC	Total methylated ratio (%) CCGG GĈGC	Full-methylated bands CCGG GĈGC	Full-methylated ratio (%) CCGG GĈGC	Hemi-methylated bands CCGG GĈGC	Hemi-methylated ratio (%) CCGG GĈGC
Watermelon	Diploid	647	181	28.00	121	18.70	60	9.27
	Triploid	655	150	22.90[a]	80	12.21	70	10.68
	Tetraploid	581	159	27.37	82	14.11	77	13.25
Salvia	Diploid	765	334	43.66	239	31.24	95	12.42
	Triploid	939	365	38.87[a]	260	27.69	105	11.18
	Tetraploid	872	347	39.79	263	30.16	84	9.63
Poplar	Diploid	1,584	255	16.10	189	11.93	66	4.17
	Triploid	1,605	387	24.11[a]	188	11.71	199	12.40
Pear	Diploid	831	129	15.52	81	9.75	48	5.78
	Triploid	886	177	20.00[a]	105	11.85	72	8.13
	Tetraploid	852	143	16.78	83	9.74	60	7.04
Loquat	Diploid	1,443	288	19.96	219	15.18	69	4.78
	Triploid	1,443	266	18.43	165	11.43	101	7.00
	Tetraploid	1,443	244	16.91	138	9.56	106	7.35

[a] Indicated that the DNA methylation levels of triploid offsprings showed significant differences compared to their diploid and tetraploid parents at the a=0.05 statistical level. Underlined cytosine is methylated

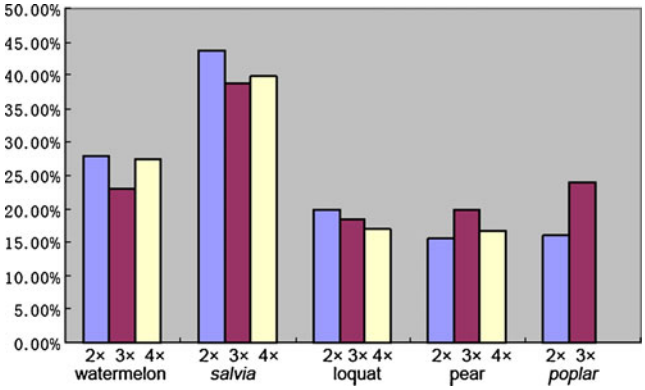

Fig. 2 Total DNA methylation levels of different ploidy watermelon, *Salvia*, loquat, pear and *Poplar*. 2×, 3×, 4× indicated diploid, triploid, tetraploid, respectively

DNA Methylation Level of Different Plants with the Same Ploidy Level

The DNA methylation levels of samples of watermelon, *Salvia*, pear, *Poplar*, and loquat with the same ploidy level were also analyzed (Fig. 4). The data indicated that the DNA methylation level in five diploid materials was varied from 15.52% to 43.66%, of which diploid *Salvia* showed the highest DNA methylation level, following by the DNA methylation level of diploid watermelon. Similar to these five diploid materials, the DNA methylation level in five triploid and four tetraploid materials were also significantly different. The extent of change in triploid materials was from 18.43% to 38.87% and from 16.78% to 39.79% in tetraploid materials. Moreover, the higher DNA methylation level was also usually detected in *Salvia* and watermelon (Table 2, Fig. 4). Further statistical analysis identified that for the same ploidy level, the annual herbaceous plants, watermelon and *Salvia*, always showed significantly higher DNA methylation levels than those of the woody perennials, pear, *Poplar*, and loquat (U values ranged from 2.38 to 14.44 and $U_{0.05}$=1.96).

DNA Methylation Patterns of Different Plants with Same Ploidy Level

The characteristics of DNA methylation patterns in five different plants with same ploidy level were explored further, especially, the DNA methylation patterns in triploid samples of all plants which were closely connected to the corresponding diploid and/or tetraploid parents. Moreover, triploid plants always show some excellent traits that are not found in diploid, tetraploid, or other polyploid plants. Then a series of comparisons between triploid plants and their corresponding diploid and/or tetraploid parents were conducted. The data

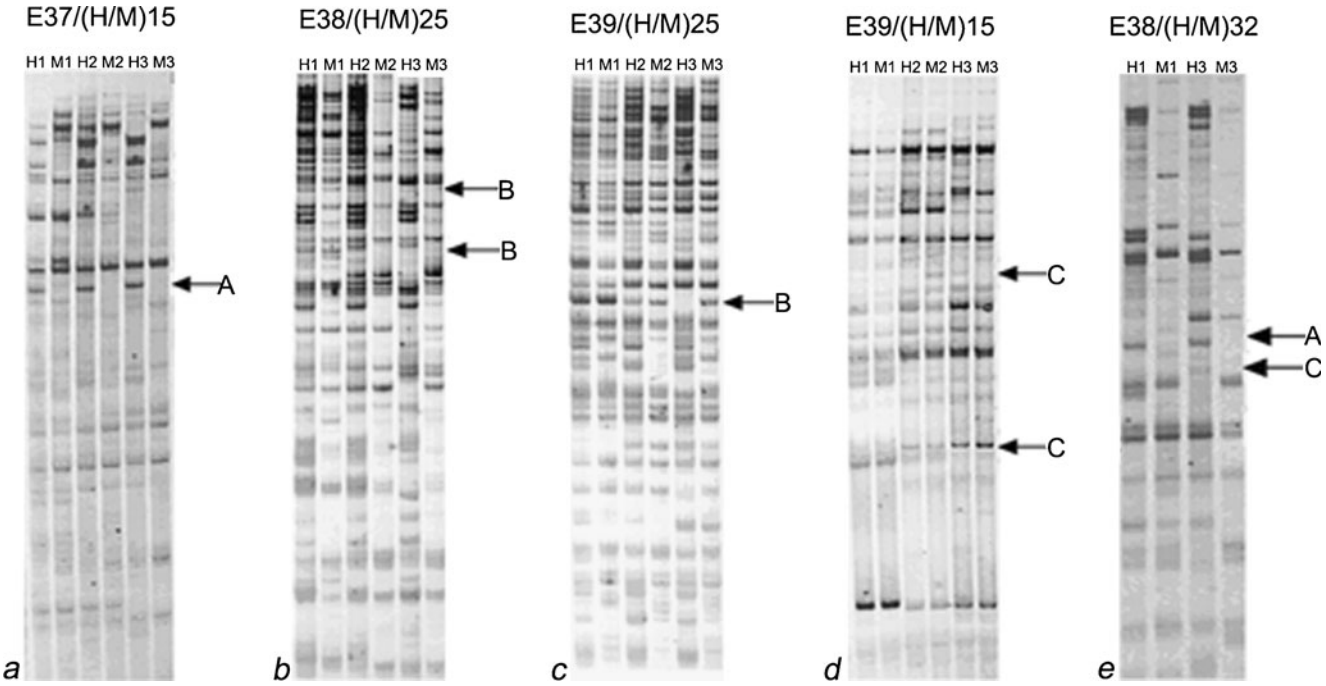

Fig. 3 Example of MSAP profiles showing the three major types of locus-specific DNA methylation patterns in triploid materials relative to their corresponding diploid and/or tetraploid parents. *M1*, *M2*, *M3* genomic DNA was digested with *MspI* and *EcoRI*; *H1*, *H2*, *H3* genomic DNA was digested with *HpaII* and *EcoRI*; *1*, *2*, *3* triploid, tetraploid, and diploid; **a**–**e** watermelon, *Salvia*, pear, loquat, and *Poplar*, respectively. *A* indicated that the DNA methylation patterns were unchanged; *B* indicated that the demethylation events occurred; *C* indicated that hypermethylation events occurred

distinctly indicated that, in triploid watermelon and *Salvia*, the DNA methylation patterns of more than 40% sites were adjusted either compared with the diploid parents or tetraploid parents (Table 3). However, in the triploid *Poplar*, pear, and loquat the changes of value were less than 34% (Table 3). It implied that in the formation of polyploids, the adjustments of DNA methylation patterns in the annual herbaceous plants (watermelon and *Salvia*) were more frequent than those in the woody perennials (*Poplar*, pear, and loquat).

Discussion

Increasing numbers of studies have demonstrated that polyploidization is a major driving force for plant genome evolution and speciation (Liu and Wendel 2003; Hegarty and Hiscock 2007; Soltis et al. 2004; Adams and Wendel 2005; Gaut and Doebley 1997; Shoemaker et al. 1996; Lagercrantz and Lydiate 1996; Blanc et al. 2000). However, a consequence of polyploidization is the breakage of cytoplasmic–nuclear balance and the conflict that results

Table 3 Changes of DNA methylation patterns in the comparison of triploid with diploid or tetraploid materials

	Watermelon		*Salvia*		Loquat		Pear		Poplar	
	3X-2X	3X-4X	3X-2X	3X-4X	3X-2X	3X-4X	3X-2X	3X-4X	3X-2X	
Total sites	735	725	728	569	1411	1411	886	878	1758	
Hypermethylation sites	143	101	151	101	204	222	77	69	299	⬆
Hypermethylation ratio	19.50%	13.90%	20.74%	17.75%	14.46%	15.73%	8.69%	7.86%	17.01%	⬆
Demethylation sites	163	228	360	200	243	203	89	85	288	⬇
Demethylation ratio	22.10%	31.50%	49.45%	35.15%	17.22%	14.39%	10.05%	9.68%	16.38%	⬇
Total changed sites	306	329	511	301	447	425	166	154	587	
Total changed ratio	41.60%	45.40%	70.20%	52.90%	31.68%	30.12%	18.74%	17.54%	33.40%	

"⬆" indicated the patterns of DNA methylation at CCGG sites were changed due to hypermethlyation events in 3X versus 2X or 4X; "⬇" indicated the patterns of DNA methylation at CCGG sites were changed due to demethylation events in 3X versus 2X or 4X

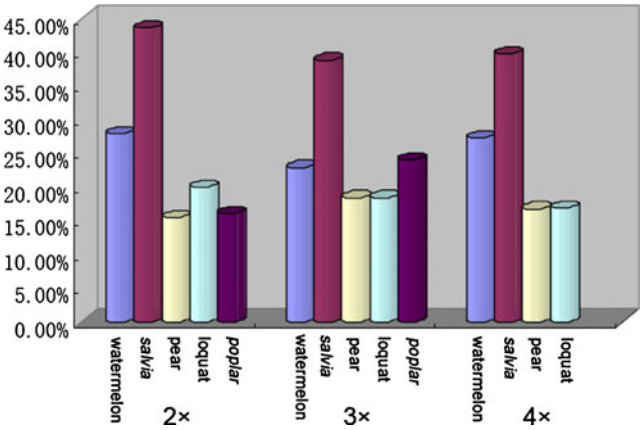

Fig. 4 Total DNA methylation levels of watermelon, *Salvia*, loquat, pear, and *polar* with the same ploidy level. 2×, 3×, 4× indicated diploid, triploid, and tetraploid, respectively

from two or more divergent and related genomes being brought into a single cell nucleus. The reestablishment of the cytoplasmic–nuclear balance and stabilization of multiple genomes in a single nucleus by polyploidy plants, and the further exhibiting of high applicability to their environment or excellent traits desired by agriculture are interesting areas of further research. A series of evidences revealed that genetic adjustments may occur during polyploidization (Rayburn et al. 2009; Chen 2007; Pires et al. 2004; Udall et al. 2005; Liu and Wendel 2003; Osborn et al. 2003), as well as the epigenetic modifications, such as DNA methylation and histone protein modification, are also important in this process (Fiuk et al. 2010; Demetriou et al. 2010; Lukens et al. 2006; Gaeta et al. 2007; Salmon et al. 2005; Shaked et al. 2001; Liu et al. 2001). In the present study, chromosome deletion was not detected in all samples (Fig. 1), implying the obvious cytogenetic variation did not occur. Moreover, several previous investigations have identified that although the changes of genomic structure, gene expression profiles, or/and quantities occurred in polyploidization, these changes appeared not to be so dramatic, and novel genes produced due to polyploidization were little reported (Albertin et al. 2005; Wang et al. 2006; Stupar et al. 2007). Consequently, genetic adjustments may also occur in the present materials, while they are not regarded as the mean emphasis for exploration. Here, we mainly focused on the characteristics of DNA methylation, which has been widely detected, and identified closely associated with genome stabilization in different polyploid plants (Chen and Chen 2008; Lukens et al. 2006; Madlung et al. 2002). The results clearly revealed that the characteristics of DNA methylation levels and patterns were different between the annual herbaceous plants (watermelon and *Salvia*) and the woody perennials (*Poplar*, pear, and loquat) during their polyploidization (Tables 2, 3). The DNA methylation levels of watermelon and *Salvia* were higher than the same ploidy of woody perennials, *Poplar*, pear, and loquat (Fig. 4). Moreover, the adjustments of DNA methylation patterns were more frequent in the two annual herbaceous plants than those of three woody perennials (Table 3). According to these observations and the materials used in the present study, a speculation is that these differences may be associated with or caused by the biological characteristics of individual species. As is well known, the annual herbaceous plants, such as watermelon and *Salvia*, usually complete a life cycle within a short time. This character can make them more sensitive to the changes of themselves and environment. Once polyploidization events occur, to cope with the genomic shock (McClintock 1984) and maintain the cytoplasmic–nuclear balance, large-scale adjustments are required such as changes of DNA methylation. In contrast, some woody perennials such as *Poplar* and pear undergo a much longer life cycle, and can bear a higher tolerance to changes within themselves and/or environment than the annual herbaceous plants. In this manner, when polyploidization events occur, dramatical genetic adjustments, even epigenetic modification is not necessary. Interestingly, although loquat is also a woody perennial, unlike *Poplar* and pear, the characteristic of DNA methylation level of triploid loquat is between those of the two annual herbaceous plants (DNA methylation level in triploid watermelon and *Salvia* was lower than their corresponding diploid parents) and the two woody perennials (DNA methylation level in triploid pear and *Poplar* was much higher than diploid parents; Table 2, Fig. 2). Further data indicated that the characteristic of DNA methylation pattern adjustments was also between the two annual herbaceous plants and the two woody perennials (Table 3). It may further convince the speculation that characteristic of DNA methylation, as well as other adjustments of genetics and epigenetics in different polyploid plants may be closely related to the biological characteristics of individual species. To a certain degree, the biological characteristics of loquat are similar to the annual herbaceous plants, while it is also distinctly different from the deciduous woody perennials such as the pear and *Poplar*.

Further data analysis indicated that the levels of DNA methylation were nonlinearly related to the ploidy level in each tested ploidy series materials (Fig. 2). It indicated that the higher ploidy level did not necessarily correspond to higher or lower levels of DNA methylation in different plant species. The similar phenomenon was detected in polyploidizaton of the *Spartina* species, in which the evolutional characteristic of the *Waxy* gene also did not show the coherence between the copy number and the ploidy level (Fortune et al. 2007). It fully exhibited the complexity of the natural basis of plant polyploidization. However, the complexity of this process, all genetic and/or

epigenetic modifications must cope with the changes of nuclear–cytoplasmic ratio and the instability of genomes, two vital events resulted by polyploidization. DNA methylation, as the most prevalent epigenetic modification of plant nuclear DNA, rightly plays a very important role in maintaining transposon inactivation, heterochromatization, and the stability of other repeat sequences (Cuozzo et al. 2007; Martienssen and Colot 2001), all which are closely related to the stability of whole genomes. Consequently, when polyploidizaton event occur, suitable DNA methylation modifications can reduce the degree of incompatibility that arises from two or more genomes in a nucleolus. The present results also suggested that although a nonlinear relationship was observed between the level of DNA methylation and ploidy level, the adjustments of DNA methylation patterns occurred widely at many genomic sites in each of the polyploid plants (Table 3). These results implied that the DNA methylation modifications surely participated in the formation of polyploid plants. However, the mechanisms by which DNA methylation modifications at the whole genomic level or at specific sites affects the stability of genomes during polyploidization still required further investigation. Another important role of DNA methylation is the regulation of gene expression. As is widely described, methylated state usually is associated with inactivation of gene expression and, conversely, gene activation is associated with demethylation (Carvalho et al. 2010; Diéguez et al. 1997; Cao and Jacobsen 2002; Wada et al. 2004; Wang et al. 2004). The methylation, hypermethylation, and demethylation events were widely observed in every ploidy level of five plant species (Table 3). However, the triploids of all materials were the most interesting. Different from tetraploids, triploids as odd-ploidy polyploids possess certain special characteristics in both DNA methylation levels and patterns. For example, the triploid watermelon and *Salvia* showed lower DNA methylation levels compared to their diploid parents (Table 2, Fig. 2), and demethylation events were predominant (Table 3). This means that numerous gene expressions were activated or upregulated in triploid watermelon and *Salvia*, which may be closely associated with the formation of some excellent traits that are usually prominently exhibited in triploid plants, such as seedlessness, increased organ size, and biomass (Hilu 1993; Liu and Wendel 2002). However, similar to two triploid annual herbaceous plants, although three triploid woody perennials, triploid pear, triploid *Poplar*, and triploid loquat, also possess excellent traits in growth, development, and organ size, as well as previous reported triploid banana, triploid apple, triploid mulberry, and triploid sugarbeet (Hamada 1963; Bhojwani and Razdan 1996; Sreekumari et al. 1999; Thomas et al. 2000). It appears that the characteristics of DNA methylation were not consistent with those in triploid watermelon and *Salvia* (Tables 2, 3). This implied that the function of DNA methylation modification may be different in the formation of triploidy effect of woody perennials from that in the annual herbaceous plants. Consequently, the underlying mechanism of triploidy effect still needs further elucidation, especially with regards to the triploidy effect in woody perennials, to our knowledge, which has never been reported. Although the present materials and data were limited, the results offer a very significant clue for future exploration of the characteristics and function of DNA methylation in the polyploidization of different plant species, especially to further understand the molecular mechanisms of the triploidy effect in different plants and its relationship with the "odd-ploidy effect" first observed by Guo et al. (1996; Auger et al. 2005; Stupar et al. 2007).

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