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Genomic evolution of *Brassica* allopolyploids revealed by ISSR marker

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Abstract

Polyploidization has been viewed as a highly dynamic process and a major force in the evolution of higher plants, including many important crops. To better understand the genomic evolution of *Brassica* polyploids, we used the *Brassica* triangle, including three allopolyploids and three diploids, to study genomic evolution after the formation of polyploids. Based on the inter-simple sequence repeat (ISSR) analysis, the different degree of A, B or C genomic modifications were observed in the three *Brassica* allopolyploids. In B-contained allopolyploids, B genome always altered less than the other genome (A or C), showing that B genome was relatively conserved in the evolution of *Brassica* allopolyploids. ISSR data supported that a higher degree of ancestral genomic divergence gave rise to a greater frequency of genomic change of polyploids. The possible mechanisms for the genomic changes and the reason for the relatively conserved B genome were discussed.

Introduction

Polyploidy is an important process in the evolution of higher plants, and probably as many as 70% of angiosperms are polyploid (Masterson 1994). There are many important crops of them, such as bread wheat, potato, tobacco, cotton, and oil seed rape. As a consequence, there has been a long history of interest in various aspects of polyploidy in plants, including classification of the various types of polyploids, mode and frequency of formation, potential evolutionary significance, and the attributes in ecological adaptation (Soltis and Soltis 1999). The present focus is on gene and genomic evolution in polyploids. Molecular data are providing new insight into the evolution of polyploids. It has been suggested that the formation of polyploid genomes may be accompanied by rapid genomic change and these genomic changes facilitated the establishment of the newly formed polyploids as successful species (Soltis and Soltis 1995; Liu et al. 1998a, b; Liu and Wendel 2002). But little is known about the genetic and functional consequences of uniting two divergent genetic systems into a common nucleus in only one of the two parental cytoplasms (Wendel 2000).

Brassica is an agriculturally important genus containing species with highly diverse morphology and wide ranging utility. The *Brassica* triangle (Figure 1) is a useful system for addressing questions of genomic evolution in polyploids. It is composed of three diploid species, *Brassica nigra*, *B. rapa*, and *B. oleracea*, and three allotetraploid (amphidiploid) species, *B. carinata*, *B. juncea*, and *B. napus*, which are produced by the crosses among the three diploid species and the following

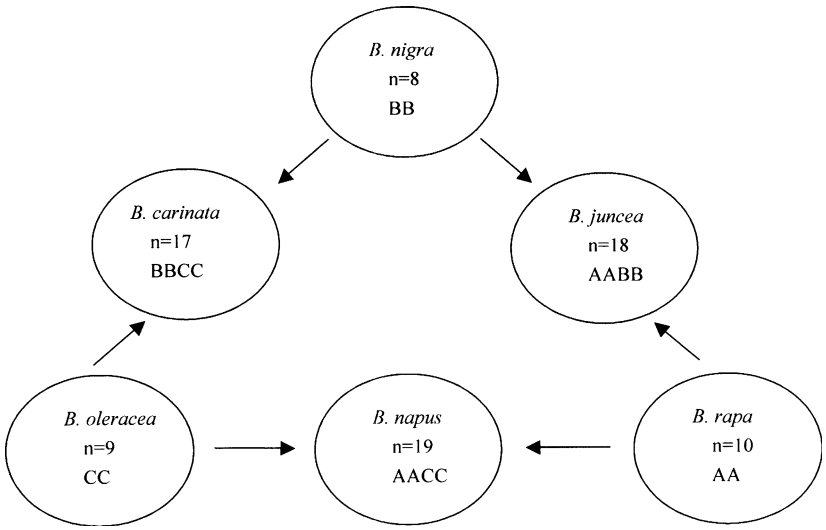

Figure 1. The *Brassica* triangle. The diploid species are in the corners of the triangle (n = haploid chromosome number). The letter A, B, and C are the genome designations (UN. 1935).

process of chromosome doubling. These species have been developed into a diverse range of important crops, including a number of oil seed and vegetable crops. Using synthetic polyploids, Song et al. (1995) reported that rapid genomic change, as assayed by loss or gain of parental restriction fragments, occurred at high frequencies in the early generations after the formation of the two polyploids, *B. napus* (genome AACC) and *B. juncea* (genome AABB). Furthermore, based on linkage map of *B. juncea* constructed by Cheung et al. (1997), they stated that complex duplications and subsequent rearrangements occurred after allopolyploidy.

In spite of these observations, mapping studies have demonstrated that the nuclear genome of *B. napus* has remained essentially unaltered since its formation (Bohuon et al. 1996). Moreover, Axelsson et al. (2000) could find no evidence of rapid genomic change in synthetic and natural *B. juncea* polyploids using restriction fragment length polymorphism (RFLP) analysis. Study on the 18S–26S rDNA, a kind of important repetitive sequences, also showed that the three allotetraploids in the *Brassica* triangle maintained parental rDNA repeats (Waters and Schaal 1996). It seems that recent investigation on the evolution of the two allotetraploids, *B. napus* and *B. juncea*, has yielded contradictory results. On the other hand, there is little evidence about the genomic evolution of the other allotetraploid, *B. carinata*. Therefore, more data on the genome structure and genomic evolution of *Brassica* amphidiploids are needed to resolve these apparently conflicting results.

A few molecular markers have been used to study genetic relationship or genomic evolution in a range of different plant species. Zietjiewicz et al. (1994) developed a new molecular marker, inter-simple sequence repeat (ISSR). This approach employs oligonucleotides, based on SSR anchored at either the 5′ or 3′ end with 2–4 purine or pyrimidine residues, as primers to amplify mainly the inter-SSR regions. SSRs or micro satellites are short tandem repeats (STRs) or variable number of tandem repeats (VNTRs) of 1–4 bases of DNA ubiquitously presented in eukaryote genomes (Tautz and Renz 1984). They dispersed throughout the genome and varied in the number of repeat units. Becacuse of the characters of SSRs, ISSR marker could give information mainly about unrepeated sequences and have the advantage of genome coverage. The utility of PCR-ISSR as phylogenetic markers for investigating evolutionary relationships among plants has been clearly established (Charpter et al. 1996; Wolfe and Liston 1998; Panda et al. 2002; Ghariani et al. 2003). Especially, the hypersensitive nuclear ISSR markers have been proved to be useful in testing hypotheses of speciation, introgression and systematic relations (Wolfe and Morgan-Richards

1998). Thus, in the present study, we used ISSR-fingerprinting data to examine the genetic relationship among the diploids in the *Brassica* triangle firstly, and secondly to make cluster analysis of all of the polyploids with their ancestral diploids in the *Brassica* triangle. In addition, we are interested in how the two divergent genomes adjusted themselves after they have merged into a common nucleus for a long evolutionary time. If expected genomic additivity has not been observed, which (or both) genome(s) will be modified? So, we also examine the different evolutionary pattern of three *Brassica* allopolyploid genomes and the evolutionary character of B genome in two B-contained allopolyploid based on ISSR data.

Materials and methods

Plant materials

The materials used in this study, including their accession numbers, ploidy level, genome constitution and sources are listed in Table 1. The 33 accessions represent the six species in the *Brassica* triangle. The nomenclature follows Gladis and Hammer (1992).

DNA isolation

Total DNA was extracted using a modified protocol of Doyle and Doyle (1987). The young leaf

Table 1. List of materials used in this study*.

Acc.No.	Species	Cultivar	Ploidy level	Genome constitution	Source
A01	*B. rapa* L. var. *chinensis* (L.) Kitam.	Shanghaiqing	2x	AA	IVW
A02	*B. rapa* L. var. *chinensis* (L.) Kitam.	Aikangqing	2x	AA	IVW
A03	*B. rapa* L. var. *glabra* E. Regel	Shandongqihao	2x	AA	IVW
A04	*B. rapa* L. var. *glabra* E. Regel	Jiaobaiwuhao	2x	AA	IVW
A05	*B. rapa* L. var. *rosularis* (Tsen et Lee) Hanelt	Wutacai	2x	AA	IVW
A06	*B. rapa* L. var. *purpuraria* (L.H. Bailey) Kitam.	Hongcaitai	2x	AA	IVW
A07	*B. rapa* L. var. *purpuraria* (L.H. Bailey) Kitam.	Shiyuehongcaitai	2x	AA	IVW
A08	*B. rapa* L. var. *oleifera* DC.	783-34918	2x	AA	IOC
B01	*B. nigra* (L.) Koch	PI169069	2x	BB	IOC
B02	*B. nigra* (L.) Koch	P1592737	2x	BB	IOC
B03	*B. nigra* (L.) Koch	BI80	2x	BB	UC
B04	*B. nigra* (L.) Koch		2x	BB	US
CO1	*B. oleracea* L. var. *botrytis* L.	Ruixueteda	2x	CC	IVW
CO2	*B. oleracea* L. var. *botrytis* L.	Jingyan45	2x	CC	IVW
CO3	*B. oleracea* L. var. *viridis* L. f. tricolor Hort.	Huangplyuyiganlan	2x	CC	WU
CO4	*B. oleracea* L. var. *viridis* L. f. tricolor Hort.	Hongpiyuyiganlan	2x	CC	WU
CO5	*B. oleracea* L. var. *capitata* L.	Jing gyihao	2x	CC	IVW
CO6	*B. oleracea* L. var. *capitata* L.	Zhongganshibahao	2x	CC	IVW
CO7	*B. oleracea* L. var. *capitata* L.	Zhongganbahao	2x	CC	IVW
CO8	*B. oleracea* L. var. *capitata* L.	Xiaguangganlan	2x	CC	IVW
CO9	*B. oleracea* L. var. *capitata* L.	Xuganyihao	2x	CC	IVW
ABI	*B. juncea* (L.) Czern. var. *multiceps* Tsen et Lee	Xuelihong	4x	AABB	IVW
AB2	*B. juncea* (L.) Czern. var. *tsatsai* Mao	Sichuanyihao	4x	AABB	IVW
AB3	*B. juncea* (L.) Czern. var. *capitata* Tsen et Lee	Baoxinjie	4x	AABB	IVW
AB4	*B. juncea* (L.) Czern. var. *megarrhiza* Tsen et Lee	Erdaomeijie	4x	AABB	IVW
AB5	*B. juncea* (L.) Czern. var. *juncea*	Guidinggaoyou	4x	AABB	IOC
AB6	*B. juncea* (L.) Czern. var. *juncea*	Tunliuhuangjie	4x	AABB	IOC
AC1	*B. napus* L.	3-KARAT 5189	4x	AACC	lOC
AC2	*B. napus* L.	Idol 5169	4x	AACC	lOC
BCI	*B. carinata* A. Braun	77-1226	4x	BBCC	lOC
BC2	*B. carinata* A. Braun	B203	4x	BBCC	lOC
BC3	*B. carinata* A. Braun		4x	BBCC	HAU
BC4	*B. carinata* A. Braun		4x	BBCC	US

*HZAU, Huazhong Agricultural University, China; lOC, Institute of Oil Crops, Chinese Academy of Agricultural Sciences, China; US, University of Silesian, Poland; UC, University of California, USA; IVW, Institute of Vegetables, Wuhan, China; WU, Wuhan University, China.

tissue was ground to a fine powder in a mortar and then transferred to a 2 mL Eppendorf tube filled with 950 μL of preheated 2 × CTAB extraction buffer containing 0.3% mercaptoethanol. After being incubated at 65 °C for 60 min, the mixture was extracted twice with 950 μL chloroform: isoamylalcohol (24:1, v/v). Samples were shaken gently for 10 min and centrifuged at 10,000 r min^{-1} for 10 min at 4 °C. The supernatant was reserved and mixed with 2/3 vol. ice-cold isopropanol. DNA was then recovered as a pellet by centrifugation at 11,000 r min^{-1} for 6 min at 4 °C, washed with 300 μL of ethanol, dried in the air, and dissolved in 200 μL of 1 × TE buffer. DNA quality and quantity were determined in 0.8% agarose gels.

ISSR fingerprinting

Twenty selected ISSR primers from primer set No. 9 (Biotechnology Laboratory, University of British Columbia, UBC) were used for PCR amplification (Table 2). These primers were mostly 17-mers to 18-mers. The amplification was carried out in a 25 μL reaction volume containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.001% gelatin, 1.3 mM $MgCl_2$, 500 nM primer, 0.3 mM each of dCTP, dGTP, dATP, dTTP, 10 ng of genomic DNA, 1 U of *Taq*-DNA polymerase (Promega). The reaction mix (25 μL) was overlaid with 50 μL mineral oil (Sigma), and PCR was performed on a MJ PTC-100 Thermocycler. Initial denaturation was for 5 min at 94 °C; followed by 40 cycles of 45 s at 94 °C, 1 min at 45 °C, and 1 min at 72 °C; with a final 10 min extension at 72 °C. The amplification products were size-separated by standard horizontal electrophoresis in 2% agarose gels run with 1 × TAE buffer (40 mM Tris–HCl, 40 mM acetic acid and 1 mM EDTA) at 120 V for 3 h. The gels were viewed and photographed by Bio Imaging Systems (Syngene, GeneGenius) after staining of the agarose gels with ethidium bromide. PCR amplifications were repeated twice with each of the primers analyzed in order to test the reproducibility of the DNA profiles. The robust bands were found to be repeatable and easily to be scored, and only these bands were considered in this study.

Table 2. The primers and their sequences*.

Primer no. (UBC)	Sequence (5′–3′)
810	$(GA)_8T$
813	$(CT)_8T$
814	$(CT)_8A$
816	$(CA)_8T$
821	$(GT)_8T$
822	$(TC)_8A$
842	$(GA)_8YG$
843	$(CT)_8RA$
846	$(CA)_8RT$
849	$(GT)_8YA$
850	$(GT)_8YC$
851	$(GT)_8YG$
853	$(TC)_8RT$
857	$(AC)_8YG$
858	$(TG)_8RT$
859	$(TG)_8RC$
860	$(TG)_8RA$
884	HBH $(AG)_7$
890	VHV $(GT)_7$
898	$(CA)_6RY$

*B = C, G or T; R = A or G; Y = C or T; H = A, C or T; V = A, C or G.

Date analysis

ISSR marker bands were scored as present (1) or absent (0), and the data obtained were used in a rectangular matrix. Such information is available with the corresponding author. The data matrix was then used to generate genetic distances, using program RAPDistance 1.04 (Armstrong et al. 1994). Cluster analysis was carried out based on genetic distance, using UPGMA (unweighted pair-group method using arithmetic averages) and the NEIGHBOR program of the software package PHILIP 3.6a2.1 (Felsenstein 2001).

Results

Primer selection

Initially, forty ISSR primers were tested for PCR amplification, and twenty of them produced good amplification products and were chosen for ISSR fingerprinting. The remaining primers either did not produce amplification products or the profiles were not distinguishable. The twenty ISSR primers produced a total of 286 bands with an average frequency of 14.3 bands per primer. The number

of the band produced by each primer ranged from 9 to 19. Of the total 286 bands produced, 275 (96.2%) were polymorphic. Figure 2 showed the amplification profiles of primer UBC 851.

Genetic distance

The genetic distance matrix for the 33 accessions was obtained based on the ISSR data (this matrix is available from the authors). For the diploid species, the average genetic distance between *B. rapa* (genome AA) and *B. oleracea* (genome CC), *B. rapa* and *B. nigra* (genome BB), *B. oleracea* and *B. nigra* were 0.499, 0.528 and 0.615, respectively, showing the divergences between B genome and A or C genome were higher than that between A and C genomes. As to the polyploid *B. juncea* (genome AABB) and one of their ancestral diploids, *B. rapa* or *B. nigra*, the average genetic distances were 0.481 or 0.474, respectively. For *B. carinata* (genome BBCC) and *B. nigra* or *B. oleracea*, the average genetic distances were 0.422 or 0.427, respectively. Finally, the average genetic distances between *B. napus* (genome AACC) and *B. rapa* or *B. oleracea* were 0.392 or 0.336, respectively. The three series of data indicated B-contained polyploids, *B. juncea* and *B. carinata*, diversified much more with their ancestral diploids than *B. napus* since their formation.

Cluster analysis

Figure 3 was the dendrogram for the polyploids and their ancestral diploids based on genetic distance. Cluster analysis generated two major groups in this dendrogram. The B-contained species (AABB, BBCC, and BB) were clustered together and the other group was composed of *B. napus* (AACC) and its ancestral diploids, *B. rapa* (AA) and *B. oleracea* (CC). All without exception, the species with same genome type clustered together.

Discussion

The evolutionary patterns of Brassica allopolyploid genomes

The results of the present work showed that the degrees of A, B or C genomic changes are different in the three natural allopolyploids. In Figure 3, *B. napus* (AACC) initially clustered with one of its ancestral species *B. oleracea* (CC), and this indicated that A genome in this allopolyploid might modified more than C genome. At the same time, *B. juncea* (AABB) and *B. carinata* (BBCC) formed a clade with one of their ancestral species *B. nigra* (BB) in Figure 3. This suggested that B genome in these two allopolyploids has undergone little modification while the other genome (A or C) has undergone substantial modification. However, Axelsson et al. (2000) could find no evidence of rapid genomic change in synthetic and natural *B. juncea* polyploids (AABB) using RFLP linkage analysis. At the same time, they could not rule the possibility that such changes occurred. Their results strongly supported that homoologous recombination, which has been suggested by Song et al. (1995) to be the main reason for the changes they observed, was minimal in the *Brassica* polyploids. At present, we do not know what are the exact mechanisms underlying the genomic changes. But we should remember that not only homologous recombination but also alternative processes, such as gene conversion or other homologous

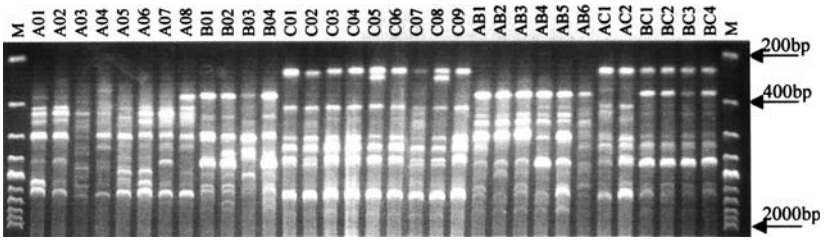

Figure 2. Inter-simple sequence repeat (ISSR) banding profile obtained on 2% agarose gel for the 33 *Brassica* accessions with the UBC primer 851. The accession numbers are listed in Table 1. *M* = molecular size marker (200 bp ladder, SABC).

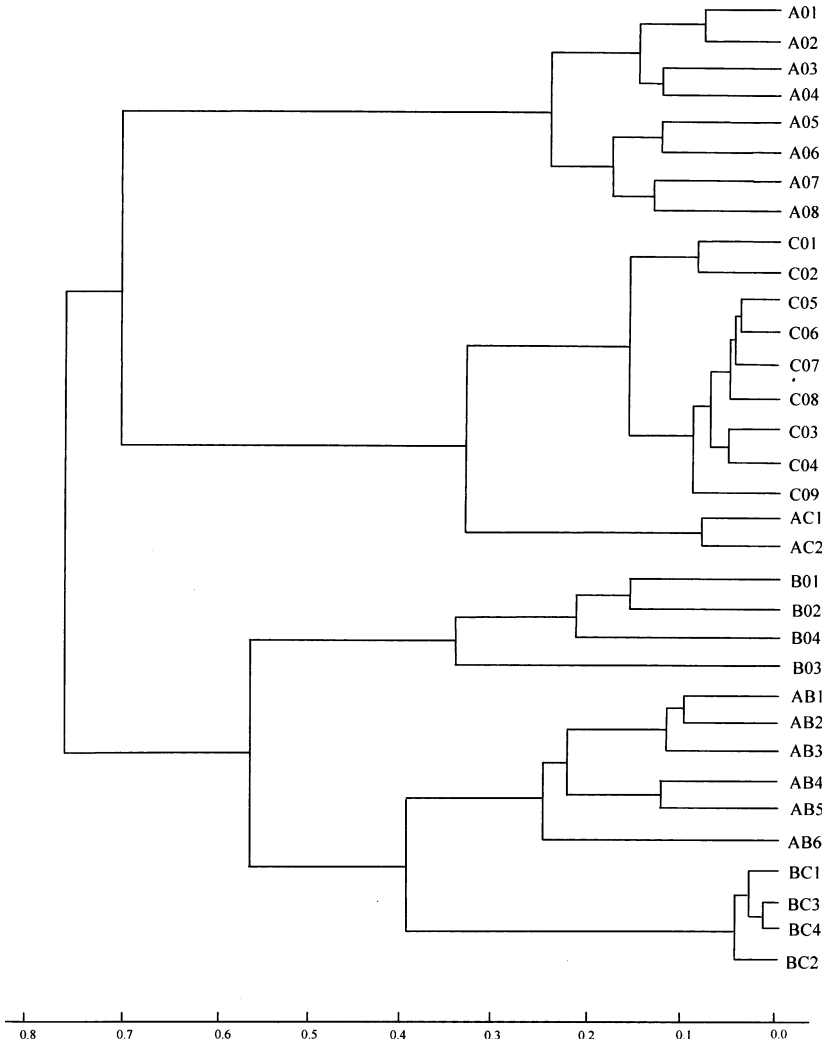

Figure 3. UPGMA dendrogram based on Nei's genetic distance for 33 diploids or polyploids in the *Brassica* triangle. The number for each accession corresponds to the listed number in Table 1. Genetic distance is given on scale below dendrogram.

genetic interaction, mutations, the selection pressure from cytoplasm, transposable element activity and a suite of more mysterious mechanisms, could cause the changes of genome after the formation of polyploid (Liu and Wendel 2002). Comai (2000) recently speculated that mismatch-repair systems in plants might be compromised when divergent genomes are brought together. He suggested that high levels of genomic mismatch might titrate available pools of mismatch-repair enzymes, and thus nonhomologous interactions would go uncorrected and be revealed as genomic instability. Shaked et al. (2001) suggested sequence elimination, as one type of genomic changes, could be caused by crossing over between direct repeats that flank the eliminated sequence and the substantial loss of the excised circle. In view of SSRs are short tandem repeats, sequence elimination might be a reasonable explanation for genomic changes observed here. Further investigations needed to determine the evolutionary details of the mechanism of genomic changes after the formation of allopolyploids.

Genomic evolution of allopolyploids by investigating on the whole genome level may be different from that on the fragment level. Because ISSR marker has the advantage of genome coverage and presumably sampled a large number of gene-rich

regions, the genomic changes here should be in the range of the whole genome for each allopolyploid. This indicated our study was in the whole genome level, which seemed to be contrary to the relative studies on the gene level. Ribosomal DNA is an important type of repeats in plant genome. Waters and Schaal (1996) reported that 18S–26S rDNA repeats in *Brassica* allopolyploids have not undergone concerted evolutionary changes after the formation of allopolyploids. May be the evolution mode of one type of repeats was different from that of the whole genome. The similar phenomena have been reported in *Aegilops* (Wang et al. 2000; Cai et al. 2001). These indications provided insight into the complex nature of genomic evolution of allopolyploids.

The above analysis is sometimes based on comparing our results with those from synthetic allopolyploids, which stood for the earlier stages of allopolyploid formation. One may argue for the different mode of genomic changes between synthetic and natural allopolyploids. The study carried out by Ozkan et al. (2001) included several wheat polyploids that arose spontaneously (without colchicines treatment) after hybridization, and no significant differences were observed in the pattern of sequence elimination in these plants vs. the synthetic polyploids. Also, the pattern of elimination in synthetic allohexaploids was similar to that of the natural hexaploid wheat. That indicated genomic changes took place in the earlier generations of wheat polyploids, which also was observed in *Brassica* (Song et al. 1995). The present work was undertaken on the natural *Brassica* allopolyploids, which have evolved for a few thousand years (Prakash and Hinata 1980). We observed these natural *Brassica* allopolyploids should have undergone genomic changes. So, by comparing our results with those from synthetic allopolyploids, it appeared that genomic changes might take place in the earlier generations after the allopolyploid formation and then these new species had to stabilize as soon as possible in order to adapt to the environment.

Genome B was relatively conserved in the genomic evolution of Brassica allopolyploids

It is very interesting that we found that genome B always altered less than another genome that had been put together with genome B to a common nucleus. The average genetic distances (0.481 and 0.474) indicated that *B. juncea* (AABB) was more divergent from *B. rapa* (AA) than the other ancestral diploid *B. nigra* (BB). Similarly, the average genetic distances (0.427 and 0.422) showed that *B. carinata* (BBCC) was more divergent from *B. oleracea* (CC) than *B. nigra* (BB). Besides, Figure 3 showed that *B. napus* (AACC) and its ancestral diploids, *B. rapa* (AA) and *B. oleracea* (CC), clustered together. The result indicated that the genome of *B. napus* was similar to that of its two ancestral diploids. In another group, *B. nigra* (BB), *B. juncea* (AABB) and *B. carinata* (BBCC) clustered together, indicating that the genomes of the two polyploids, *B. juncea* and *B. carinata*, were both similar to their common ancestral diploid *B. nigra*. In other words, genome B altered less than genome A or C after the formation of the two B-contained polyploids.

This phenomenon is independent of genome dosage. According to Arumuganathan and Earle (1991), the genome size was 507–516 Mbp (million base pairs) for *B. rapa* (CC), 468 Mbp for *B. nigra* (BB) and 599–662 Mbp for *B. oleracea* (CC), respectively. Obviously, the genome size of *B. nigra* is not larger than *B. oleracea* or *B. rapa*.

Probably, there were some evolutionary mechanisms and significance underlying this phenomenon. Some studies have reported that the genome B of the three B-contained species contains interesting disease resistance genes against, for example *phoma lingam*, which is absent in *B. napus* and results in significant loss in its yield (Plieske et al. 1998; Dixelius and Wahlberg 1999). Integrating disease resistance genes from B-contained species to *B. napus* is under way in many projects at present. In a particular way, B-genome was relatively conserved presumably due to selection pressure in view of genome B containing the important disease resistance genes. This means the organization of B-genome may play an important role in the genomic evolution of *Brassica* allopolyploids.

The cytoplasmic effect was not observed here. In the reciprocal synthetic *B. rapa* × *B. nigra* allopolyploids (AABB), Song et al. (1995) found the disappeared RFLP bands in *B. juncea* were prone to be from paternal parent genome. Restriction pattern analysis of chloroplast DNA (Palmer et al. 1983) has provided information on the maternal

contributors of various amphidiploids, specifically that *B. juncea* (AABB) has the cytoplasm of *B. rapa* (AA) and *B. carinata* (BBCC) has the cytoplasm of *B. nigra* (BB) whereas the cytoplasm of *B. napus* is of a more complex origin. In our results (Figure 3), the B-contained allopolyploids (AABB and BBCC) only clustered with *B. nigra* (BB). Therefore, the ISSR analysis indicated that genomic changes did not always take place in the paternal parent genome. On the other hand, we noted that Song et al. (1995) used 89 randomly nuclear DNA clones (19 anonymous clones, 63 cDNA clones, and seven nuclear genes of known function). So, it is difficult to say those clones could provide enough information with genomic coverage. From the above, we could find that no direct evidence showed the cytoplasm was the most important effect factor that determined the mode of evolution of *Brassica* allopolyploids. May be there were other factors, for example, the constitution of genome as discussed above, also played important roles in the process.

The divergences between ancestral genomes affect the degree of genomic changes of Brassica allopolyploids

Song et al. (1995) suggested that a higher degree of sequence divergence may give rise to a greater frequency of genomic change, but they only investigated the two synthesized polyploids, *B. juncea* and *B. napus*, with their parental diploids. At present study, ISSR marker was used to analyze three natural allopolyploids, including *B. carinata*, with their ancestral diploids.

From genetic distance matrix, the average genetic distances among the diploids were 0.499 (A and C), 0.528 (A and B) and 0.615 (B and C), respectively. Furthermore, in Figure 3, the accessions of *B. rapa* (AA) *B. oleracea* (CC) and the accessions of *B. nigra* (BB) belonged to two different groups, respectively. This result agreed with the earlier studies (Quiros et al. 1991; Truco et al. 1996). These authors suggested that the current diploid cultivars were evolved from two lineages, the *B. rapa*/*B. oleracea* lineage and the *B. nigra* lineage.

We also observed that the B-contained allopolyploids, AABB or BBCC, modified more than AACC after their formations. The average genetic distances between B-contained allopolyploids and their ancestral diploids (0.481, 0.474, 0.422 and 0.427) are all far than that between *B. napus* (AACC) and its ancestral diploids (0.392 and 0.336). Moreover, the B-contained species (BB, AABB and BBCC) clustered together and *B. napus* clustered with its two ancestral diploids in the dendrogram (Figure 3). Connecting this phenomenon with the previous results about the relationship of the three ancestral genomes, we suggested that the more divergent one ancestral genome was from the other, the more modified the polyploid genomes would be after their formation. In other words, when the two genomes merged into a common nucleus, they have to adjust themselves to the new environment. If they are similar, it will be easier for them to suit each other. Thus, little changes are needed to adapt to the new environment. There might be a difference between genomes in their ability to be modified in the polyploid background.

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