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DNA methylation alteration is a major consequence of genome doubling in autotetraploid *Brassica rapa*

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Abstract: Polyploids are typically classified as autopolyploids or allopolyploids based on the origin of their chromosome sets. Autopolyploidy is much more common than traditionally believed. Allopolyploidization, accompanied by genomic and transcriptomic changes, has been well investigated. In this study, genetic, DNA methylation and gene expression changes in autotetraploid *Brassica rapa* were investigated. No genetic alteration was detected using an amplified fragment length polymorphism (AFLP) approach. Using a cDNA-AFLP approach, approximately 0.58% of fragments showed changes in gene expression in autotetraploid *B. rapa*. The methylation-sensitive amplification polymorphism (MSAP) analysis showed that approximately 1.7% of the fragments underwent DNA methylation changes upon genome doubling, with hypermethylation and demethylation changes equally affected. Fragments displaying changes in gene expression and methylation status were isolated and then sequenced and characterized, respectively. This study showed that variation in cytosine methylation is a major consequence of genome doubling in autotetraploid *Brassica rapa*.

Key words: autotetraploid; *Brassica rapa*; DNA methylation; gene expression; genome doubling

INTRODUCTION

Two major types of polyploidy are known: autopolyploidy and allopolyploidy. Autopolyploids are generally considered to be derived from multiplication of the genome within a single genome, whereas allopolyploids arise via interspecific hybridization and chromosome doubling [1]. Autopolyploids have long been considered rare in natural populations and viewed as evolutionary dead-ends [1]. A dramatic resurgence in the study of polyploidy has increased our knowledge of autopolypoid dynamics and has elevated estimates of the frequency of autopolyploids in natural populations [1,2]. Thus, autopolyploidy may contribute more to evolution and species diversification than was traditionally thought [1,3].

Genomic shock, including chromosomal rearrangement, the gain and loss of chromosome segments, gene repression and activation, subfunctionalization and transposon activation, as well as epigenetic changes, has been investigated repeatedly in allopolyploids [4]. In autopolyploids, changes to the nuclear environment are not as profound as in allopolyploids [5,6]. The extent to which autopolyploidy induces genetic, epigenetic and gene expression changes in different autopolyploids remains debatable.

Contrasting results have been reported for the effects of autopolyploidization upon genetic change. Losses of 17% and 10% of total DNA content have been detected in autopolyploid *Phlox drummondii* [7] and *Elymus elongates* [8], respectively. However, a

study using amplified fragment length polymorphism (AFLP) reported approximately 1.5% genetic change in autopolyploid *Phlox drummondii* [9]. In *Paspalum notatum* autopolyploids, approximately 10% sequence elimination was detected during conversion from diploid to autotetraploid. [10]. Chromosomal rearrangement was first reported in synthetic autotetraploid *Arabidopsis thaliana* [11]. In contrast, Santos et al. (2003) [12] and Ozkan et al. (2006) [13] reported no major chromosomal rearrangements or DNA loss in a synthetic autopolyploid of *Arabidopsis thaliana*.

Compelling evidence for epigenetic changes, particularly for cytosine methylation, has been investigated in dandelions [14], *Eragrostis curvula* [15], *Phlox drummondii* [9], *Solanum* [16] and *Cymbopogon* [17] autopolyploids, which demonstrated striking ploidy epigenetic responses. However, there is little evidence for any rapid methylation changes in watermelon of higher ploidy [18].

Gene expression changes are another prominent consequence of genome doubling. Non-additive gene expression and functional plasticity were investigated in autopolyploids of *Arabidopsis* [19], maize [20], *Isatis indigotica* [21], *Pasalum notatum* [22], *Eragrostis curvula* [23], *Paulownia fortune* [24], potato (*Solanum phureja*) [25], an autopolyploid sunflower series [26] and rice [27]. However, there is little information on autotetraploid *Brassica rapa*.

The genus *Brassica* has long served as a model system for studying the molecular and phenotypic changes associated with both recent and ancient polyploidization events. Previous studies have reported rapid genetic, epigenetic, and expression changes in *Brassica* allopolyploids and found that homoeologous recombination is the major mechanism underlying these variations [6,28-30]. Studies aimed at separating hybridization and genome doubling effects have confirmed that genome doubling can ameliorate genomic and transcriptomic alterations induced by hybridization and can instigate additional alterations [6,29]. No significant proteomic changes were detected between diploid and autopolyploid *Brassica oleracea* [31]. Therefore, independent studies are necessary to clarify the genetic, epigenetic and gene expression responses in *Brassica* autopolyploids.

The objective of this work was to uncover genetic, DNA methylation and gene expression changes in *Brassica* autopolyploids. AFLP, methylation-sensitive amplification polymorphism (MSAP) and cDNA-AFLP approaches were applied in autotetraploid *Brassica rapa* and its diploid progenitors. Sequences showing changes in DNA methylation and gene expression in the autotetraploids were cloned and characterized, respectively.

MATERIALS AND METHODS

Plant materials

Autotetraploid *Brassica rapa* was generated with colchicine treatment of fully homozygous diploid *Brassica rapa* L. cv. Duanbaigeng. Thirty days after germination, the plantlets were flooded with a 0.1% colchicine solution for approximately 6 h. The plantlets were rinsed in distilled H_2O and grown to maturity. From each colchicine-treated plant (C1 generation of autotetraploids), seeds (C2 generation of autotetraploids) were harvested and sown. The ploidy of the C2 generation of autotetraploids was assessed using chromosome number and morphological characteristics (the size of and the number of chloroplasts in the guard cells of stoma).

The C2 generation of autotetraploids and the diploid progenitor were grown in a greenhouse under the same conditions. Young leaves (5 cm in length) of mature plants and similarly sized flowers (~0.5 cm in length, before opening) were collected from different autotetraploid and diploid individuals at comparable developmental stages.

DNA and RNA extraction and cDNA synthesis

Total DNA was isolated using the CTAB procedure. Total RNA was extracted using TRIzol reagent (Invitrogen, cat. no. 15596026). The reverse transcription, synthesis of second-strand cDNA and purification of cDNA were performed by strictly following previously described procedures [6].

AFLP analysis

AFLP was performed according to previous research [6]. The adaptors and primers are listed in Supple-

mentary Table S1. Selective PCR products were mixed with 25 μl of formamide dye (98% formamide, 10 mM EDTA, 0.05% w/v bromophenol blue and xylene cyanol), denatured at 95°C for 4 min and separated by electrophoresis on 6% denaturing polyacrylamide (20:1 acrylamide:bisacrylamide, 7.5 M urea, and 1× Tris-borate-EDTA buffer, pH 7.8). The gels were prerun at 100 W for approximately 30 min before 4.5 μl of the mix was loaded; the gels were then run at 65 W for approximately 2 h. The gels were silver-stained using a DNA silver staining system (Promega, catalog number: Q4132). Gel images were taken using a CCD camera.

cDNA-AFLP analysis

The cDNA-AFLP procedure was described in previous research [29] and modified from a published method [32]. The adaptors and primers are listed in Supplementary Table S1. Electrophoresis and staining were performed as AFLP analysis. To ensure that there was no DNA contamination in our RNA samples, a negative control was prepared without reverse transcriptase (RNA samples treated with RNase-free DNase I, starting from the digestion–ligation step). A clear cDNA-AFLP gel with no bands was obtained.

MSAP analysis

MSAP analysis was performed following the procedure described in previous research [29], which was modified from the general procedure [33]. The adaptors and primers are listed in Supplementary Table S1. The electrophoresis and staining were performed as for AFLP analysis above.

Scoring of AFLP, cDNA-AFLP and MSAP bands

For AFLP, cDNA-AFLP and MSAP analyses, one biological replicate and three technical replicates were performed. The technical replicates were run from the same cDNA and DNA sample but from a different digestion-ligation-amplification reaction. Only clear and reproducible bands between the three replicates were used for scoring. Moreover, with AFLP, cDNA-AFLP and MSAP gels, the upper and lower regions of the gels, where resolution was not satisfactory, were not used for band scoring. The scored AFLP, cDNA-AFLP and MSAP bands were transformed into a binary character matrix, using "1" or "0" to indicate the presence or absence of a band at a particular position, respectively.

Cloning and characterization of differentially methylated and expressed fragments

Fragments that showed evidence of alteration in MSAP gels and cDNA-AFLP gels in the autotetraploids were excised, eluted and amplified with the same primer combinations used in the selective amplification. The PCR products were cloned with the pCR2.1-TOPO TA cloning kit (Invitrogen) and sequenced. The sequences obtained were analyzed for similarity to the *Brassica rapa* genome sequence via Phytozome 12 (https://phytozome.jgi.doe.gov/pz/portal.html).

RESULTS

The absence of genetic changes in autotetraploids

To assess genome doubling-induced genetic changes in a quantitative manner, AFLP was performed on DNA from leaves to track genetic changes in the autotetraploids compared to the diploid plants. The band patterns in the autotetraploids were expected to be similar to the diploids, and all cases of deviation from such additivity were scored as genetic changes induced by genome doubling. A total of 2069 AFLP fragments were obtained using 48 pairs of selective primer combinations from leaves. No novel or missing bands were detected between diploid and autotetraploid plants. It should be noted that the AFLP approach would mask any absence of a single gene copy in autotetraploids.

Alterations in gene expression in autotetraploids

To investigate the consequences of gene expression on genome doubling, cDNA-AFLP display was performed on the autotetraploids and their diploid progenitors. Using 48 different selective primer pairs, 1966 and 2033 transcripts were detected in leaves and flowers, respectively (Fig. 1). Of the 1966 transcripts that were detected in leaves, 7 transcripts were silenced in autotetraploids and 3 transcripts were ac-

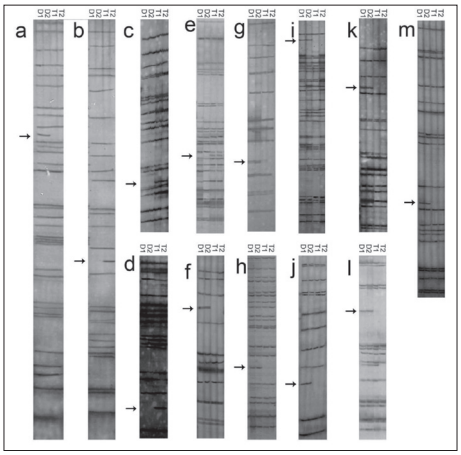

Fig. 1. Lanes showing differential banding in flowers between diploid and autotetraploid *Brassica rapa* detected by cDNA-AFLP analysis. Arrows indicate transcriptional changes as follows **a:** arrow indicates gene silencing in autotetraploid samples; **b-e:** arrows indicate gene activation in autotetreaploid samples; **f-m:** arrows indicate gene silencing in autotetreaploids. D1 – diploid sample 1; D2 – diploid sample 2; T1 – autotetraploid sample 1; T2 – autotetraploid sample 2.

Table 1. Summary of cDNA-AFLP analysis in autotetraploids and their diploid progenitor.

Organ	Total transcripts	Expression changes in autotetraploids		
		Silence (%)	Novel (%)	Total change (%)
Leaves	1966	7 (0.36)	3 (0.15)	10 (0.51)
Flowers	2033	9 (0.44)	4 (0.20)	13 (0.64)
Total	3999	16 (0.40)	7 (0.18)	23 (0.58)

tivated in autotetraploids. In all, 0.51% of the transcripts showed altered expression in leaves (Table 1). Of the 2033 transcripts that were detected in flowers, 9 transcripts were silenced in autotetraploids and 4 transcripts were activated in autotetraploids. In all, 0.64% of the transcripts showed altered expression in flowers (Table 1).

Alterations in rapid DNA methylation in autotetraploids

The MSAP technique was performed by incorporating a pair of isoschizomers, *Hpa*II/*Msp*I, which possess differential sensitivity to cytosine methylation at a CCGG site. The MSAP band patterns were compared between the diploid and the autotetraploid samples to detect cytosine methylation changes at CCGG sites throughout the genome. In the absence of methylation changes, the autotetraploid lines were expected to have the same MSAP patterns as the diploid parent. Any deviation from this expected additivity was considered to be the result of an alteration in methylation pattern related to genome doubling, in which the autotetraploid plants significantly differed from the diploid parent plants. In the autotetraploid and diploid *B. rapa*, 1798 and 1636 clear and reproducible bands were amplified, respectively, from leaves of mature plants and from flowers using 48 pairs of *Eco*RI + *Hpa*II/*Msp*I selective primer combinations (Fig. 2).

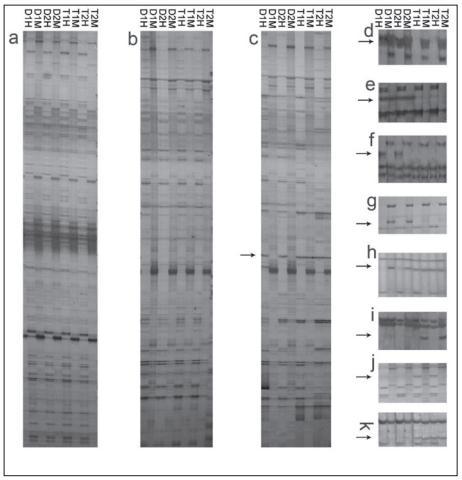

Fig. 2. Examples of MSAP analysis in flowers of diploid and autotetraploid of *Brassica rapa*. Arrow indicates methylation changes. **a, b**: Additive DNA methylation patterns; **c:** arrow indicates C2 MSAP band pattern listed in Table 2; **d:** arrow indicates B1 MSAP band pattern listed in Table 2; **e:** arrow indicates B2 MSAP band pattern listed in Table 2; **f:** arrow indicates C1 MSAP band pattern listed in Table 2; **g:** Arrow indicates D1 MSAP band pattern listed in Table 2; **h:** Arrow indicates D2 MSAP band pattern listed in Table 2; **i:** Arrow indicates E1 MSAP band pattern listed in Table 2; **j:** Arrow indicates E2 MSAP band pattern listed in Table 2; **c:** Arrow indicates E3 MSAP band pattern listed in Table 2. D1H: diploid sample 1 *Eco*RI-*Hpa*II lane; D1M: diploid sample 1 *Eco*RI-*Msp*I lane; D2H: diploid sample 2 *Eco*RI-*Hpa*II lane; D2M: diploid sample 2 *Eco*RI-*Msp*I lane; T1H: autotetraploid sample 1 *Eco*RI-*Hpa*II lane; T1M: autotetraploid sample 1 *Eco*RI-*Msp*I lane; T2H: autotetraploid sample 2 *Eco*RI-*Hpa*II lane; T2M: autotetraploid sample 1 *Eco*RI-*Msp*I lane.

Five major groups were identified according to methylation status in the autotetraploids and the diploids, as shown in Table 2. The first group (group A) contains additive band patterns between the autotetraploids and the diploids, among which 1270 and 1160 nonmethylation bands (A1), 120 and 103 hemi-methylation bands (A2), and 383 and 338 full

Table 2. Frequencies of MSAP band patterns and cytosine methylation alterations in various autotetraploid and diploid organs.

	Group	MSAP band patterns*				Organs		Total	Methylation status**
		Diploid		Autotetraploid					
		*Hpa*II	*Msp*I	*Hpa*II	*Msp*I	Leaves	Flowers		
Methylation additive	A1	+	+	+	+	1270	1160	2430	U
	A2	+	-	+	-	120	103	223	Hemi
	A3	-	+	-	+	383	338	721	Full
Methylation non-additive	B1	+	+	-	+	1	2	3	Hyper
	B2	+	+	-	-	9	12	17	Hyper
	C1	+	-	-	-	2	3	5	Hyper
	C2	+	-	+	+	1	1	2	De
	D1	-	+	-	-	2	5	7	Hyper
	D2	-	+	+	+	3	3	6	De
	E1	-	-	-	+	1	2	3	De
	E2	-	-	+	-	2	3	5	De
	E3	-	-	+	+	4	4	8	De
	Subtotal					25	35	60	
Total						1798 (1.39%)	1636 (2.14%)	3434 (1.75%)	

*+ Band present, - band absent; *Hpa*II: fragments obtained after digestion with *Eco*RI-*Hpa*II; *Msp*I: fragments obtained after digestion with *Eco*RI-*Msp*I

** U: un-methylation; Hemi: hemi-methylation; Full: full methylation; Hyper: hypermethylation; De:demethylation

methylation bands (A3) were detected in leaves and flowers, respectively.

Four additional groups of non-additive band patterns were observed. Ten and fourteen non-methylated bands from diploid leaves and flowers were found to be hypermethylated in the autotetraploids (group B). Three and four hemi-methylated bands from diploid leaves and flowers displayed different methylation status in the autotetraploids (group C). Five and eight fully methylated bands from diploid leaves and flowers showed different methylation status in the autotetraploids (group D). Seven and nine novel methylation bands were detected from the diploid parental "00" band pattern in leaves and flowers, respectively (group E). This type of methylation change could be caused by full methylation of both cytosine residues or full methylation of the external cytosine to another methylation status, rather than by sequence mutation at non-CCGG to CCGG sites. In all, 25 of 1798 (1.39%) CCGG sites displayed altered DNA methylation states in leaves, and 35 of 1636 (2.14%) CCGG sites displayed altered DNA methylation states in flowers.

Methylation changes in autotetraploids were caused by either hypermethylation or demethylation. A band appearing in the *Msp*I lane or *Hpa*II lane indicated possible demethylation alteration and vice versa; a band missing in the *Msp*I lane or *Hpa*II lane indicated possible hypermethylation alteration. It should be noted that if internal CCGG site(s) exist in the MSAP fragments, the methylation states cannot be calculated according to the above rationale [34]. However, most studies have indicated that internal CCGG site(s) in MSAP fragments occur infrequently [29,35]. In this study, the B, C1 and D1 groups indicated hypermethylation changes, and the C2, D2 and E groups indicated demethylation changes. Thus, 14 bands were hypermethylated and 11 bands were demethylated in leaves, and 22 bands were hypermethylated and 13 bands were demethylated in flowers. It is interesting that the diploid genome methylation CCGG sites (10 and 14 in leaves and flowers, respectively) and diploid genome non-methylation CCGG sites (8 and 12 in leaves and flowers, respectively) had equal ability to undergo methylation status shifts during the process of genome doubling.

Molecular characterization of fragments showing changes in DNA methylation and gene expression

Transcript-derived fragments (TDFs) and MSAP-isolated fragments (MIFs) showing alterations in the flowers of the autotetraploids were cloned and sequenced. The homologies of the MIFs and TDFs to

Table 3. Molecular characterization of fragments showing different expression in autotetraploids according to cDNA-AFLP analysis

Clone ID	Expression pattern*		*Brassica rapa* genome position	Sequence similarity	Gene ID	E-value
TDF1	+	-	A01:9398979..9399170	Unknown protein	Brara.A01680	7E-92
TDF2	+	-	A01:7946545..7946814	MAC/Perforin domain	Brara.A01437	5.829E-48
TDF3	+	-	A09:41306531..41306737	Dip2/Utp12 Family (Utp12);	Brara.I04927	3.625E-36
TDF4	+	-	A09:25416622..25418926	RNA recognition motif	Brara.I02677	4.8E-32
TDF5	+	-	A04:8387546..8387672	4.1.1.39 – Ribulose - bisphosphate carboxylase	Brara.D00895	2.091E-57
TDF6	+	-	A05:3372571..3372625	26S proteasome regulatory complex, ATPase RPT4	Brara.E00605	3.991E-18
TDF7	+	-	A02:12599412..12599477	Osativa_PAC2_0_323_peptide	Orysa|LOC_Os03g29360.1	7.651E-27
TDF8	+	-	A06:19246913..1924701	RING/FYVE/PHD ZINC FINGER SUPERFAMILY PROTEIN	Brara.F02259	1.004E-47
TDT9	+	-	A04:25417714..25417802	MuDR family transposase	-	4.776E-32
TDF10	-	+	A05:14408003..14410191	SWIM zinc finger (SWIM) // MULE transposase domain	Brara.E00513	1.1E-36
TDF11	-	+	A08:9090224..9090343	Unknown protein	Brara.H00700	8.615E-56
TDF12	-	+	A10:10953706..10953906	PTHR11977//PTHR11977:SF47 - VILLIN	Brara.J01154	1.341E-33
TDF13	-	+	A03:25417714..25417802	C2H2-type zinc finger	Brara.C00672	4.776E-32

* Band pattern in cDNA-AFLP gels as follows: diploid lane, autotetraploid lane; + band present, - band absent

Table 4. Molecular characterization of fragments showing methylation changes in autotetraploids relative to their diploid progenitors.

Clone ID	MSAP pattern[a]				Group	*Brassica rapa* genome position	Sequence similarity	Sequence ID[b]	E-value
MIF1	+	+	-	+	B1	A01:5955031..5955193	Subtilase family protein AT4G20430	Brara.A01115	4.77589E-32
MIF2	+	+	-	+	B1	A02:9166023..9166328	TRANSCRIPTION ELONGATION FACTOR SPT6	Brara.B01624	2.73E-156
MIF3	+	+	-	-	B2	A01:2814255..2814337	_	_	2.83E-28
MIF4	+	+	-	-	B2	A02:6984173..6984298	Membrane-bound O-acyl transferase family	Brara.B01324	5.07E-59
MIF5	+	+	-	-	B2	A08:13021727..13021908	_	_	3.1E-89
MIF6	+	+	-	-	B2	A10:9949588..9949695	Cytidylyltransferase-like	Brara.J01021	2.45E-49
MIF7	+	+	-	-	B2	A07:17479904..17480097	_	_	1E-95
MIF8	+	+	-	-	B2	A03:24971900..2497286	Unknown protein	Brara.C04670	9.6E-92
MIF9	+	+	-	-	B2	A09:9405270..9405326	_	_	2.76219E-16
MIF10	+	+	-	-	B2	Scaffold64657:263..516	_	_	1.3695E-114
MIF11	+	+	-	-	B2	Scaffold24905:906..1044	_	_	8.19743E-32
MIF12	+	+	-	-	B2	A09:37949847..37950746	CYTOCHROME C OXIDASE SUBUNIT 5C-2-RELATED	Brara.I04312	1.1E-40
MIF13	+	+	-	-	B2	A09:41149745..41149839	SNARE protein TLG1/Syntaxin 6	Brara.I04890	6.2E-37
MIF14	+	+	-	-	B2	A02:4584974..4586401	Unknown protein	Brara.B00959	1E-84
MIF15	+	+	-	-	B2	A10:8491553..8491739	_	_	6.25E-92
MIF16	+	-	-	-	C1	Scaffold46535:86938..87021	_	_	1.0055E-18
MIF17	+	-	-	-	C1	A04:10818655..10818736	_	_	1.981E-32
MIF18	+	-	-	-	C1	A05:12404953..12405252	_	_	1.8304E-38
MIF19	+	-	+	+	C2	A09:14620344..14620851	_	_	8.5762E-113
MIF20	-	+	-	-	D1	A03:13346017..13346231	Aquaporin (major intrinsic protein family)	Brara.C02640	4.64E-107
MIF21	-	+	-	-	D1	Scaffold63484:27675..27813	_	_	1.4E-48
MIF22	-	+	-	-	D1	Scaffold56651:284..517	_	_	1.2824E-114

Table 4. continued

MIF23	-	+	-	-	D1	A07:4490127..4490688	_	_	0
MIF24	-	+	-	-	D1	A07:17847803..17847962	Prolyl-tRNA synthetase	Cre09.g406200.t1.2_GX3M	5.31757E-54
MIF25	-	+	+	+	D2	A04:9080385..9080525	MuDR family transposase AT1G64255.1	Arath|AT1G64255.1_GX7P	4.00815E-42
MIF26	-	+	+	+	D2	A10:5217533..5217749	_	_	2.7719E-78
MIF27	-	+	+	+	D2	A02:9080385..9080525	_	_	4.01E-42
MIF28	-	-	-	+	E1	A06:12667306..12667407	_	_	4.11E-46
MIF29	-	-	-	+	E1	A06:14485430..14485515	_	_	3.2E-26
MIF30	-	-	+	-	E2	A04:10179600..10179740	MuDR family transposase AT1G64255.1	Arath|AT1G64255.1_GX7P	2.08E-39
MIF31	-	-	+	-	E2	A05:13780048..13780149	_	_	4.11E-46
MIF32	-	-	+	-	E2	A09:9080385..9080525	_	_	4.01E-42
MIF33	-	-	+	+	E3	A08:10648108..10648657	_	_	4.53838E-68
MIF34	-	-	+	+	E3	A07:6659442..6659553	Zinc knuckle (CCHC-type) family protein	Arath|AT5G32613.1	3.87E-47
MIF35	-	-	+	+	E3	A06:9080385..9080525	Chloroplast Ycf2;ATPase	Arath|ATCG01280.1_GX11P	4.00815E-42

a. banding pattern in MSAP gels as follows (+, band present; -, band absent): diploid *Eco*RI-*Hpa*II lane, diploid *Eco*RI-*Msp*I lane; autotetraploid *Eco*RI-*Hpa*II lane, autotetraploid *Eco*RI-*Msp*I lane
b. _ indicates no annotated function for the isolated fragments

the *Brassica rapa* genome sequence were detected via Phytozome 12. Among the 13 TDFs, two transposase-like proteins were detected (TDF9 and TDF10) (Table 3). The molecular characterizations of the MIFs are listed in Table 4. Of the 35 sequenced MIFs, 14 sequences showed similarity to known annotated genes in *Brassica rapa* (Table 4). MIF10, MIF25 and MIF30 had similarity to known retroelement sequences.

DISCUSSION

This study provides a comprehensive portrayal of genome doubling effects in autotetraploid *B. rapa*. No genetic alteration was detected in autotetraploid *B. rapa* using an AFLP approach. Approximately 0.58% and 1.7% of fragments showed changes in expression and changes in DNA methylation, respectively. This may underestimate genetic alterations in autotetraploids; because genome doubling increases the level of genomic redundancy and AFLP is a dominant marker, some recessive alterations may be masked by the dominant alleles. AFLP-derived cDNA-AFLP and MSAP approaches may underestimate gene expression and DNA methylation alteration in autotetraploids. Only approximately 0.02% of sites in the genome, 0.06% of cytosine sites in the genome and 4.2% of the total transcript fragments were analyzed, compared to whole genome sequencing, and thus some variation underlying genome doubling is missed. However, MSAP and AFLP can still provide insight into genetic and epigenetic changes in plant biology [36,37].

Gene expression alterations in autopolyploids

About 0.58% of gene expression changes occurred in autotetraploid *B. rapa*. This result is consistent with a previous study in amphihaploid and amphidiploid *B. napus* [29], trigenomic allohexaploid *Brassica carinata* × *Brassica rapa* [6] and autopolyploid *Pasalum notatum* [22], which confirmed that genome doubling per se could induce small but distinct gene expression alterations during polyploid formation.

The proportion of alterations in genome doubling-induced gene expression varied among autopolyploids. Approximately 1.2% to 7.2% of gene expression changes were detected in *Isatis indigotica* [21], *Paulownia fortune* [24], *Eragrostis curvula* [23] and rice [27]. In maize [20], potato (*Solanum phureja*) [25] and *Arabi-*

dopsis [19], almost no ploidy-dependent changes in gene regulation were detected. A study in an autopolyploid sunflower series [26] reported that gene expression varied among different autopolyploid individuals.

Cytosine methylation changes in autopolyploids

MSAP alterations, either from genetic variation or from methylation status, shift the marker locus. AFLPs are typically interpreted as genetic variation. In this study, the MSAP deviation from Mendelian expectation is much higher than that of the AFLP markers. This indicated that most DNA methylation changes in the autotetraploids were caused by a methylation status shift in the pre-existing CCGG sites. Compared to genetic and gene expression changes detected in this study, DNA methylation changes are a major consequence in autotetraploid *B. rapa*. A similar phenomenon was also detected in amphihaploid and amphidiploid *B. napus* [29]. Studies in trigenomic allohexaploid *Brassica carinata* × *Brassica rapa* indicated that genomes *per se* could induce half of the methylation changes in the conversion from triploid to hexaploid [6]. The DNA methylation-sensitive enzymes used in the MSAP assay are biased towards DNA methylation at CGG and CCG sites, while DNA methylation changes at CG, CHG and CHH sites are not detected by the MSAP assay.

Studies in *Cymbopogon* [17] and *Eragrostis curvula* [15] autopolyploids reported that autopolyploidy caused increased cytosine methylation. Variation in DNA methylation during the tetraploid–diploid conversion reverted during the diploid–tetraploid conversion in *Eragrostis curvula* [15]. A cross between dandelion ploidy levels triggered de novo methylation variation [14]. A study in *Phlox drummondii* provided evidence for an increase in DNA methylation changes through subsequent generations [9]. Stochastic changes among individuals were observed in *Solanum* [16]. However, no methylation changes were detected in watermelon with differing ploidy [18]. The above data supported the hypothesis that variation in cytosine methylation might be a major consequence during genome doubling in polyploids.

Epigenetic landscape changes can affect gene expression. The role of epigenetic changes during polyploid formation and diploidization is still not completely clear. Furthermore, the DNA methylation status of newly formed species appears to change consistently through several early generations [9]. All these studies focused on signal cytosine methylation polymorphisms (SMP), which may not be linked to genotype per se. Future work should analyze differentially methylated regions (DMR) during genome doubling [38].

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Supplementary Material:

Table S1. AFLP, MSAP and cDNA-AFLP adapters, primers and selective primer combinations used in this study: http://serbiosoc.org.rs/sup/TableS1.pdf