Journal of Integrative Agriculture 2016, 15(6): 1228–1238

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RESEARCH ARTICLE

Analysis of cytosine methylation in early generations of resynthesized *Brassica napus*

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Abstract

DNA methylation, an important epigenetic modification, serves as a key function in the polyploidization of numerous crops. In this study, early generations of resynthesized *Brassica napus* (F_1, S_1–S_3), ancestral parents *B. rapa* and *B. oleracea* were analyzed to characterize their DNA methylation status during polyploidization, applying DNA methylation-sensitive amplification polymorphism (MSAP) and high-performance liquid chromatography methods. In F_1, 53.4% fragments were inherited from both A- and C-genomes. Besides, 5.04 and 8.87% fragments in F_1 were inherited from A- and C- genome, respectively. 5.85 and 0.8% fragments were newly appeared and disappeared in resynthesized *B. napus*, respectively. 13.1% of these gene sites were identified with methylation changes in F_1, namely, hypermethylation (7.86%) and hypomethylation (5.24%). The lowest methylation status was detected in F_1 (38.7%) compared with in S_1–S_3. In S_3, 40.32% genes were methylated according to MSAP analysis. Sequencing of methylated fragments indicated that genes involved in multiple biological processes were modified, including transcription factors, protein modification, and transporters. Expression ananlysis of *DNA methyltransferase 1* and *DNA methyltransferase chromomethylase 3* in different materials was consistent to the DNA methylation status. These results can generally facilitate dissection of how DNA methylation contributes to genetic stability and improvement of *B. napus* during polyploidization.

Keywords: resynthesized *Brassica napus*, DNA methylation, epigenetics, polyploidization

1. Introduction

Polyploidization has been considered as an important motivation during the evolution and formation of higher plants. 70% of angiosperm has been proven to have undergone polyploidization event during evolutionary history (Ramsey 2011). Many crops with significant economic values, such as rapeseed (Song *et al*. 1995), wheat (Shaked *et al*. 2001; Kashkush *et al*. 2003), and cotton (Liu *et al.* 2001), are polyploids. Genomic shock, which occurs during distant hybridization and polyploidization of plants, can facilitate many important genetic events, including genomic rearrangement (Udall *et al*. 2005; Kong *et al*. 2011), gene insertion-deletion (Chen 2007; Jiang *et al*. 2013), and activation of transposons (Kashkush *et al*. 2003). These dynamic changes during plant evolution would significantly contribute to modifications of the genome structure and expressional pattern (Chen and Ni 2006; Gaeta *et al*. 2007; Feldman and Levy 2012), as well as improve the adaptability of plants to diverse environments.

Received 1 July, 2015 Accepted 24 December, 2015
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doi: 10.1016/S2095-3119(15)61277-1

Global changes in gene expression normally occur during polyploidization, and most genes are considered to have a non-additive expression pattern in polyploids (Birchler *et al*. 2003; Wang *et al*. 2006; Xu *et al*. 2009; Jackson and Chen 2010; Qi *et al*. 2012; Jiang *et al*. 2015). For instance, one out of 10 and one out of three genes of newly resynthesized hexaploid wheat (2*n*=42, BBAADD) were changed compared with the female and male parents, respectively. Furthermore, preference of gene expression in wheat tended to cluster in specific genomes (Akhunova *et al*. 2010). Similar expressional variations were observed in natural and resynthesized allotetraploid cotton (Yoo *et al*. 2013). However, not all variations can be explained by the genetic law of Mendel. These unclassified regulatory modifications during polyploidization can be explained and facilitated by epigenetic mechanisms (Finnegan 2001; Bird 2007), which have been proven as inheritable events during plant evolution. Epigenetic regulation, including DNA methylation, histone modification, chromosome reshaping, and non-coding RNA regulation, contributed to plant polyploidization and has been passed onto progenies by mitosis and meiosis (Chinnusamy and Zhu 2009). Interactions of homologous and homeologous genes during the integration of different genomes would definitely result in variation and genome preference of specific gene expressions (Gaeta *et al*. 2007; Xu *et al*. 2009). Recently, considerable researches on DNA methylation status of resynthesized polyploids have been reported. The outline of network regulation in plant methylation was reported with the inclusion of many important methyltransferases, such as methyltransferase (MET), chromomethylase (CMT), and domains rearranged methyltransferase (DRM), among which MET and CMT function in sustaining symmetrical and asymmetrical cytosine methylation, respectively (Law and Jacobsen 2010; Apashkin *et al*. 2011; Noy-Malka *et al*. 2014).

A comparison of DNA methylation status in allopolyploid *Arabidopsis* and the ancestral parents showed that 8.3% genes changed during polyploidization (Madlung *et al*. 2002). In resynthesized *B*. *napus*, 0.73 to 1.93% genes were detected with methylation variation (Lukens *et al*. 2006; Gaeta *et al*. 2007; Xu *et al*. 2009). Salmon *et al*. (2005), applying the amplified fragment length polymorphism (AFLP) and methylation-sensitive amplification polymorphism (MSAP) methods, reported that 30% of the parental methylation patterns were altered in hybrids of *Spartina*. Similar methylation changes were observed during polyploidization of hexaploid wheat (Qi *et al*. 2010; Zhao *et al*. 2011). Wang *et al*. (2014) reported extensive epigenetic changes in newly synthesized amphidiploid *Asteraceae*. Epigenetic alteration (e.g., DNA methylation) under intergenetic genomic shock, which is still poorly understood, was lower in the amphihaploid than in the parental genome, but increased in the early generations of hybrids. This probably corresponds to the instability of polyploids.

Brassica, one of the important members of the family *Cruciferae*, is of significant economical and nutritional value to human life. Allopolyploids in *Brassica* act as important resources for the evolutionary analysis of polyploidization. Many synthesized *Brassica* polyploids (AABBCC, AABC, BBAC, and CCAB) have been generated to study the genetic interactions of different genomes during polyploidization (Lukens *et al*. 2006; Gaeta *et al*. 2007; Xu *et al*. 2009; Pradhan *et al*. 2010; Mason *et al*. 2012). Genetic and epigenetic variations occurred during polyploidization of resynthesized *B*. *napus* have been reported, which are considered a necessary evolutionary event for the stability of new polyploids. Song *et al*. (1995) first reported the relationship between DNA methylation and polyploidization of *B*. *napus*. In this study, we used early generations of resynthesized *B*. *napus* (F_1, S_1–S_3) and their ancestral parents *B*. *rapa* and *B*. *oleracea* to analyze DNA methylation status during polyploidization, applying the MSAP and high-performance liquid chromatography (HPLC) methods. The results would facilitate thorough analysis of how DNA methylation contributes to genetic stability and improvement of *B*. *napus* during polyploidization.

2. Results

2.1. Variation of DNA methylation levels in F_1 of resynthesized *B. napus* compared with diploids

Polyploidization is a complex evolutionary event involving many genetic and epigenetic changes. In the present study, we applied resynthesized *B*. *napus* and diploid parents to assess DNA methylation in the entire genome level using the HPLC method. Chromatogram of standard and plant materials was listed in Fig. 1. Higher methylation status was detected in *B*. *oleracea* (CC) compared with *B*. *rapa* (AA) (Fig. 2). The DNA methylation levels in F_1, S_1–S_3 and between the two diploid parents varied significantly. Moreover, methylation level increased gradually in early generations of *B*. *napus*, of which F_1 was detected to have the lowest methylation level of 4%. By contrast, 6.98% of the S_3 genome was methylated (Fig. 2). Besides, we classified the polypmorphic fragments from MSAP analysis in consideration of both genetic and methylation changes. The 20 different combinations of selective amplification primers used for MSAP analysis and the fragments are classified and shown in Table 1. As shown in Fig. 3, five types of polymorphic bands were detected. Type A includes polymorphic fragments inherited from both A- and C-genomes, types B and C stand for fragments inherited from A-genome and C-genome, respectively. Types D and E stand for new

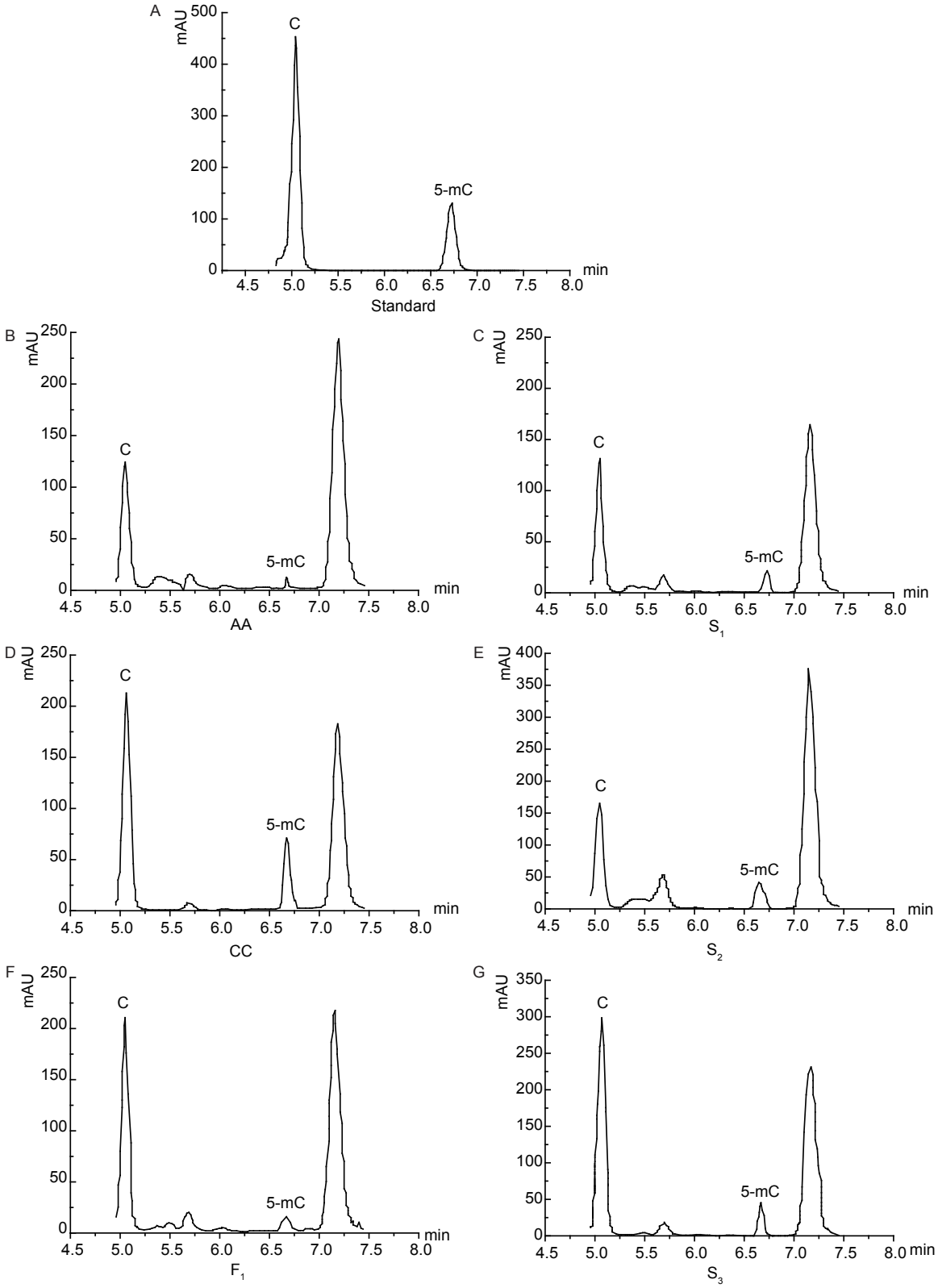

Fig. 1 Chromatogram of 5-methylcytosine (5-mC) methylation level in standard (A), *Brassica rapa* (B), *Brassica oleracea* (C), F_1 generation (D), S_1 generation (E), S_2 generation (F), and S_3 generation (G). AA, *B. rapa* (*cv*. Aikangqing); CC, *B. oleracea* (*cv*. Zhonghua Jielan); F_1, resynthesized *Brassica napus*; S_1–S_3, successive selfing generations. Relative quantification was conducted by comparing the peak areas of similar retention times using the calibration curves of available cytosine (C) and 5-mC as standard. The same as below.

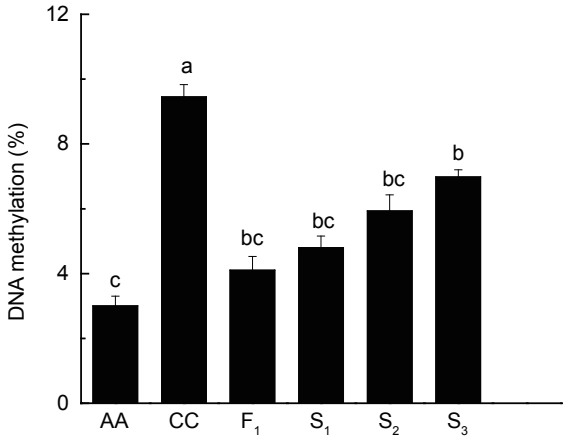

Fig. 2 HPLC analysis of DNA methylation levels in early generations of resynthesized *B. napus* and diploid parents. a, b and c above each column indicate significant difference (P<0.05).

fragments and disappeared fragments in resynthesized *B*. *napus*, respectively. In comparison with *B*. *rapa* and *B*. *oleracea*, the profile of the F_1 generation of resynthesized *B*. *napus* include 394 type A, 25 type B, 44 type C, 29 type D, and 4 type E fragments. In consideration of the characteristics of *Hpa*II and *Msp*I, we classified (+, +) as nonmethylation fragments, (−, −) and (+, −) as hypermethylation, (−, +) as hypomethylation. Nearly 53.4% of the fragments were identified without methylation changes among F_1, *B*. *rapa*, and *B*. *oleracea*, including three subtypes (A1, B1, C1, E1–E2). B2, B3, B6, C6–C8, D1–D4, D7 presents fragments that were hypermethylated in F_1 compared with diploid parents (*B*. *rapa* and *B*. *oleracea*). B4–B5, C4–C5, and D5–D6 include fragments that were hypomethylated in F_1 compared with diploid parents (Table 1).

Table 1 Variation of DNA methylation in S_1 of resynthesized *Brassica napus* compared with diploids

Type	Diploid				Allopolyploid		No. of band	Percentage
	AA		CC		F_1			
	M	H	M	H	M	H		
A1	+	+	+	+	+	+	241	394
A2	+	–	+	–	+	–	87	(79.44%)
A3	–	+	–	+	–	+	66	
B1	+	+	–	–	+	+	6	25
B2	–	–	–	+	–	–	3	(5.04%)
B3	–	–	+	–	–	–	2	
B4	–	+	–	–	–	+	4	
B5	–	+	+	+	–	+	2	
B6	–	–	+	+	–	–	8	
C1	–	–	+	+	+	+	14	44
C2	–	–	+	–	+	–	7	(8.87%)
C3	+	+	+	–	+	–	3	
C4	–	–	–	+	–	+	4	
C5	+	+	–	+	–	+	3	
C6	–	+	–	–	–	–	5	
C7	+	–	–	–	–	–	2	
C8	+	+	–	–	–	–	6	
D1	+	+	+	+	–	–	2	29
D2	+	+	–	+	–	–	2	(5.85%)
D3	–	+	+	+	–	–	2	
D4	+	–	+	+	–	–	3	
D5	+	+	+	+	–	+	6	
D6	–	–	–	–	–	+	7	
D7	–	+	–	+	–	–	4	
D8	–	–	–	–	+	–	3	
E1	–	+	–	–	+	+	2	4
E2	–	–	–	–	+	+	2	(0.8%)
Total							496	

M indicates the selective amplication results using the genomic DNA as template, which was digested by *Eco*RI and *Msp*I. H indicates the selective amplication results using the genomic DNA as template, which was digested by *Eco*RI and *Hpa*II. AA, *Brassica rapa* (*cv*. Aikangqing); CC, *B. oleracea* (*cv*. Zhonghua Jielan); F_1, resynthesized *Brassica napus*. The same as below.

2.2. Variation of DNA methylation levels in early generations of resynthesized *B. napus*

The results of DNA methylation patterns in the F_1, S_1–S_3 generations of resynthesized *B*. *napus* are shown in Table 2. According to the characteristics of *Hpa*II and *Msp*I in MSAP analysis, polymorphic fragments presented in digestion by *Msp*I but not by *Hpa*II was taken as CG methylation, whereas, fragments presented in *Hpa*II-digest but absent in *Msp*I-digest was taken as CHG methylation (Qi *et al*. 2010). The highest methylation status was observed in S_3 (40.32%), whereas the lowest methylation status was observed in F_1 (38.70%). Variation of DNA methylation in different generations of *B*. *napus* corresponded with the HPLC data mentioned previously. Specifically, 20.16, 19.35, 19.52, and 19.56% methylation in F_1, S_1–S_3 were identified at CG sites, whereas 18.54, 19.95, 20.56, and 20.76% methylation in F_1, S_1–S_3 were identified at CHG sites, respectively (Table 2). Different methylation statuses in early generations of resynthesized *B*. *napus* might be an important driving force of polyploidization events.

2.3. Sequence analysis of polymorphic MSAP fragments

Seven repeatable MSAP fragments, ranging from 157 to 350 bp, and represented for 5 classes of methylation/demethylation were sequenced and predicted for their biological functions (Table 3). Basic Local Alignment Search Tool analysis of these sequences revealed their similarity with many reported gene functions in multiple biological processes. For instance, M1 was identified as homologous to *BnaA06g00900D*, which encodes a putative

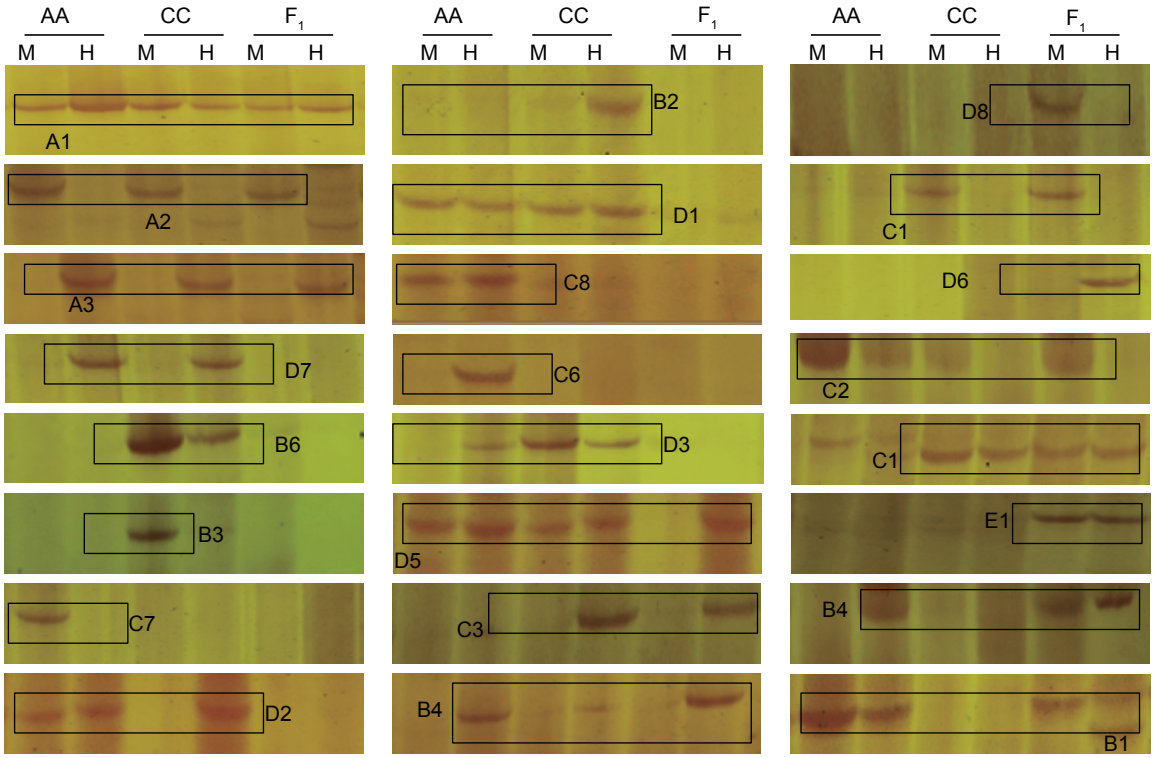

Fig. 3 DNA methylation patterns in early generations of resynthesized *B. napus* and diploid parents. A1–D4 stand for four types of methylation patterns identified (see Table 1). H, fragments obtained after digestion with *Eco*RI–*Hpa*II; M, fragments obtained after digestion with *Eco*RI–*Msp*I.

Table 2 DNA methylation pattern in early generations of resynthesized *B. napus*

Samples	Total sites	No. of CG-methylated sites and ratio	No. of CHG-methylated sites and ratio	Total methylation level (%)
F_1	496	100 (20.16%)	92 (18.54%)	38.70
S_1	497	96 (19.35%)	99 (19.95%)	39.30
S_2	497	97 (19.52%)	102 (20.56%)	40.08
S_3	496	97 (19.56%)	103 (20.76%)	40.32

NDP-L-rhamnose synthase, and is involved in seed coat mucilage cell development. M2, M3, M4, M5 and M6 were identified as homologous sequences to *BnaA07g31670D*, *BnaA08g11270D*, *BnaC06g27490D*, *BnaA09g00510D*, and *BnaA09g36550D*, respectively. These fragments were homologous to proteins transferring glycosyl groups, oxysterol binding protein, proteins with transcript processing functions. Nevertheless, identification of these DNA fragments validated MSAP as an important method for the thorough analysis of DNA methylation during polyploidization of *B. napus*.

2.4. Gene expressional analysis during polyploidization of *B. napus*

We analyzed *MET1* and *CMT3* expressions in the F_1, S_1–S_3 generations of resynthesized *B. napus*, *B. rapa*, and *B. oleracea* (Fig. 4-A and B). *MET1* was highly expressed in different generations of resynthesized *B. napus*, compared with *B. rapa* and *B. oleracea*. F_1 showed the highest expression value, indicating that methylation at CG sites extensively occurred during the interaction of the A and C genomes. By contrast, the expression of *CMT3* is lower in allopolyploids than that in diploid parents, of which F_1 showed the lowest expression of *CMT3*. Given that *CMT3* is involved in regulating methylation at CHG sites, different expression levels of *CMT3* might be responsible for the variations of CHG methylation in different materials. In particular, expressional variations of *MET1* and *CMT3* corresponded with the methylation differences identified by MSAP analysis (Table 2). Based on the sequencing result of polymorphic fragments in MSAP analysis, relative expression of homologous to these fragments was also verified (Fig. 4-C–F). Unfortunately, not all the expressional variation was coherent to the MSAP results. For instance, both M1 (*BnaA06g00900D*) and M2 (*BnaA07g31670D*) were identified with hypomethylation pattern in *B. oleracea*, but

Table 3 Sequence analysis of polymorphic fragments resulted from methylation-sensitive amplification polymorphism (MSAP) analysis

Pattern	Diploid				Allopolyploid								Length (bp)	Reference accession in *B. napus*	Reference accession in *Arabidopsis*	Description
	AA		CC		F_1		S_1		S_2		S_3					
	M	H	M	H	M	H	M	H	M	H	M	H				
M1	–	–	–	+	–	–	–	–	–	–	–	–	170	BnaA06g00900D	AT1G53500.1	MUCILAGE-MODIFIED 4 (MUM4), encodes a putative NDP-L-rhamnose synthase
M2	–	–	–	+	–	–	–	–	–	–	–	–	165	BnaA07g31670D	AT1G74600.1	ORGANELLE TRANSCRIPT PROCESSING 87, a trans-factor recognizing two editing sites in mitochondrial genes nad7-C24 and atp1-C1178
M3	–	–	–	–	–	–	–	+	–	+	–	+	210	BnaA08g11270D	AT4G08180.1	OSBP (oxysterol binding protein)-related protein 1C (ORP1C)
M4	–	–	–	–	–	+	–	–	–	–	–	–	350	BnaC06g27490D	AT5G43620.1	Pre-mRNA cleavage complex II
M5	–	–	–	–	+	–	–	–	–	–	–	–	157	BnaA09g00510D	AT4G02030.2	VACUOLAR PROTEIN SORTING 51(VPS51), Vps51/Vps67 family protein
M6	–	–	–	–	–	–	–	+	–	–	–	–	367	BnaA09g36550D	AT3G57200.1	Unknown protein, transferase activity, transferring glycosyl groups

expression of M1 in *B. oleracea* and F_1, S_1–S_3 were much lower than in *B. rapa*. Adversely, expression of M2 in *B. oleracea* is relatively higher than *B. rapa* and amphidiploids (except for S_1). As known, hypomethylation in plant genome normally accompanies with upregulation of gene expression (Tang *et al*. 2014). But due to dramatic genomic changes during diversification and polyploidization of *Brassica* species, gene expression variation in different genomes might also be affected by genome-biased expression and silencing of genes, interaction of cytoplasm and nuclear genome during polyploidization (Zhao *et al*. 2013). M3 was identified with hypomethylation in S_1–S_3, expression of *BnaA08g11270D* was higher in S_2 and S_3 than in diploid parents, but the highest expression of M3 was observed in F_1. As to M5, which was hypomethylated in F_1, its expression in F_1 was lower than in diploids and S_2, S_3. But the lowest expression of M5 was presented in S_1, which can not be explained only by DNA methylation.

3. Discussion

Many crops with significant economic value and polyploidization history, such as wheat, cotton, and rapeseed, were obtained from interspecific hybridization and subsequent chromosomal doubling (Leitch and Leitch 2008; Soltis and Soltis 2009; Chen 2010; Zhang *et al*. 2013). With regard to natural polyploids, the efficiencies and contributions of genetic variation during polyploidization are hardly evaluable because of the complexity of genomic interaction and successive self-crossing. Epigenetic changes (e.g., DNA methylation) during polyploidization are necessary for gene expression regulation in polyploids (Chinnusamy and Zhu 2009). Given that DNA methylation has been verified as a heritable modification in plants, considerable attention has been focused on the molecular mechanisms of methylation during polyploidization (Chinnusamy and Zhu 2009). Genomic changes including DNA elimination and genome rearrangement also affect genome stability (Lysak *et al*. 2005). Many reports on polyploidization have used AFLP to study the rapid genetic changes occurred in polyploids, including loss of parental band in hybrids (Qi *et al*. 2010). As for MSAP analysis, the polymorphic band resulted from *Hpa*II and *Msp*I-digest should indicate different cytosine methylation at the 5´-CCGG sites. But according to MSAP analysis, we can not exclude these bands occurred or disappeared during early polyploidization from genetic variation. In this study, we have classified the polymorphic bands into different types according to their inheritance from parent lines (Table 1). Type A includes polymorphic fragments inherited from both A- and C-genomes, types B and C stand for fragments inherited from A- and C-genome, respectively. Types D and E stand for new fragments and disappeared fragments in resynthesized *B. napus*, respectively. In consideration of the characteristics of *Hpa*II and *Msp*I, we identified 53.4% fragments without methylation changes among F_1, *B. rapa*, and *B. oleracea*, including subtypes (A1, B1, C1, E1–E2). 13.1% of gene sites were identified with methylation in F_1, which were classified into three methylation types, namely, hypermethylation (types B2, B3, B6, C6–C8, D1–D4, D7, 7.86%) and hypomethylation (types B4–B5, C4–C5, D5–D6, 5.24%) (Table 1, Fig. 3). Based on the methylation variations in *B. napus*, *B. rapa*, and *B. oleracea*, we propose that these variations

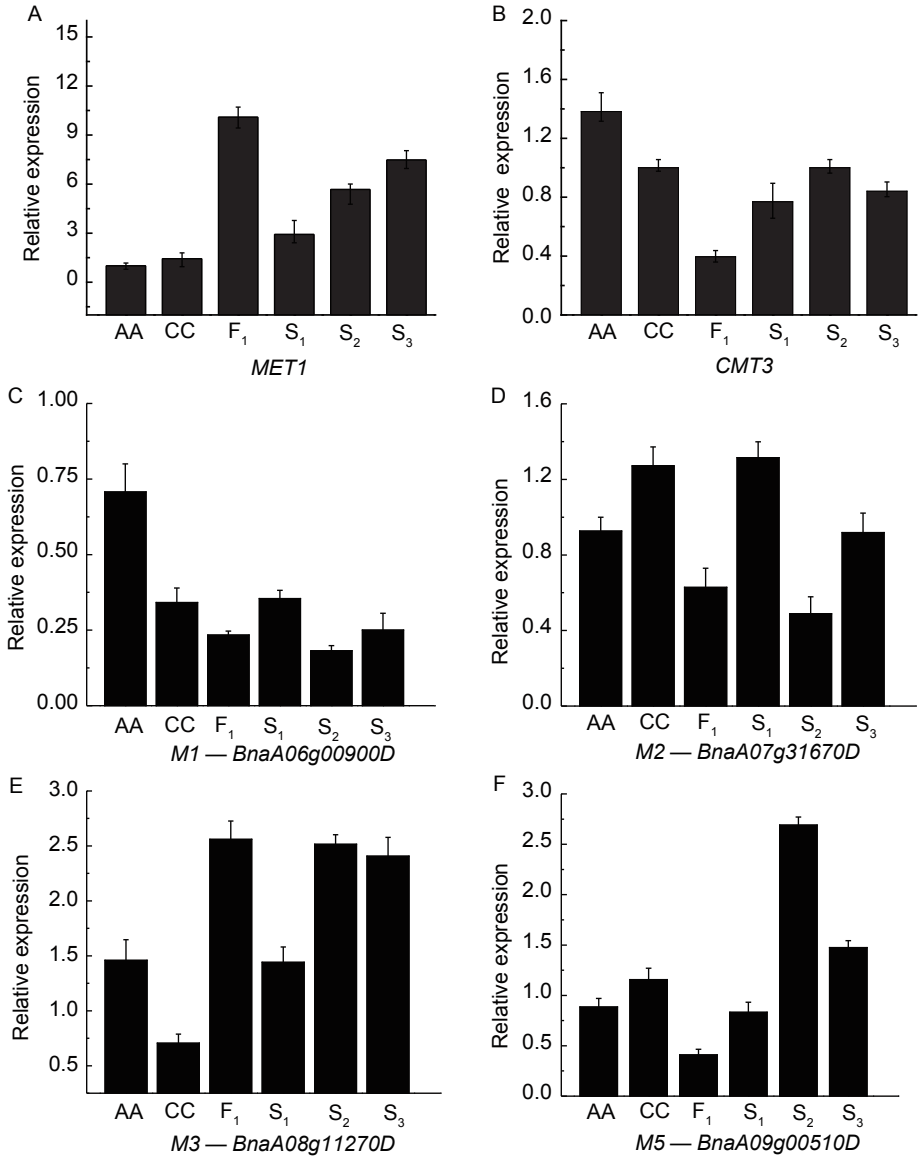

Fig. 4 Quantitative RT-PCR analysis of the DNA methyltransferase genes (A, B) and polymorphic fragments in methylation-sensitive amplification polymorphism (MSAP) analysis (C–F) in the early generations of resynthesized *B. napus* and diploid parents. *MET1*, *DNA methyltransferase 1*; *CMT3*, *DNA methyltransferase chromomethylase 3*; *M1–M3 and M5*, *BnaA06g00900D*, *BnaA07g31670D*, *BnaA08g11270D*, and *BnaA09g00510D*. Error bars represent the standard error for three independent experimental replicates.

might be necessary to maintain genome stability during the interaction of A and C genomes. These DNA level variations, as well as different phenotypes and agronomic characteristics among *B*. *napus*, *B*. *rapa*, and *B*. *oleracea*, are important for polyploidization of *B*. *napus*. Epigenetic changes during polyploidization of *B*. *napus* are similar to cotton and *Spartina* (Liu *et al*. 2001; Salmon *et al*. 2005). Combining the results of MSAP and HPLC analyses, we observed coherent variations of methylation in F_1, S_1–S_3, which are considered important epigenetic alterations under intergeneric genomic shock and might be responsible for heterosis of resynthesized F_1, as well as genomic stability of later generations (Tables 1 and 2, Fig. 2). Interestingly, polymorphic fragments classified in type C were identified with C-genome preference. Xu *et al*. (2009) have also reported genomic preference in *B*. *napus*, and C-genome-specific gene silencing and methylation alterations were more frequent than A-genome. Similar genomic preference has been reported in cotton and wheat (Shaked *et al*. 2001; Keyte *et al*. 2006). Notably, the percentage of CHG-methylated sites in resynthesized *B*. *napus* is generally higher than that of CG-methylated sites. All epigenetic changes occurring during polyploidization are necessary to genome stability (Wang *et al*. 2013). The variation of DNA methylation level

in early generations of resynthesized *B*. *napus* agreed to the report by Wang *et al*. (2014), who identified that DNA methylation in newly synthesized amphidiploid *Asteraceae* was lower than in the parental genome, but increased in the early generations of hybrids. Epigenetic events associated with intergeneric genomic shock during A and C genome combinations are significant to explain gene expression and phenotype variation in allopolyploid hybrids (Li *et al*. 2014).

The functions of regulatory factors in epigenetic events, such as DNA methylation and histone modification, are crucial in evaluating methylation changes during polyploidization, including *MET* and *CMT* (Apashkin *et al*. 2011; Noy-Malka *et al*. 2014). Transcript expression analysis of *MET1* and *CMT3* revealed that the expression of *MET1* was upregulated in *B*. *napus* than in diploids, whereas *CMT3* was downregulated in resynthesized rapeseeds compared with *B*. *rapa* and *B*. *oleracea* (Fig. 4). Li *et al*. (2014) reported that *MET1* and *CMT3* were mainly involved in controlling the fully methylated status of CCGG. Our results also revealed ~20% of CG methylation in polyploids, which corresponds with the expressional variations of *MET1* and *CMT3* (Table 2, Fig. 4). Further analysis of these interesting polymorphic MSAP fragments will be conducted to enrich our knowledge on the epigenetic regulation of *B*. *napus* during polyploidization (Kong *et al*. 2011; Jiang *et al*. 2013). In the present study, we identified the gene expression pattern of several demethylation fragments listed in Table 3. Unfortunately, variation in gene expression of these polymorphic fragments in resynthesized *B*. *napus* and diploid parents were not consisted to the methylation pattern (Fig. 4). We suspect that dramatic genomic changes during diversification of A and C genomes, as well as genetic changes during combination of A and C genomes in *B*. *napus* would be partially responsible for this (Jiang *et al*. 2013; Zhao *et al*. 2013).

Brassica, as a model for polyploidization analysis in plant evolution, is an important crop with a significant economic value and close relationship with *Arabidopsis* (Wu *et al*. 2014*)*. Recently, considerable information on genomes and transcriptomes has been published, which would facilitate further research on different aspects of *Brassica*, including evolutionary research and improvement of agronomic traits. The DNA methylation profile of resynthesized *B*. *napus* is necessary to reveal the evolutionary framework of *B*. *napus*. The DNA methylation profile of resynthesized *B*. *napus* would significantly update our knowledge on improving the agronomic and economic value of rapeseed, with the use of genetic and epigenetic information.

4. Conclusion

The lowest methylation status was detected in F_1 (38.7%) compared with S_1–S_3. In S_3, 40.32% genes were methylated according to MSAP analysis. Sequencing of methylated fragments indicated that genes involved in multiple biological processes were modified, including transcription factors, protein modification related genes, and transporters. Expression ananlysis of *DNA methyltransferase 1* and *DNA methyltransferase chromomethylase 3* in different materials was consistent to the DNA methylation status. These results can generally facilitate dissection of how DNA methylation contributes to genetic stability and improvement of *B*. *napus* during polyploidization.

5. Materials and methods

5.1. Plant materials

Seeds of resynthesized *Brassica napus* (F_1 generation), successive selfing generations (S_1–S_3), female parent *B*. *rapa* (*cv*. Aikangqing) (2*n*=20, AA) and male parent *B*. *oleracea* (*cv*. Zhonghua Jielan) (2*n*=18, CC) were cultivated in climate chambers at 25°C, a 16 h light/8 h dark photoperiod, and 70% relative humidity. The F_1 hybrid between *B*. *rapa* and *B*. *olearcea* was obtained by pre-culture of amphihaploid and chromosome doubling (Li *et al*. 2010). The third true leaves from three plants of each genotype were pooled at the same physiological stage (40-day-old seedlings), three replicates were preserved and frozen at −80°C for use.

5.2. DNA and RNA isolation

The protocol for DNA extraction was a modification of the cetyl trimethylammonium bromide (CTAB) method of Doyle and Doyle (1990). Chloroform and CTAB were used to eliminate proteins and carbohydrates, whereas CTAB-DNA was precipitated and collected. Extracted DNA was dissolved using ddH_2O containing RNase. The concentration and purity of DNA was determined by electrophoresis and spectrophotometry. DNA samples were preserved at −20°C for use. Total RNA was extracted from leaves using RNAiso Plus (Vazyme, China) based on the protocol of the manufacturers. After removing the contaminated DNA by DNaseI (RNase free) treatment, purified total RNA (5 μg) was used as template for cDNA synthesis using a Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland). cDNA was then stored at −20°C for use.

5.3. HPLC analysis of DNA methylation

Based on the method of Sotgia *et al*. (2010) with few modifications, approximately 50 μg of DNA was hydrolyzed using 200 μL of 70% perchloric acid for 1 h at 100°C. Then, the pH was adjusted from 3 to 5 with 1 mol L^{-1} of KOH. Finally, the incoming $KClO_4$ precipitate was centrifuged at 12 000

r min^{-1} for 5 min, and the hydrolysate was collected and automatically injected into an HPLC system (Agilent 1200, USA) coupled with an Alltima C18 column (with a granulation and size of 5 μm and 250 mm×4.6 mm, respectively). Chromatographic separation was conducted with a flow rate of 0.8 mL min^{-1} and oven temperature of 40°C using a mixture of two solvents, namely, 10% methanol and 0.1 mol L^{-1} of sodium pentanesulfonate, in 0.2% triethylamine. The UV spectra was recorded at 273 nm. Relative quantification was conducted by comparing the peak areas of similar retention times using the calibration curves of available cytosine and 5-mC (5-methylcytosine) as standard. The percentage of 5-mC in each sample was calculated as follows: Concentration of 5-methylcytosine/(Concentration of 5-methylcytosine+Cytosine). All analyses were repeated three times, and the mean±standard error was calculated.

5.4. MSAP assay and sequence analysis of MSAP fragments

MSAP analysis was conducted based on the method of Xiong *et al*. (1999) with few modifications, with 15 U *Eco*RI and 10 U *Hpa*II or 10 U *Msp*I used to digest DNA at 37°C for 12 h. *Hpa*II and *Msp*I are methylation-sensitive restriction endonucleases both recognizing CCGG sequences. *Hpa*II can only cleave non-methylated CCGG and hemimethylated mCCGG sequences, *Msp*I can digest non-methylated CCGG and hemi or fully methylated CmCGG sequences but not hemi and fully methylated mCCGG and mCmCGG sequences (Fulnecek and Kovarik 2014). The digested fragments were then ligated to specific adaptors using T_4 ligase (Takara, Japan). After ligation at 16°C for 4 h, pre-amplification and selective amplification were conducted using dilutes of ligates as template. The adaptors and primers used for pre-amplification and selective amplification were listed in Table 4. Finally, polymerase chain reaction (PCR) products were separated on 6% denaturing polyacrylamide gel and stained with silver. DNA fragments were scored, with "+" and "−" denoting the presence and absence of bands, respectively. All the identified bands can generally be classified into four types, namely, (+, +), (+, −), (−, +) and (−, −). Interesting polymorphic fragments with different methylation patterns between hybrids and parents were excised from gels and recycled for re-amplification. The re-amplified fragments were cloned into the pMD19-T vector for sequencing.

5.5. Gene expressional analysis of DNA methyltransferase and polymorphic fragments in MSAP analysis

Expression of *DNA methyltransferase 1* (*MET1*) and *DNA methyltransferase chromomethylase 3* (*CMT3*), as well as polymorphic fragment from MSAP analysis were analyzed by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) using a FastStart Universal SYBR Green Master (ROX; Roche, Switzerland). Approximately 20 μL of the reaction mixture containing 0.3 μmol L^{-1} of gene-specific forward and reverse primers, 1 μL of cDNA, and 10 μL of SYBR Green reagent was used. qRT-PCR was conducted using an ABI 7500 system (Applied Biosystems, USA) under the following conditions: 2 min at 50°C; 1 min at 95°C; and 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s in a 96-well optical reaction plate (Bio-Rad Laboratories, USA). For each pair of primers, gel electrophoresis and

Table 4 Sequences of adaptors and primers used for pre-amplification and selective amplification in MSAP analysis

Adaptors/Primers	Sequence (5′→3′)
*Eco*RI adaptor 1	CTCGTAGACTGCGTACC
*Eco*RI adaptor 2	AATTGGTACGCAGTCTAC
*Msp*I/*Hpa*II adaptor 1	GATCATGAGTCCTGCT
*Msp*I/*Hpa*II adaptor 2	CGAGCAGGACTCATGA
*Eco*RI pre-amplification	GACTGCGTACCAATTC
*Msp*I/*Hpa*II pre-amplification	ATCATGAGTCCTGCTCGG
*Eco*RI selective primer 1	GACTGCGTACCAATTCAAC
*Eco*RI selective primer 2	GACTGCGTACCAATTCACG
*Eco*RI selective primer 3	GACTGCGTACCAATTCACT
*Eco*RI selective primer 4	GACTGCGTACCAATTCAGT
*Eco*RI selective primer 5	GACTGCGTACCAATTCAAG
*Eco*RI selective primer 6	GACTGCGTACCAATTCACA
*Eco*RI selective primer 7	GACTGCGTACCAATTCACC
*Hpa*II/*Msp*I selective primer 1	ATCATGAGTCCTGCTCGGTAA
*Hpa*II/*Msp*I selective primer 2	ATCATGAGTCCTGCTCGGTCC
*Hpa*II/*Msp*I selective primer 3	ATCATGAGTCCTGCTCGGTTC
*Hpa*II/*Msp*I selective primer 4	ATCATGAGTCCTGCTCGGTAC
*Hpa*II/*Msp*I selective primer 5	ATCATGAGTCCTGCTCGGTGC
*Hpa*II/*Msp*I selective primer 6	ATCATGAGTCCTGCTCGGTAG
*Hpa*II/*Msp*I selective primer 7	ATCATGAGTCCTGCTCGGTTG
*Hpa*II/*Msp*I selective primer 8	ATCATGAGTCCTGCTCGGTCA
*Hpa*II/*Msp*I selective primer 9	ATCATGAGTCCTGCTCGGACA
*Hpa*II/*Msp*Iselective primer 10	ATCATGAGTCCTGCTCGGATT
*Hpa*II/*Msp*I selective primer 11	ATCATGAGTCCTGCTCGGAAT

Table 5 Primers used for qRT-PCR analysis

Genes	Primer sequence (5′→3′)
β-actin	Forward: 5′-TCTTCCTCACGCTATCCTCCG-3′ Reverse: 5′-AGCCGTCTCCAGCTCTTGC-3
MET1	Forward: 5′-CGAGGCAGAAGTAGTAGAAGCG-3′ Reverse: 5′-GGAGGAGAAAGAAGCCAAGC-3′
CMT3	Forward: 5′-GTGTCAAACAACGAAACCCG-3′ Reverse: 5′-TCAGTCGCAGAGCCAGTCAT-3′
BnaA06g00900D	Forward: 5′-GAAAGGACGGTTGGAGGA-3′ Reverse: 5′-CCAGCGACATTGACACGA-3′
BnaA07g31670D	Forward: 5′-CCTGAGAACCGCCATTAT-3′ Reverse: 5′-TCTTCCCATTCACCAACCT-3′
BnaA08g11270D	Forward: 5′-TGGGAAACCAAACAAGCAA-3′ Reverse: 5′-CGAACCTCAACACCCGTAG-3′
BnaA09g00510D	Forward: 5′-CAGTTAGATGTCCCAGTTGATAG-3′ Reverse: 5′-CGATAAGCACGCATAGCCT-3′

melting curve analyses were conducted to ensure that only one expected PCR amplicon was generated. *B. napus β-actin* (NCBI $AS_1$11812) was used as a control to quantify the PCR products. A comparative C_T method ($\Delta\Delta C_T$) was used in quantification analysis. Primers used for qRT-PCR analysis were listed in Table 5.

Acknowledgements

This study was supported by the National Key Basic Research Program of China (2015CB150201), the National Natural Science Foundation of China (NSFC, 31330057, 31401414), the Priority Academic Program Development of Jiangsu Higher Education Institutions of China, the Jiangsu Province Science Foundation, China (BK20140478, 14KJB210008), and the Jiangsu Province Graduate Innovation Fund, China (KYZZ15_0367).

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(Managing editor WENG Ling-yun)