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Production and genetic analysis of resynthesized *Brassica napus* from a *B. rapa* landrace from the Qinghai-Tibet Plateau and *B. alboglabra*

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ABSTRACT. This study aimed to reveal the genetic and epigenetic variations involved in a resynthesized *Brassica napus* (AACC) generated from a hybridization between a *B. rapa* (AA) landrace and *B. alboglabra* (CC). Amplified fragment length polymorphism (AFLP), methylation-sensitive amplified polymorphism, and the cDNA-AFLP technique were performed to detect changes between different generations at the genome, methylation, and transcription levels. We obtained 30 lines of resynthesized *B. napus* with a mean 1000-seed weight of over 7.50 g. All of the lines were self-compatible, probably because both parents were self-compatible. At the genome level, the S0 generation had the lowest frequency of variations (0.18%) and the S3 generation had the highest (6.07%). The main variation pattern was the elimination of amplified restriction fragments on the CC genome from the S0 to the S4 generations. At the methylation level, we

found three loci that exhibited altered methylation patterns on the parental A genome; the variance rate was 1.35%. At the transcription level, we detected 43.77% reverse mutations and 37.56% deletion mutations that mainly occurred on the A and C genomes, respectively, in the S3 generation. Our results highlight the genetic variations that occur during the diploidization of resynthesized *B. napus*.

Key words: Allopolyploidization; Resynthesized *Brassica napus*; Genetic variation; Qinghai-Tibet Plateau

INTRODUCTION

Material with an excellent phenotype can be used as a parental line for a resynthesized species, laying a foundation for improving germplasm resources. In this study, one of the parents was 'Qinghai Dahuang' rape, which originated on the Qinghai-Tibet Plateau. It has a yellow seed coat, is self-compatible, has large, heavy grains (approximately 6-7 g per 1000 grains), a high oil content (41%), and exhibits greater lodging and shattering resistance than other *Brassica rapa* varieties (Xiao et al., 2012). Polyploidization underlies species formation, provides the driving force for the evolution of species (Hegarty and Hiscock, 2008), and is widespread in angiosperms (Leitch and Bennett, 1997; Soltis and Soltis, 1999). Polyploids can be divided into autopolyploids and allopolyploids. Autopolyploids are derived from the doubling of diploids, while allopolyploids are formed by the hybridization of two or more diploids from different species and genera, followed by doubling. Although allopolyploidy was very common during eukaryote evolution, the molecular mechanisms involved in allopolyploidization in plants are still poorly understood (Allard et al., 1993).

Previous studies have shown that allopolyploidization can cause variations in the genome and gene expression. Variations at the genomic level mainly include chromosomal rearrangements, inversions, transpositions, and fragment eliminations; variations in gene expression include gene silencing, gene activation, methylation changes, transposon activation, and nucleolar dominance (Attia and Röbbelen, 1986; Kenton et al., 1993; Chen and Pikaard, 1997; Cui et al., 2006). Numerous studies have demonstrated that sequence elimination was one of the main modes of genomic variation during the evolution of synthetic allopolyploids (Shaked et al., 2001; Kashkush et al., 2002; Scannell et al., 2006; Xu et al., 2009). Wang et al. (2004) generated four resynthesized Arabidopsis strains using different parents, and found that 4 and 11% of the parental genes were silenced in the progeny of the two strains. In allopolyploid crops, variations at the transcription level have frequently been reported, and they mainly involve the emergence of new transcripts and the loss of original transcripts (Liu et al., 1998; Kashkush et al., 2002; Adams and Wendel, 2005a; Chen et al., 2007).

Cytosine methylation is common in allopolyploids, and includes the restriction or modification of genomic structures and epigenetic regulation (Liu and Wendel, 2003). The plant genome contains 20-30% of 5'-end cytosine methylation. New evidence suggests that in the nuclear genome of Arabidopsis, changes in cytosine methylation and methylation patterns can affect gene activity (Richards, 1997; Zhang et al., 2010). Widespread changes in methylation patterns can emerge in newly synthesized allopolyploids, and Madlung et al. (2002) found that 8.3% of loci in the genomes of Arabidopsis allopolyploids exhibited altered methylation patterns. Xu et al. (2009) found that approximately 6.84% of restriction sites exhibited different methylation patterns in synthetic *B. napus*. Shaked et al. (2001) used the methylation-sensitive amplified polymorphism (MSAP) technique to analyze genome-wide DNA methylation conditions during the evolution of an

allotetraploid synthesized from Aegilops and Triticum dicoccoides, and found that approximately 13% of the loci in both the F1 diploid and the tetraploid were methylated.

Most polyploid ancestors in nature either are unknown or have demised, and it is difficult to study their evolution. However, resynthesized allopolyploids have known parents, which can facilitate the comparative analysis of genomes and gene expression between the synthetic species and their parents. Therefore, synthetic allopolyploids are ideal materials for the investigation of the molecular mechanisms of early allopolyploidization. *B. napus* (2n = 38, AACC) evolved from a natural hybridization between B. oleracea (2n = 18, CC) and *B. rapa* (2n = 20, AA), or B. oleracea and B. campestris (2n = 20, AA), followed by natural chromosome doubling, and is diploid (AACC). Resynthesized *B. napus* is an important way to broaden *Brassica* resources, and is also an effective means of studying the evolution of *Brassica* allopolyploids. In addition, the gene pool formed by allotypes provides a basis for *Brassica* crossbreeding and new germplasm resources (Chen and Ni, 2006; Chen et al., 2008; Jesske et al., 2013).

We studied the reciprocal hybridization of *B. rapa* 'Qinghai Dahuang' rape (2n = 20, AA) and B. oleracea 'Middle-late maturing Chinese kale' (2n = 18, CC) and performed immature embryo rescue, ovary culture, and chromosome doubling to resynthesize a batch of *B. napus*. The first objective of the study was to investigate genomic variation between generations (S0, S1, S3, and S4) of synthetic *B. napus* allopolyploids using amplified fragment length polymorphism (AFLP) markers. The second objective was to use MSAP markers to elucidate how genomic polyploidization affects DNA methylation changes between diploid parents and the tetraploid S0 generation. In addition, we used the cDNA-AFLP technique to examine variations in gene expression between the diploid parents and the S0 generation of allotetraploids, and between the S0 generation and the S3 generation.

MATERIAL AND METHODS

Material

The maternal line was *B. rapa* L., 'Qinghai Dahuang' (AA, labeled as QD) and the paternal line was B. oleracea var. alboglabra L., 'Zhongchi Jielan' (CC, labeled ZC). Hybridization between the two species yielded a number of new *B. napus* L. (AACC). 'Qinghai Dahuang' is a *B. rapa* cultivar that has yellow seeds, and originated on the Qinghai-Tibet Plateau; it was provided by the Institute of Spring Rape, Qinghai Academy of Agriculture and Forestry, Qinghai, Xining, China. The 'Zhongchi Jielan' was provided by the Oil Crops Research Institute of the Chinese Academy of Agricultural Sciences, Wuhan, Hubei, China. Both of the parents were homozygous varieties (lines) from many generations of inbreeding.

The phenotypes of synthesized S0, S1, S3, and S4 generations of *B. napus* and their original diploid parents were investigated. The cultivars, Qingza2 and Qingyou14, and another artificially synthesized *B. napus* S1, which was derived from Menyuan rapeseed, a landrace of *B. rapa* and ZC; the self-crossing S2 generation was used as comparison material. We randomly surveyed 30 individuals from each line; if there was at least one self-compatible plant in a line, the line was considered self-compatible and marked as YES; if not, it was marked as NO. Through microspore culture for each plant with 1000-grain weight greater than 7.5 g per plant, we obtained doubled haploid plants. Analysis of variance was conducted using SPASS version 3.5 (Weidenbach et al., 2009).

The experimental materials for AFLP testing included plants of the synthesized S0, S1, S3, and S4 generations of *B. napus*. The S0 generation was obtained by the colchicine-mediated doubling of F1 plants derived from the interspecific hybridization of QD and ZC. The selfing of

each plant in the S1 generation yielded the S2 generation, the selfing of each plant in the S2 generation yielded the S3 generation, and the selfing of each plant in the S3 generation yielded the S4 generation. Fourteen plants from each generation were characterized by their numbers of chromosomes, pollen viability, and phenotypic features. The cDNA-AFLP testing materials included one plant from the S0 generation, four plants from the S3 generation, and their original parents, QD and ZC. The test materials for the MSAP analysis were 14 plants from the tetraploid S0 generation and their diploid parents, QD and ZC.

Methods

DNA and RNA extraction and cDNA analysis

Fresh young leaves were collected from plants of the S0, S1, S3, and S4 generations and the parental lines (QD and ZC) at the seedling stage, and the cetyltrimethylammonium (CTAB) method was used for DNA extraction (Doyle, 1990). RNA extraction was performed using a TRIzol kit according to the manufacturer instructions (Invitrogen), and cDNA synthesis was performed using an M-MLV RTase cDNA synthesis kit (Takara, Otsu, Japan) following the procedures described in the manual.

AFLP analysis

AFLP analysis was performed as described by Vos et al. (1995). We used 5 U *Eco*RI and 2 U MseI (with a reaction volume of 25 mL) to digest DNA at 37°C, and the ends of the restriction digest fragments were ligated to the following artificial adaptors: E-F, 5'-CTCGTAGACTGCGTAC C-3' and E-R, 5'-AATTGGTACGCAGTC-3'; M-F, 5'-GACGATGAGTCCTGAG-3' and M-R, 5'-TACTC AGGACTCATC-3'.

The ligation products were amplified using preamplification primers (EA/MC), the preamplified products were diluted (1:30), and were then amplified using selective primers. The selective amplification products were denatured, underwent polyacrylamide gel electrophoresis, and were developed by silver staining. The gel images were then used in statistical analysis (Vos et al., 1995). We selected 45 pairs of highly polymorphic AFLP primer EA/MC combinations for the whole-genome scanning of plants in the S0, S1, S3, and S4 generations.

cDNA-AFLP analysis

The cDNA-AFLP reactions were conducted in an identical manner to the AFLP, except that the templates for the reaction were cDNAs. We chose cDNAs synthesized from RNA that was extracted from plants of the parental QD and ZC lines and the S0 and S3 tetraploid progenies as the templates. Thirty of the 45 pairs of primer combinations were used to scan the transcriptome by AFLP analysis.

MSAP analysis

MSAP is a methylation detection method that is based on AFLP molecular markers. Two isoschizomers of *Hpa*II and *Msp*I were used in combination with *Eco*RI for the double digestion of DNA. Both *Hpa*II and *Msp*I can recognize the CCGG site, but they have different sensitivities

to methylation. In the absence of methylation, these two enzymes can both cut the CCGG sites, whereas they exhibit differential recognition and reaction to certain methylation patterns. *Hpa*II can cut hemimethylated CCGG sites but not fully methylated sites. *Msp*I can recognize the sequence if the internal cytosine residue is methylated, but cannot recognize it if the external cytosine is methylated, i.e., *Msp*I cannot digest sites containing mCCGG (McClelland et al., 1994). Because the restriction enzymes had different sensitivities to DNA methylation, the same sequence could yield different band patterns, and, consequently, we could determine the DNA methylation status. The reaction procedures were as described by Xiong et al. (1999). The EA adaptors were 5'-CTCGTAGACTGCGTACC-3' (E-F) and 5'-AATTGGTACGCAGTC-3' (E-R), and the H/M adaptors were 5'-GATCATGAGTCCTGCT-3' (H/M-F) and 5'-CGAGCAGGACTCATGA-3' (H/M-R). The *Eco*RI preamplification primer was 5'-GACTGCGTACCAATTC-3', and the H/M I preamplification primer was 5'-ATCATGAGTCCTGCTCGG-3'. We used 16 pairs of selective amplification primers; some of the primer pairs were selected from those reported by Xu et al. (2012) in their study of synthetic *B. napus*. We designed three primer pairs: E + CA, E + AG, and E + AA (Table 1). We used combinations of *Eco*RI/*Hpa*II and *Eco*RI/*Msp*I to perform the enzyme digestion of genomic DNA, which was followed by adaptor ligation, preamplification, and selective amplification using the AFLP program. The selective amplification products were mixed with sample loading buffer, denatured, and detected on a 6% polyacrylamide gel. All of the primers were synthesized by the Shanghai Sangon Biological Engineering Co. Ltd. (Shanghai, China), and all of the tests were conducted twice.

Table 1. Selective primers for methylation-sensitive amplified polymorphism analysis.

*Eco*RI selective primer		H/M selective primer	
Primer	*Eco*RI pre-selection primer + three selective nucleotides	Primer	*Hpa*II/*Msp*I pre-selection primer + three selective nucleotides
EA 1	EA0 + AAA	H/M 1	H/M0 + TAA
EA 2	EA0 + AAT	H/M 2	H/M0 + TCC
EA 3	EA0 + AAC	H/M 3	H/M0 + TGG
EA 4	EA0 + AAG	H/M 4	H/M0 + TAC
EA 5	EA0 + ATA	H/M 5	H/M0 + TAG
EA 6	EA0 + ATT	H/M 6	H/M0 + TCA
EA 7	EA0 + ATC	H/M 7	H/M0 + TCG
EA 8	EA0 + ATG	H/M 8	H/M0 + TGA
EA 9	EA0 + ACA	H/M 9	H/M0 + TGC
EA 10	EA0 + ACT	H/M 10	H/M0 + TAT
EA 11	EA0 + ACC	H/M 11	H/M0 + TCT
EA 12	EA0 + ACG	H/M 12	H/M0 + TGT
EA 13	EA0 + AGA	H/M 13	H/M0 + TTA
EA 14	EA0 + AGT	H/M 14	H/M0 + TTC
EA 15	EA0 + AGC	H/M 15	H/M0 + TTG
EA 16	EA0 + AGG	H/M 16	H/M0 + TTT

Statistical analysis

The AFLP and cDNA-AFLP electrophoresis bands were statistically analyzed using “1” and “0”, with “1” indicating the presence of bands and “0” indicating the absence of bands. For the MSAP analysis, isoschizomers of *Hpa*II and *Msp*I were used in combination with *Eco*RI for double digestion, and “+” and “-” were assigned according to the presence or absence of bands. Each sample was run in two lanes; in the first lane, *Eco*RI/*Hpa*II was digested and recorded as H, and in the second lane, *Eco*RI/*Msp*I was restriction-digested and recorded as M. The band patterns could

be divided into three types: type I, with bands in both H and M (+, +), indicating no methylation; type II, with bands in H and no bands in M (+, -), indicating external methylation on a single strand; and type III, with no bands in H and with bands in M (-, +), indicating double-stranded internal methylation.

RESULTS

Seed weight and self-compatibility of resynthesized *B. napus* allopolyploids

Compared to the S2 (Menyuan rapeseed x ZC), the S0-S4 generations had stable self-compatibility. We obtained 30 single plants with a 1000-grain weight of over 7.5 g. Both the 1000-seed weight and yield of each generation were significantly higher than those of the control varieties ($P < 0.01$). The 1000-seed weight and yield decreased with increasing allopolyploid generation, which indicated selfing depression (Table 2). The doubled haploid plants had significantly larger grains than the Qing14 and Qingza2 plants (Figure 1).

Table 2. Self-compatibility and grain weight and yield performance of different synthetic *Brassica napus* generations and comparison lines.

	N	SC (Y/N)	SW (g)[A]	YP (g)[A]
Material				
QD	30	Y	7.53 ± 0.12[a]	15.21 ± 0.78[a]
ZC	30	Y	3.21 ± 0.07[b]	12.19 ± 0.54[b]
S0	30	Y	7.58 ± 0.26[ac]	37.19 ± 1.39[c]
S1	30	Y	6.90 ± 0.16[c]	20.91 ± 1.25[d]
S3	30	Y	6.64 ± 0.25[c]	16.41 ± 1.21[ae]
S4	30	Y	6.00 ± 0.17[d]	14.73 ± 1.04[af]
Control material				
Qingyou14	30	Y	3.53 ± 0.13[be]	23.13 ± 0.93[g]
Qingza2	30	Y	4.22 ± 0.21[e]	27.82 ± 1.32[h]
(Menyuan rapeseed x ZC) S1	30	Y	4.23 ± 0.19[e]	11.45 ± 1.10[bi]
(Menyuan rapeseed x ZC) S2	0	N	0	0

[A]Means ± standard error. Different lower-case letters in the same column indicate a significant difference at the 0.01 level. SC, self-compatibility (Y, self-compatible; N, self-incompatible). SW, mean 1000-seed weight. YP, mean grain weight per plant. QD, 'Qinghai Dahuang'; ZC, 'Zhongchi Jielan'.

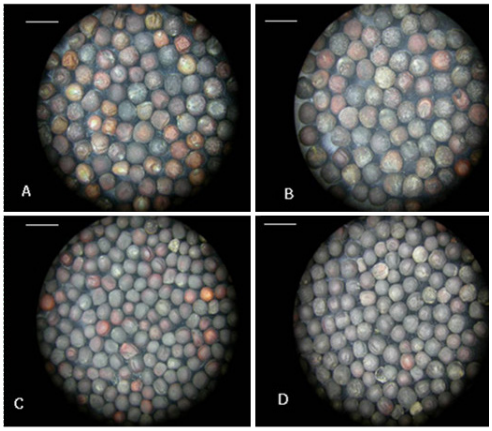

Figure 1. Rapeseed grains under an electron microscope. A, S0 doubled haploid plant 677-2. B, DH plant 677-10. C, Qingyou14. D, Qingza2.

Variation at the genomic level among different generations of allopolyploids

We used 45 pairs of AFLP primers to analyze plants from the S0, S1, S3, and S4 generations and their parents, QD (*B. rapa*, AA) and ZC (B. oleracea, CC). We found that there were two types of variation in the polyploid progeny: the addition of new fragments and the elimination of specific parental fragments. The S0 generation had 1093 bands after the amplifications; two loci had variations, and the variation rate was 0.18%. One of the variations was the absence of a specific locus from the maternal line, QD (AA), in S0, and the other was a newly added specific locus in S0 (Figure 2). The S1 generation had 1092 bands from the amplifications and 10 sites had variations, which accounted for 0.91% of the total sites. Of these, nine (0.82%) loci with deletion variations were detected in the C genome. The S1 generation also exhibited a new band. The S3 generation had 774 allelic loci from the amplifications, and the mutation rate of the loci was 6.07%; all of these loci exhibited deletion variations. A total of 1.67% of the allelic loci were deleted from the A genome, 2.45% were deleted from the C genome, and 0.645% exhibited the elimination of common bands from the AC genomes. In the S4 generation, 555 bands were amplified; 4.50% of the sites exhibited changes, and the variation type was the elimination of fragments. The rate of fragment loss of the A genome was 1.44%, that of the C genome was 2.88%, and that of the AC genomes was only 0.18% (Table 3).

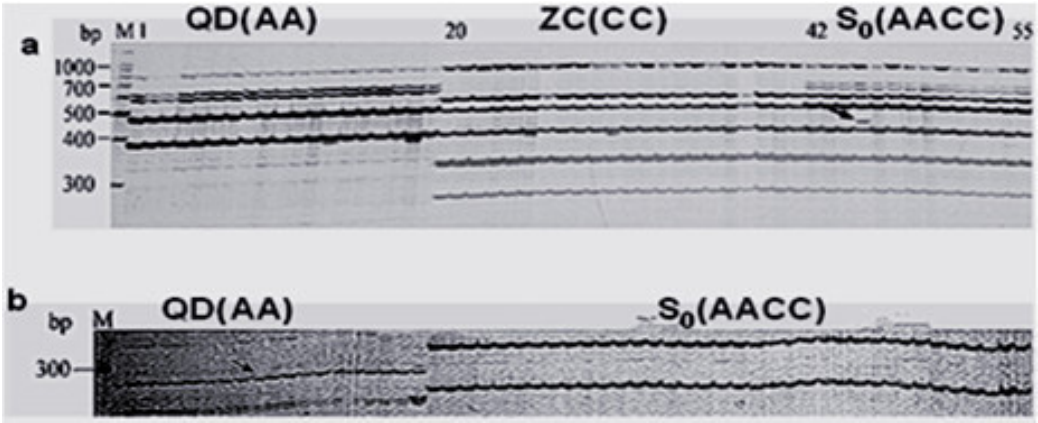

Figure 2. New and missing bands detected by amplified fragment length polymorphism. **a.** 1-19, maternal line 'Qinghai Dahuang'; 20-41, paternal line 'Zhongchi Jielan'; 42-55, S0. Arrow indicates new bands in the S0 generation. **b.** Arrow indicates the maternal band at 280 bp that was missing in the S0 generation.

Table 3. Amplified fragment length polymorphism detection of variations in different generations.

	S0	S1	S3	S4	Total
Total number of bands	1093	1092	774	555	3514
Overall variation rate (%)	2 (0.18)	10 (0.95)	47 (6.07)	25 (4.50)	84 (11.66)
Loss of amplified fragments from the A genome (%)[a]	1 (0.09)		13 (1.67)	8 (1.44)	21 (3.12)
Loss of amplified fragments from the C genome (%)[b]		9 (0.82)	19 (2.45)	16 (2.88)	45 (6.25)
Loss of amplified fragments common to the A and C genomes (%)[c]			5 (0.64)	1 (0.18)	6 (0.82)
New fragments (%)	1 (0.09)	1 (0.09)			2 (0.18)
Loss of amplified fragments of unknown origin (%)			10 (1.29)		10 (1.29)

[a]Loss of amplified fragments from the AA genome (*Brassica rapa*). [b]Loss of amplified fragments from the CC genome (*Brassica oleracea*). [c]Loss of amplified fragments common to the AA and CC genomes (*Brassica napus*).

Variations in gene expression between S0 and S3 generations of resynthesized *B. napus*

To reveal the role of polyploidization in generating differences at the transcriptional level between *B. napus* allopolyploids and their diploid parents, this study utilized 30 cDNA-AFLP primer pairs to examine the S0 and S3 generations and the diploid parents. There were eight variation types (Figure 3). A total of 167 specific amplified fragments were detected in the maternal QD (AA) cDNA, 197 were detected in the paternal ZC (CC) cDNA, and the S3 generation mainly exhibited reverse mutations and deletion mutations. Compared with the S0 generation, the S3 generation gained 434 specific amplified fragments, 190 of which were absent in the S0 generation but were reversed in the S3 generation, with a reverse mutation rate of 43.77%. There were 46 reversed fragments from the A genome, with a reverse mutation rate of 10.59%; there were 38 reversed fragments from the C genome, accounting for 8.75% of the total reversed fragments; and there were 17 reversed fragments that were common in the A-C genome, with a reverse mutation rate of 3.91%. In addition, there were 89 new amplified fragments with undetermined sources of variation in the S3 generation, accounting for 20.50% of the total reversed transcripts (Table 4). The 163 amplified bands inherited in the S0 generation disappeared in the S3 generation, with a mutation rate of 37.55%. A total of 25 amplified fragments from the A genome and 54 amplified fragments from the C genome were eliminated, and the mutation rates were 5.76 and 12.44%, respectively. A total of 13 specific fragments from the A-C genome in the S0 generation were eliminated in the S3 generation, accounting for 2.99% of the total eliminated bands. In addition, we could not determine the source of variation for another 71 amplified fragments, which accounted for 16.35% of the total amplified bands (Table 4).

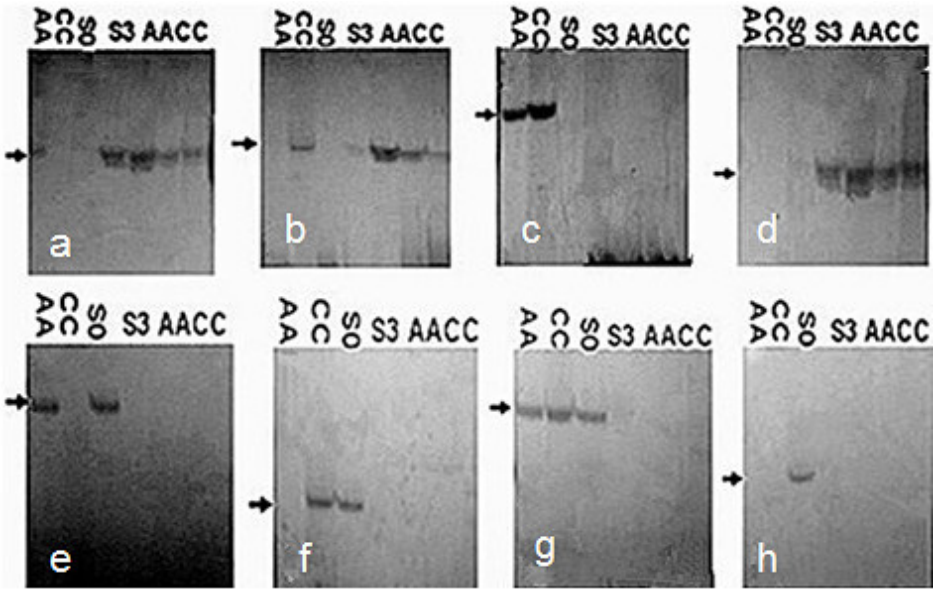

Figure 3. Variation types detected by cDNA-amplified fragment length polymorphism. AA, maternal 'Qinghai Dahuang' genome; CC, paternal 'Zhongchi Jielan' genome; S0 and S3 generations, AACC genome. Arrows indicate different patterns of variation (a, 1001; b, 0101; c, 1101; d, 0001; e, 1010; f, 0110; g, 1110; and h, 0010). 1, bands present; 0, bands absent.

Table 4. Summary of cDNA-amplified fragment length polymorphism analysis results of S0, S3, and parents.

Variation source	QD (AA)	ZC (CC)	S0	S3	Rate of variation type (%)	Total variation rate (%)
			(AACC)			
Number of amplified fragments	167	197	597	434		
Reverse of A genomic amplified fragment elimination[a]	1	0	0	1	46 (10.59)	190 (43.77)
Reverse of C genomic amplified fragment elimination[b]	0	1	0	1	38 (8.75)	
Reverse of A-C genomic amplified fragment elimination[c]	1	1	0	1	17 (3.91)	
Undetermined source	0	0	0	1	89 (20.50)	
A genomic amplified fragment elimination[d]	1	0	1	0	25 (5.76)	163 (37.55)
C genomic amplified fragment elimination[e]	0	1	1	0	54 (12.44)	
A-C genomic amplified fragment elimination[f]	1	1	1	0	13 (2.99)	
Undetermined source	0	0	1	0	71 (16.35)	

[a]Specific fragments from the A genome (*Brassica rapa*) were eliminated in the S0 generation and reversed in the S3 generation. [b]Specific fragments from the C genome (*Brassica oleracea*) were eliminated in the S0 generation and reversed in the S3 generation. [c]Specific fragments from the A and C genomes were eliminated in the S0 generation and reversed in the S3 generation. [d]Specific fragments from the A genome (*Brassica rapa*) appeared in the S0 generation and were eliminated in the S3 generation. [e]Specific fragments from the C genome (*Brassica oleracea*) appeared in the S0 generation and were eliminated in the S3 generation. [f]Specific fragments from the A and C genomes appeared in the S0 generation and were eliminated in the S3 generation. 1, bands present; 0, bands absent. Each cDNA-amplified fragment length polymorphism band was considered a specific transcript. QD, 'Qinghai Dahuang'; ZC, 'Zhongchi Jielan'.

The probability of reverse mutations for cDNA-amplified fragments from the A genome was slightly higher than that from the C genome. However, the cDNA-amplified fragment elimination ratio was significantly higher for amplified fragments from the C genome than that from the A genome. Additionally, the reverse mutation rate in the S3 generation was higher than the deletion mutation rate.

DNA methylation detection in the S0 generation and diploid parents

We randomly selected 18 pairs of MSAP primers to detect the methylation conditions of the diploid parents and S0 plants. We detected three sites with altered methylation patterns. The EA2HM4 and EA3HM4 primer combinations revealed that one (-, +) band type from the maternal 'Qinghai Dahuang' rape became (-, -) in the S0 generation (Table 5, pattern 4). After the synthesis of *B. napus*, methyl transfer reactions probably occurred at this site in the A genome, i.e., the original double-stranded internal methylation was changed into double-stranded external methylation, and the CCGG sequences in the corresponding sites may have been changed. The EA5HM14 primer combinations amplified the band type (-, +) in the maternal line and the band type (+, -) in the paternal line, and the band type amplified in the S0 plants was the same as that in the paternal line (+, -) (Table 5, pattern 7), indicating that the corresponding loci in the A genome of the hybrids were not cut by *Msp*I. This pattern may have been caused by the switch from internal methylation to external methylation or changes in the CCGG sequence. No changes in the methylation patterns were detected in genome C of the hybrids, and bands with variations accounted for 1.35% of the total bands in the S0 generation. The MSAP band types for the S0 generation and the two parents could be divided into 14 patterns, 4 and 7 of which had changed methylation patterns (Table 5).

The maternal QD had 42 methylated loci, the methylation ratio was 27.27%, and full-methylation loci and hemimethylation loci accounted for 26.62 and 0.65%, respectively, of all the amplified loci. The paternal ZC had 40 methylation sites, the methylation ratio was 23.12%, and full-methylation loci and hemimethylation loci accounted for 18.50 and 4.62%, respectively, of all

the amplified loci. The parents shared 68 unmethylated bands and 12 bands with full methylation, and no hemimethylation bands were detected in either parent. A total of 219 effective band types were amplified from 10 plants of the S0 generation. The methylation sites from one parent (the H and M band types were '-, +' or '+, -') were usually masked by the non-methylated band types from the other parent (the H and M types were +, +). Therefore, we only detected 41 methylation sites, and the methylation rate was 18.72%; the rates of full methylation and hemimethylation were 15.98 and 2.74%, respectively (Table 6).

Table 5. Methylation patterns detected by methylation-sensitive amplified polymorphism.

Band type/combination pattern	Band type for methylation patterns						Total band No.	Methylation variation rate (%)
	QD (AA)		ZC (CC)		S0 plants (AACC)			
	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	*Hpa*II	*Msp*I		
1	-	-	-	+	-	+	13	5.88
2	-	-	+	-	+	-	4	1.81
3	-	-	+	+	+	+	50	22.62
4	-	+	-	-	-	-	2	0.90
5	-	+	-	-	-	+	10	4.52
6	-	+	-	+	-	+	12	5.43
7	-	+	+	-	+	-	1	0.45
8	-	+	+	-	+	+	1	0.45
9	-	+	+	+	+	+	15	6.79
10	+	-	-	-	+	-	1	0.45
11	+	+	-	-	+	+	35	15.84
12	+	+	-	+	+	+	7	3.17
13	+	+	+	-	+	+	2	0.90
14	+	+	+	+	+	+	68	30.77

+, bands present; -, bands absent. QD, 'Qinghai Dahuang'; ZC, 'Zhongchi Jielan'.

Table 6. Methylation patterns in parents and the S0 generation.

Methylation pattern	Female parent QD (AA)	Male parent ZC (CC)	S0 plants (AACC)
Total bands	154	173	219
Full methylation (%)[a]	41 (26.62)	32 (18.50)	35 (15.98)
Hemimethylation (%)[b]	1 (0.65)	8 (4.62)	6 (2.74)
Total methylation rate (%)	42 (27.27)	40 (23.12)	41 (18.72)

[a]Two restriction sites of *Hpa* II and *Msp* I were methylated. [b]One site of *Hpa*II or *Msp*I was methylated. QD, 'Qinghai Dahuang'; ZC, 'Zhongchi Jielan'.

DISCUSSION

Genomic changes after allopolyploidization

Polyploidization is an important mechanism in plant evolution, and plays an important role in increasing variation in plants. With the development of molecular biology techniques in recent years, a variety of genomic changes related to polyploidization, particularly allopolyploidization, have been reported (Liu and Wendel, 2002, 2003; Lukens et al., 2004; Adams and Wendel, 2005b). As determined by the AFLP technique, the possible reasons for the emergence or disappearance of bands in hybrid progeny in comparison to the parents might be the occurrence of genetic events such as DNA sequence elimination, genomic rearrangements, and transposable element activation in the newly synthesized allopolyploids, leading to a disappearance or shift of the original restriction

sites (Shan et al., 2005; Tu et al., 2009). Therefore, differential bands in the resynthesized *B. napus* that were not present in the parents suggest that certain variations exist in the corresponding restriction sites. We found that the S0 generation had the lowest variation rate (0.182%), the S3 had the highest (6.07%), and the level of genomic variation continued to increase from the S0 to the S3 before gradually decreasing in the S4 (4.50%). From the S1 to the S4 generation, the gene fragment loss rate of the A genome was 3.12%, that of the C genome was 6.25%, and the main type of variation was the loss of restriction sites in the C genome. These results demonstrate that variations occurred in early generations of the artificially synthesized *B. napus* allopolyploids, but the variation frequency was significantly lower in the S0 generation than in the S3 generation. Ozkan et al. (2001) reported that in artificially synthesized wheat, several specific sequences from the B genome were lost in the F1 generation, and the sequence elimination events mainly took place in the first three generations. The authors suggest that the elimination of specific sequences of the parental genome is conducive to the rapid genetic stabilization of artificially synthesized species, which is a mechanism of adaptation during evolution. In the present study, sequence elimination mainly occurred in the first four generations, supporting the results of Ozkan et al. (2001). Lukens et al. (2004) and Gaeta et al. (2007) analyzed genomic variations in the S0 and S5 generations of 50 artificially synthesized *B. napus* strains, and found that there were very few variations in the DNA sequences of the S0 generation, whereas very high genetic variation frequencies were detected in 47 plants of the S5 generation. Sites with variations were detected in 33% of the restriction fragment length polymorphism markers and 71% of the simple-sequence repeat markers, and most of the missing fragments were caused by unidirectional translocations between non-homologous chromosomes (A1-C1, A2-C2, A3-C3, etc.). We found that the S3 generation of artificially synthesized *B. napus* had the highest variation rate, which decreased in the S4 generation. Gaeta and Chris Pires (2010) named this phenomenon of genetic variation progressively increasing with the progression of generations after the artificial synthesis of allopolyploids the “polyploidization gear effect”. In other words, genetic recombination or unidirectional translocation events occurring between some homologous chromosomes after allopolyploidization result in higher frequencies of mismatches between some homologous chromosomes in the prophase of meiosis in the next generation of plants, giving rise to a higher frequency of genomic variation in the offspring.

Genomic sources of genetic and epigenetic variation in artificially synthesized species

In comparison to the S0 generation, the S4 mainly exhibited deletion variations at the genomic level, and these variation sites were mainly from the C genome. MSAP analysis showed that the three loci with altered methylation patterns were all located in the A genome. The methylation level of each generation did not exhibit many differences compared to the previous generation. However, changes in methylation patterns favored the C genome, which is consistent with the results of Xu et al. (2009). Song et al. (1995) studied artificially synthesized *B. napus* (AACC and CCAA) derived from reciprocal crosses, and did not find that any particular genome was more likely to exhibit variations than any other. This result suggests that the resynthesized *B. napus* combination that the authors investigated had fairly good nuclear-cytoplasmic compatibility. Gaeta et al. (2007) found that in the S0 generation of artificially synthesized *B. napus* that used B. oleracea as the maternal line, the fragment loss frequencies of the A and C genomes were 0.2 and 0.3%, respectively, with no significant differences. They found that in the S5 generation of the same combination, the C genome had more frequent DNA variations and the A genome had a higher frequency of methylation pattern changes, which is consistent with our findings. In allotetraploids

from Cucumis sativus (2n = 2x = 14) and C. hystrix (2n = 2x = 24), Chen et al. (2007) found that in both reciprocal crosses, C. sativus exhibited approximately twice as many genomic fragment deletions than did the C. hystrix genome. This result indicates that genomic directional variation is not affected by the cytoplasm, but is instead affected by the number of parental chromosomes: the parental genome with the fewest chromosomes is more likely to exhibit variation. In summary, the mechanism of genomic directional variation may involve many factors, such as cytoplasm influence, differences in parental chromosome numbers, and parental genetic differences. The variations detected in this study were mainly derived from the C genome, possibly due to the dual influences of two factors, cytoplasm and chromosome number.

In the S3 generation, the loss rate of genomic fragments from the C genome was 2.45%, and the cDNA-specific fragment deletion rate was 12.44%. The loss rate of genomic fragments from the A genome fragment was 1.67%, and the cDNA-specific fragment deletion rate was 5.76%. These results indicate that variations at the genomic level can affect the expression levels of genes in that genome. Previous studies have found that the most important variation mode in allopolyploidy is gene elimination (Chen et al., 2007; Xu et al., 2009, 2012). Interestingly, our study demonstrated that in *B. napus* allopolyploids, reverse mutations of amplified bands from either the A or C genomes were also a major source of variation.

In summary, this study generated large grain and self-compatible *B. napus* allopolyploid resources, and elucidated variations between different generations of artificially synthesized *B. napus* allopolyploids in the genome, in gene expression, and in epigenetics. The AFLP test results showed that genomic variations could occur in early generations of artificially synthesized *B. napus*. However, the mutation frequency was relatively low in the S0 generation, and was the highest in the S3 generation. Variations between generations mainly included the elimination of restriction sites in the C genome. Variations in gene expression mainly included the deletion and reversion of specific transcripts, and there was a positive correlation between the gene fragment loss frequency and the transcript deletion frequency in the same genome. There were various patterns of methylation; however, the frequency of methylation pattern changes was low from the parents to the S0 generation, which was supported by the fact that changes in the methylation patterns from the A genome were detected only when three pairs of MSAP primers (EA3HM4, EA2HM4, and EA5HM14) were used.

Conflicts of interest

The authors declare no conflict of interest.

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