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Analysis of Structure Variations and Expression Characteristics of *DMP8* and *DMP9* Genes in Brassicaceae

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Abstract: Doubled haploid (DH) technology based on in vivo haploid induction (HI), which is used to obtain true-breeding lines within a single generation, is a technique that significantly increases modern crop-breeding efficiency. Recently, dicot *Arabidopsis thaliana* lines containing mutations in *DMP8/9* were used as haploid inducer lines, but the use of this new HI mechanism is limited in Brassicaceae species, which include many important vegetable, oil, and fodder crops. Here, we investigated the phylogenetic distribution of the *DMP8* and *DMP9* homologous genes from 26 sequenced Brassicaceae species. We found that *DMP8* only exists in the tribe Arabideae, while multiple copies of the *DMP9* gene are presenting in all the investigated Brassicaceae species. The syntenic *DMP9* genes were divided into two groups derived from the S genomic block and R genomic block, respectively. We further investigated the duplication, structure variations, and expression of the *DMP9* genes in *Brassica* species that had undergone an extra whole-genome triplication. Our results revealed that *DMP9* was lost in the most fractionated (MF2) subgenome, and the retained *DMP9s* in the least fractionated (LF) subgenome and medium fractionated (MF1) subgenome showed diversified expression patterns, indicating their functional diversification. Our results will be useful for obtaining the target *DMP* genes for the establishing of HI lines in Brassicaceae crops.

Keywords: *DMP8*; *DMP9*; Brassicaceae; HI; *Brassica rapa*

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1. Introduction

Haploid induction (HI) technology is used to obtain homozygous lines in a single generation. Compared with the traditional breeding approach, which requires multiple generations of selfing or backcrossing, the approach employing doubled haploids increases crop-breeding efficiency [1]. As important cultivated crops, haploids of *Brassica* species could be obtained through in vitro anther or microspore cultures; however, the procedure is time-consuming and complex, and the induction is genotype-dependent [2,3].

In vivo HI technology, which has been widely used in monocot breeding, has revolutionized traditional crop breeding, especially in maize (*Zea mays*). The loss of function of *ZmPLA1/MTL/NLD* (patatin-like phospholipase A1/MATRILINEAL/NOT LIKE DAD) in maize, which encodes a sperm cell-expressed phospholipase [4–6], can trigger maternal HI, and the HI rate (HIR) can reach 2–3%. This new approach has been extended to other monocots, including rice [7] and wheat [8,9], but has not been extensively applied to dicots owing to the lack of identifiable *ZmPLA1/MTL/NLD* orthologues [10]. In contrast, another maternal haploid inducer gene identified in maize, *Zea mays* DOMAIN OF UNKNOWN FUNCTION 679 membrane protein (*ZmDMP*), belonging to an unknown plant-specific protein family, has a conserved amino acid sequence in both monocots and dicots. The HIR in *ZmDMP* mutants is 0.1–0.3%, which can be increased 5–6-fold when *zmdmp* is combined with *mtl/zmpla1/nld* alleles. In *Arabidopsis*, the DMP protein family has ten members (DMP1 to 10), and all DMP proteins have four transmembrane domains. However, a phylogenetic analysis showed that *AtDMP8* and *AtDMP9* had the closest relation with

ZmDMP. The loss of function of *AtDMP8* and *AtDMP9* triggers in vivo maternal HI in *Arabidopsis thaliana* [10].

A previous study found that *AtDMP8* and *AtDMP9*, expressed explicitly in both generative and sperm cells, are involved in a gamete interaction that leads to correct double fertilization in flowering plants [11]. To date, haploid inducer systems with *DMP* genes have been reported for several other dicot plants, including tomato (*Solanum lycopersicum*), tobacco (*Nicotiana tabacum*), *Medicago truncatula*, *Brassica napus*, and *Brassica oleracea* [12–15].

The Brassicaceae family, containing 340 genera and 4636 species, is a very important economic plant family [16]. The Brassicaceae family, which includes many important vegetable, oil, and fodder crops [17], provides nutrients and phytochemicals for human health. The Brassicaceae family has undergone three major ancient whole-genome duplication (WGD) events: a paleohexaploidy (γ) event and two paleotetraploidy (β and α) events, which are referred to as the *At-γ*, *At-β*, and *At-α* of the core Brassicaceae [3,18]. A key agricultural genus of the Brassicaceae family, the *Brassica* genus, had undergone an extra whole-genome triplication (WGT) event. These duplication events, followed by rapid DNA sequence divergence and fractionation of the ancestral gene sequence, reduced the gene similarity and increased the adaptive ability to survive and diversify in the changing environment [18,19]. Furthermore, WGDs tend to cause expression divergence within and between species, contributing to morphological diversity and expanding regulatory networks [20–22].

Because of the complex genome of Brassicaceae, it is essential to analyze the gene evolution and expression characteristics, especially in those crops for which it is difficult to obtain transgenic plants, such as *Brassica rapa*. Herein, we investigated the evolutionary history of the *DMP8* and *DMP9* genes in 26 sequenced Brassicaceae species by constructing phylogenetic trees and examining the syntenic relationship of the *DMP9* genes. Specifically, we tested whether the duplicated *DMP9* genes represented expression divergence within or between *Brassica* species. It is essential to study their evolution to accurately find the functional target of *DMP9* genes, and such a study also paves the way for applying this HI system in *B. rapa*.

2. Materials and Methods

2.1. Plant Materials

The germinated seeds of *B. rapa* "Chiifu", *B. rapa* "B9008", and *B. oleracea* "JZS" were sown in pots in a greenhouse without climate control in Beijing. Plants were grown during the spring of 2021 under normal growth conditions. Pollen was collected from these plants for DNA isolation, RNA isolation, and RNA-seq.

2.2. Identification and Phylogenetic Analysis of DMP9 Homologous Genes

Genes and genome datasets for 26 *Brassicaceae* species, including *Brassica rapa* (v3.0), *Brassica oleracea*, *Brassica nigra*, *Brassica juncea*, *Brassica napus*, *Brassica carinata*, *Boechera retrofracta*, *Boechera stricta*, *Crucihimalaya himaliaca*, *Capsella grandiflora*, *Capsella rubella*, *Camelina sativa*, *Arabidopsis lyrata*, *Arabidopsis halleri*, *Arabidopsis thaliana*, *Descurainia sophia*, *Cardamine hirsuta*, *Leavenworthia alabamica*, *Raphanus sativus*, *Sisymbrium irio*, *Isatis indigotica*, *Arabis alpina*, *Schrenkiella parvula*, *Thlaspi arvense*, *Thellungiella halophila*, and *Aethionema arabicum*, were downloaded from the BRAD (http://brassicadb.cn, accessed on 10 November 2022). The 18 *B. rapa* genomes included in the pan-genome were obtained from Cai et al. [23]. Based on the results of all 26 *Brassicaceae* species and a *B. rapa* pan-genome multiple sequence alignment, a neighbor-joining tree was constructed using a bootstrap test with 1000 replicates. The phylogenetic tree was visualized in MEGA 7.0 [24]. For identifying syntenic genes, we used the function of "Syntenic Gene @ Subgenomes" of the BRAD database (http://brassicadb.cn/syntenic-gene/, accessed on 10 November 2022), which identifies syntenic genes in *Brassicaceae* species based on SynOrths [25,26].

2.3. Transcriptome Sequencing

To investigate the expression patterns of *DMP9* homologous genes, we collected flower buds at differential development stages from *B. rapa* "Chiifu", *B. rapa* "B9008", and *B. oleracea* "JZS" plants, each having three biological replicates. Pollen was collected from flower buds of different lengths and observed under a microscope (Carl Zeiss Primo Star 415500-0057-000).

Pollen development was divided into six stages: diploid nucleus, haploid nucleus, microspore, uninuclear pollen, bicellular pollen, and tricellular pollen (mature pollen), according to observation by a microscope. In total, 54 samples were collected and used for RNA-seq via the Illumina platform.

2.4. Conserved Domain and Gene Structure Analysis

The gene structures of *DMP9* copies in the *B. rapa* pan-genome were generated using TBtools software [27]. The parameters were set to the default values [27].

2.5. RNA-Seq Data Analysis

The total RNA was extracted with a Trizol reagent from the samples of flower buds at the six pollen developmental stages collected from *B. rapa* "B9008", *B. rapa* "Chiifu", and *B. oleracea* "JZS". The RNA was used for RNA-seq via the Illumina platform. All raw reads were filtered by fastp [28] using the parameter "-z 4 -q 20 -u 30 -n 5". All clean reads were mapped onto the corresponding genome using Hisat2 (version 2.2.0) [29]. We calculated the TPM (transcripts per million) value of each gene using StringTie (version 2.1.3b) [30]. Data from different experimental sets were analyzed for statistical significance using one-way analysis of variance (ANOVA) with GraphPad Prism 7.0 software. A p-value < 0.05 was considered significant.

3. Results

3.1. AtDMP8 and AtDMP9 Homologous Genes in 26 Brassicaceae Genomes

The full-length DNA sequences of *AtDMP8* and *AtDMP9* were used for a BLASTP search (http://brassicadb.cn/#/BLAST/, accessed on 10 November 2022) to identify homologous genes with the identity $\geq$ 85% in Brassicaceae species. The results showed that a few homologous segments, showing a high identity with the *DMP9* gene, were not annotated in the genomes (Supplementary Table S1). Thereafter, we performed a syntenic analysis for the *DMP8* and *DMP9* homologous genes using SynOrths [25]. The homologous genes were divided into two parts: syntenic genes and non-syntenic genes. Syntenic genes from different species are orthologs located on syntenic fragments. Therefore, they often share similar functions and originate from a common ancestor [25,31]. Here, we analyzed the evolution of syntenic homologous genes of *DMP8* and *DMP9* in the 26 species of the Brassicaceae family.

A previous study showed that the *DMP8* in the *Brassica* genus was lost [15]. Our results showed that the *DMP8* genes located in the A block, showing a good syntenic relationship, only existed in *A. thaliana* and *A. lyrata* (Supplementary Table S1, Figure 1a). The *DMP8* duplication is a unique duplication event that occurs in the genus *Arabidopsis*. Moreover, we identified 50 *AtDMP9*-like genes whose identities were greater than 85% in the 26 sequenced Brassicaceae genomes (Supplementary Table S1). This result is consistent with the expectation of multiple *DMP9* copies in the Brassicaceae species, derived from multiple whole-genome duplications of the Brassicaceae ancestor and the triploidization event during the genome evolution of Brassiceae. The number of homologous *DMP9* genes varied in different Brassicaceae species, with two *DMP9* homologs in 18 species, one *DMP9* homolog in five species, and four *DMP9* homologs in three species, accounting for 69.23, 19.23, and 11.54% of all the Brassicaceae species, respectively.

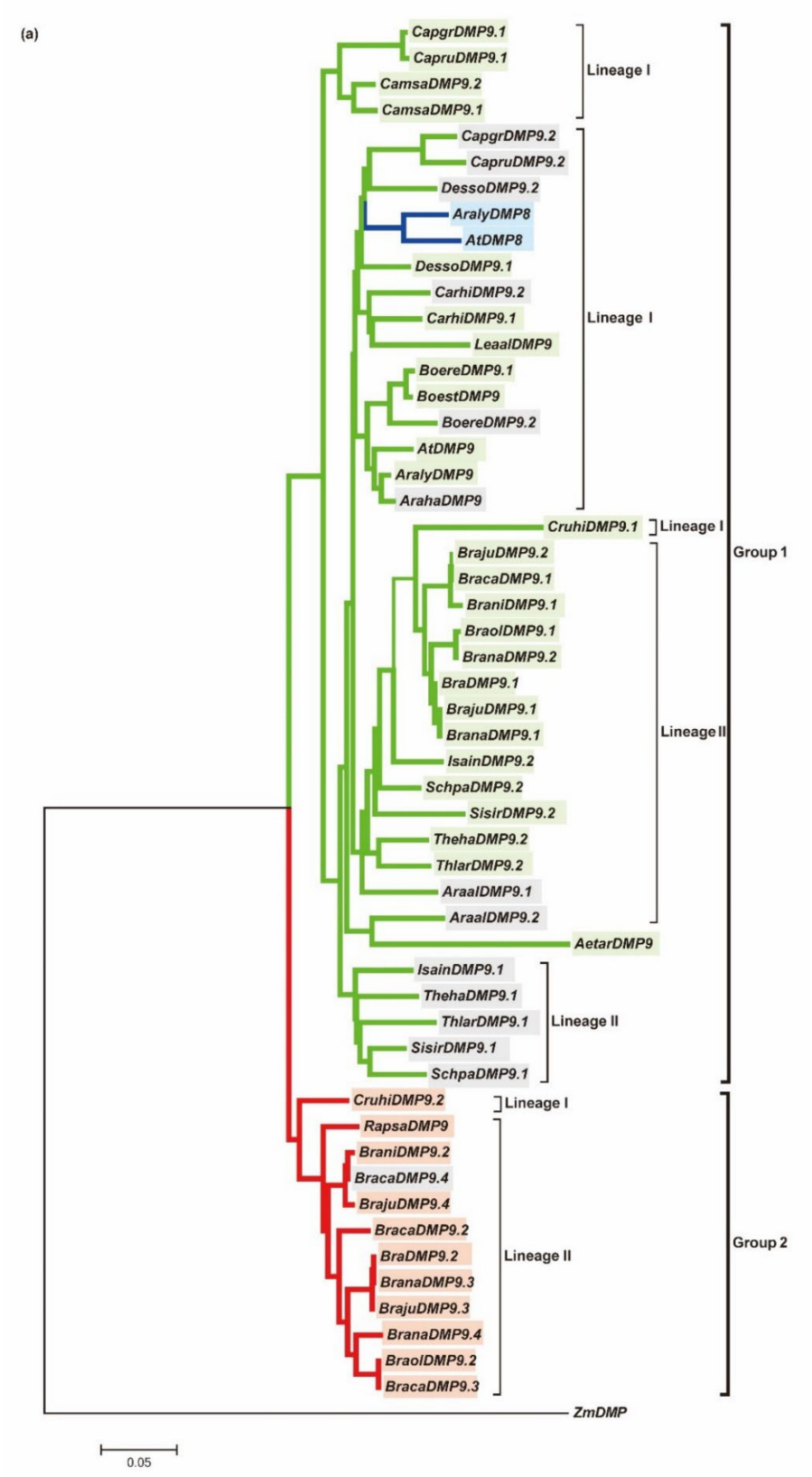

Figure 1. *Cont.*

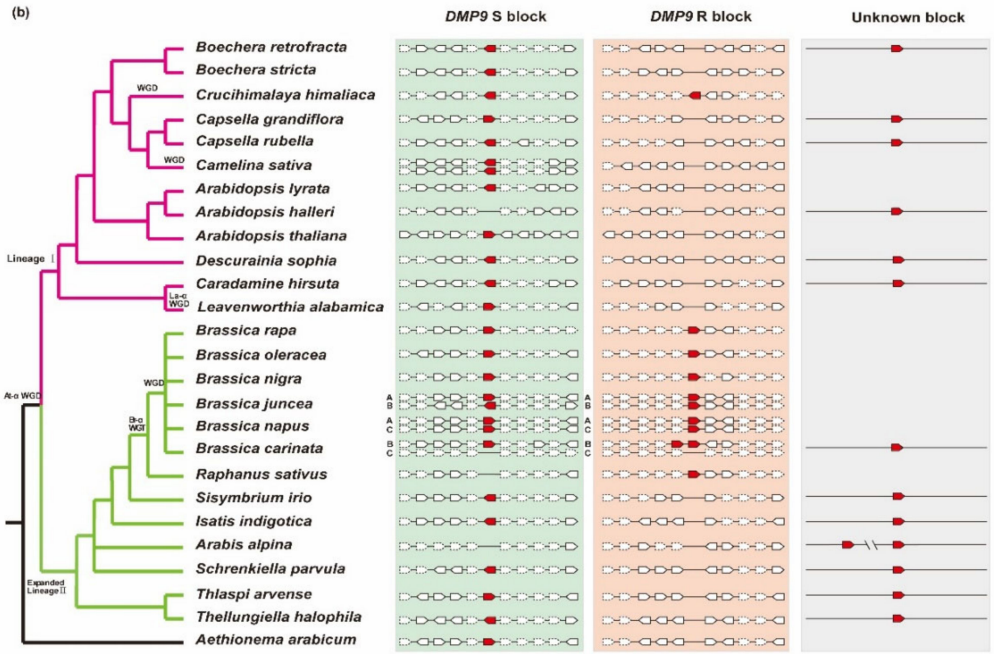

Figure 1. Synteny and phylogenetic evolution analysis of *DMP9* homologous genes in 26 Brassicaceae species. (**a**) An unrooted neighbor-joining phylogenetic tree of 54 *DMP9* homologous genes from 26 Brassicaceae species and maize. *DMP9* homologous genes in Brassicaceae species clustered in two groups: Group 1 (green branch) and Group 2 (red branch). The blue background represents the gene of A block. The green background represents the gene of S block. The red background represents the gene of R block. The gray background represents the gene that has no syntenic relationship. The *ZmDMP* gene of maize was used as the outgroup. Numbers on branches are bootstrap support percentages based on 1000 replicates. The phylogenetic tree was constructed with MEGA7.0 using the maximum likelihood algorithm with default parameters [24]. (**b**) Schematic representation of synteny of *DMP9* homologous genes among 26 Brassicaceae species. The background colors represent different genomic blocks; green is the S block, red is the R block, and gray is the non-syntenic genes. The white box with a full line represents a syntenic gene. The white box with a dotted line represents no gene.

Taking the maize *ZmDMP* gene as an outgroup, we constructed a phylogenetic tree of the *DMP8* and *DMP9* homologous genes of the 26 sequenced Brassicaceae species based on their DNA sequences (Figure 1a). The phylogenetic tree was visualized in MEGA 7.0 [24]. The phylogeny results showed that the homologous genes of *DMP8* and *DMP9* clustered into two groups: Group 1 and Group 2. Almost all sequenced Brassicaceae species in Group 1 (excluding *R. sativus*) retained *DMP9* genes, while only *A. thaliana* and *A. lyrata* retained *DMP8* genes. In contrast, in Group 2, only the genus *Brassica* and *C. himaliaca* possessed *DMP9* genes (Figure 1a). Overall, the phylogenetic analysis revealed that all sequenced Brassicaceae species retained *DMP9* genes.

Based on the 24 basic genomic blocks (GBs, A-X) of Brassicaceae genomes, we performed the syntenic analysis of *DMP9* homologous genes using SynOrths [25,32] (Figure 1b). Our study revealed that the *DMP9* homologous genes in Brassicaceae were clustered into two groups: Group 1 (green branch) and Group 2 (red branch). In addition, the genes in Group 1 were mainly derived from the S block, and those in Group 2 were mainly derived from the R block. Alternatively, the numbers of the *DMP9* homologous genes varied across the genomic blocks of 26 Brassicaceae species. Most Brassicaceae species possessed *DMP9* genes in the S block (excluding *A. halleri*, *R. sativus*, *and A. alpina*).

In the R block, the *DMP9* homologous genes only presented in the genus *Brassica*, *R. sativus*, and *C. himaliaca*. Alternatively, 13 *DMP9* homologous genes had no syntenic relationship located in Group 1, and one of *B. carinata* was located in Group 2 (Figure 1). There was only one exception for *DMP9* in *B. carinata*, where tandem duplicated copies located in the R block rather than homoeologous copies were observed. Among the 26 Brassicaceae species, only *R. sativus* retained one *DMP9* paralogous copy after the WGD and WGT events.

3.2. The Evolution of DMP9 Homologous Genes between Species with an Extra WGT

As the genus *Brassica* had undergone WGT, and the genome possesses three subgenomes—LF (least fractionated), MF1 (medium fractionated 1), and MF2 (most fractionated 2)—it was expected that there would be three *DMP9* copies in the genomes of *Brassica* species [33]. To verify the impact of WGT on the *DMP9* evolution in Brassicaceae, we analyzed the loss of *DMP9* copies in each of the three subgenomes in the genus *Brassica* (*B. rapa*, *B. oleracea*, *B. nigra*, *B. juncea*, *B. napus*, and *B. carinata*), and *R. sativus*. The results showed that the *DMP9* copy in the MF2 subgenome had been lost in all the analyzed *Brassica* species. All the diploid *Brassica* genus species, *B. rapa* (AA), *B. oleracea* (CC), and *B. nigra* (BB), retained two *DMP9* copies, while in *R. sativus*, it remained a single duplicate. Among the three allotetraploid species, both *B. juncea* (AABB) and *B. napus* (AACC) contained four *DMP9* copies, and each of the two diploid ancestors contributed two copies located in the LF and MF1 subgenomes; however, *B. carinata* (BBCC) kept four copies only in the B subgenome, including one copy located in the LF subgenome, two copies in a tandem duplication located in the MF1 subgenome, and the last one being a nonsyntenic gene.

Interestingly, we found that the *DMP9* homologous genes of species in the genus *Brassica* located in the S block belonged to the LF subgenome, and the copy located in the R block belonged to the MF1 subgenome. Specifically, the LF and MF1 subgenomes retained only one S block and one R block in the diploid *Brassica* species (*B. rapa*, *B. oleracea*, and *B. nigra*), respectively (Figure 1b). This result demonstrated that the WGT event accelerated the loss of *DMP9* copies.

Moreover, during the formation of the *DMP9* loci in *B. carinata*, the *DMP9* gene was amplified by a tandem duplication (TD) event and a segmental duplication, which were smaller-scale duplications (including TD, segmental duplication, and gene transposition duplication) [34,35]. These distributions are shown in Figure 1.

Note that all these analyses on the evolutionary history of *DMP9* homologous genes in the Brassicaceae species are based on one reference genome per species, and that the gene copy number and functionality of some gene copies may be different within a species.

3.3. DMP9 Homologous Genes in B. Rapa

To investigate the impact of the *DMP9* gene evolution within a species, we used *B. rapa* as a model. *B. rapa* shares a common ancient hexaploid ancestor with *Brassica* plants and has undergone an additional WGT, which has played an essential role in the speciation and morphotype diversification of *Brassica* plants [2].

B. rapa has a rich variety of morphotypes showing extreme traits, such as the leafy heads of Chinese cabbage, the enlarged roots or stem tubers of turnips, and the enlarged stems of caixin. In this study, we examined the *B. rapa* pan-genome derived from the genome assembly of the 18-accession evolution of *DMP9* genes. The result showed that there were 41 *DMP9* homologous genes in the *B. rapa* pan-genome. Among the 18 *B. rapa* genomes, 14 contained two *DMP9* homologs, accounting for 83.33%. Three genomes contained three *DMP9* homologous genes, and one genome (TBA) contained four *DMP9* homologs (Table 1, Figure 2a). We noticed that the homologs of *DMP9* were tandemly duplicated in the R block of two genomes (Figure 2b). The results showed that the *DMP9* homologous genes had a relatively complex evolution route in *B. rapa*.

Table 1. List of *DMP9* homologous genes of 18 *B. rapa* varieties and subspecies.

Sample	Name	Description	E-Value	Identity	Genomic Block	Accession
CCA	ssp. *pekinensis*	Chinese cabbage	0	80.30%	S	*CCADMP9.1*
			0	68.30%	S	*CCADMP9.2*
			0	85.8%	R	*CCADMP9.3*
CCB	ssp. *pekinensis*	Chinese cabbage	0	91.00%	S	*CCBDMP9.1*
			0	85.60%	R	*CCBDMP9.2*
Chiifu #	ssp. *pekinensis*	Chinese cabbage	0	91.00%	S	*BraDMP9.1*
			0	85.80%	R	*BraDMP9.2*
CXA	ssp. *parachinensis*	Caixin	0	90.30%	S	*CXADMP9.1*
			0	85.60%	R	*CXADMP9.2*
CXB	ssp. *parachinensis*	Caixin	0	90.30%	S	*CXBDMP9.1*
			0	85.80%	R	*CXBDMP9.2*
OIA	ssp. *oleifera*	Oil seeds	0	91.00%	S	*OIADMP9.1*
			0	85.80%	R	*OIADMP9.2*
OIB	ssp. *oleifera*	Rapid cycling	0	90.30%	S	*OIBDMP9.1*
			0	90.30%	S	*OIBDMP9.2*
			0	85.60%	R	*OIBDMP9.3*
OIC	ssp. *oleifera*	Rapid cycling	0	90.30%	S	*OICDMP9.1*
			0	85.60%	R	*OICDMP9.2*
Z1	ssp. *oleifera*	Sarson type	0	90.30%	S	*Z1DMP9.1*
			0	85.60%	R	*Z1DMP9.2*
TUA	ssp. *rapa*	Turnip	0	90.70%	S	*TUADMP9.1*
			0	85.80%	R	*TUADMP9.2*
TUE	ssp. *rapa*	Turnip	0	91.00%	S	*TUEDMP9.1*
			0	85.60%	R	*TUEDMP9.2*
PCA	ssp. *chinensis*	Pak choi	0	90.30%	S	*PCADMP9.1*
			0	85.80%	R	*PCADMP9.2*
PCB	ssp. *chinensis*	Pak choi	0	90.30%	S	*PCBDMP9.1*
			0	85.70%	R	*PCBDMP9.2*
WTC	ssp. *narinosa*	Wutacai	0	91.00%	S	*WTCDMP9.1*
			0	85.60%	R	*WTCDMP9.2*
TCA	ssp. *chinensis var.tai-tsai*	Taicai	0	91.00%	S	*TCADMP9.1*
			0	85.80%	R	*TCADMP9.2*
BRO	*Broccolieto*	Broccolleto	0	68.30%	S	*BroDMP9.1*
			0	90.70%	S	*BroDMP9.2*
			0	85.80%	R	*BroDMP9.3*
MIZ	ssp. *nipposinica*	Mizuna	0	91.00%	S	*MIZDMP9.1*
			0	85.70%	R	*MIZDMP9.2*
TBA (WLD)	*unknown*	From Tibet, China	0	90.70%	S	*TBADMP9.1*
			0	85.80%	R	*TBADMP9.2*
			0	85.60%	unknown	*TBADMP9.3* *
			0	85.60%	unknown	*TBADMP9.4* *

Notes: # The accession was used as *B. rapa* reference genome. * indicates non-syntenic genes.

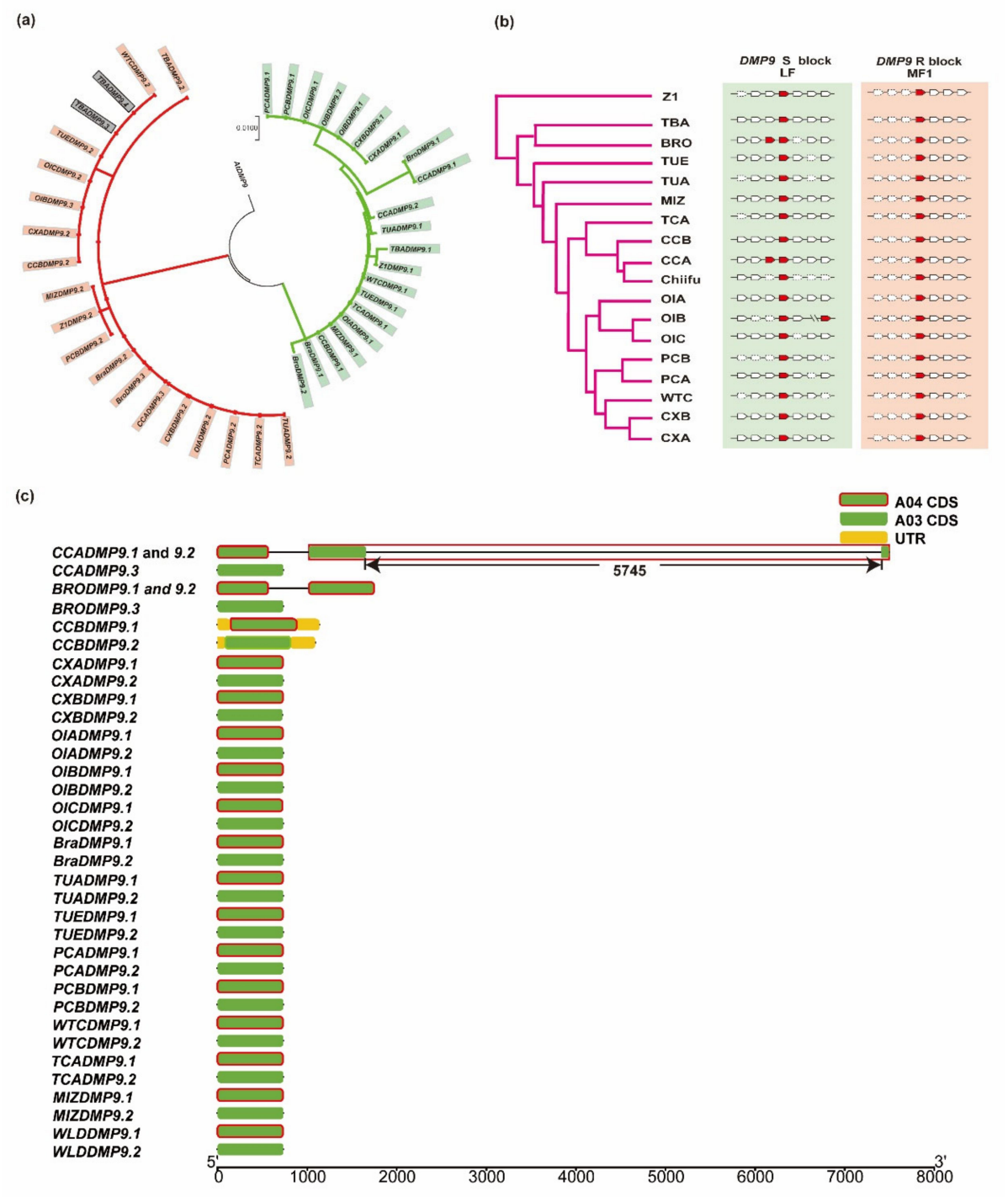

Figure 2. Evolution of *DMP9* homologous genes in *B. rapa* pan-genome. (**a**) Phylogeny relationships of *DMP9* genes in *B. rapa* pan-genome. An unrooted neighbor-joining phylogenetic tree of 41 *DMPs* from *B. rapa* pan-genome. *DMPs* belonged to two branches. The green background represents the S block. The red background represents the R block. The gray background represents the non-syntenic gene of the A block. The *AtDMP9* gene was used as the outgroup. (**b**) The syntenic relationship of *DMP9* genes in *B. rapa* pan-genome. The green background represents the S block. The red background represents the R block. The white box with a full line represents the syntenic gene. The white box with the dotted line represents no gene. (**c**) Gene structures of syntenic *DMP9* genes in the *B. rapa* pan-genome. The green boxes represent CDS, and the yellow boxes indicate an untranslated region.

Taking the *A. thaliana AtDMP9* gene as an outgroup, we constructed a phylogenetic tree of the *DMP9* homologous genes identified from the 18 *B. rapa* genomes based on their DNA sequences (Figure 2a). A syntenic relationship analysis showed that the *DMP9* homologous genes were retained in the LF (S block) and MF1 (R block) subgenomes, but were lost in the MF2 subgenome (Figure 2b). The homologous genes were more conserved in the R block of the MF1 subgenome compared to the S block in the LF subgenome. Although most of the *B. rapa* genomes retained two *DMP9* homologous genes, four (CCA, BRO, OIB, and TBA) retained more *DMP9* copies during their evolution. However, it seemed that the tandem duplication of *DMP9* was not subspecies-specific, as the two Chinese cabbage genomes were different at this locus. CCA and BRO retained a tandem duplicated gene, while CCB did not. TBA retained four copies, but only two of them were annotated. To explore the features and potential functions of *DMP9* copies, we performed a gene structural analysis based on the gene sequence similarity of the encoded proteins (Figure 2c). The results showed that most *DMP9* copies indicated high conservation. The tandem duplication in *B. rapa* CCA and BRO shared a high similarity, but there was a 5745 bp insertion in the tandemly duplicated copy of CCA. In short, we proposed that the *DMP9* gene not only diversified among the Brassicaceae species, but also within species, such as in the *B. rapa* pan-genome.

3.4. Transcriptome Analysis of DMP9 Homologous Genes in B. Rapa and B. Oleracea

Expression divergence between duplicated genes has long been of interest to geneticists and evolutionary biologists because it is considered the first step in functional divergence between duplicate genes, which increases the chance of the retention of duplicated genes in a genome [36,37]. In a previous study, it has been found that *DMP8* was lost and *DMP9* retained two homologous genes in *B. oleracea*. The *BolDMP9.1* gene was nonfunctional, owing to a 1 bp deletion in the exon, while *BolDMP9.2* mutants could induce a maternal haploid with an HIR of about 2.35% [15]. *B. oleracea* and *B. rapa* showed a close relationship with *A. thaliana*. Based on the evolution analysis and previous studies of the *DMP9* homologous genes in Brassicaceae, we used *B. oleracea* "JZS", *B. rapa* "Chiifu" (*B. rapa* reference genome Chiifu), and *B. rapa* "B9008" (CCA genome) to further study the expression patterns of *DMP9* genes in *B. rapa* (Table 2 and Figure 3a).

Table 2. *DMP9* homologous gene information of *B. oleracea* "JZS", *B. rapa* "Chiifu", and *B. rapa* "B9008".

Gene Name	Chr	Gene ID	Start	End	Gene Block	Species	Varieties/ Subspecies
AtDMP9	A05	*At5g39650*	15875199	15876116	*S*	*A. thaliana*	*A. thaliana*
BraDMP9.1	A04	*BraA04g012030.3C*	9524183	9524908	*S*	*B. rapa*	Chiifu
BraDMP9.2	A03	*BraA03g003970.3C*	1691698	1692426	*R*	*B. rapa*	Chiifu
CCADMP9.1 *	A04	*A04p12560.1_BraCCA*	8999489	9000040	*S*	*B. rapa*	B9008
CCADMP9.2 *	A04	*A04p12570.1_BraCCA*	9000509	9001147	*S*	*B. rapa*	B9008
CCADMP9.3	A03	*A03p04040.1_BraCCA*	1746905	1747628	*R*	*B. rapa*	B9008
BolDMP9.1	C04	*BolC04g044930.2J*	46361838	46362575	*S*	*B. oleracea*	JZS
BolDMP9.2	C03	*BolC03g004320.2J*	2118435	2119158	*R*	*B. oleracea*	JZS

Note: * indicates tandem duplication.

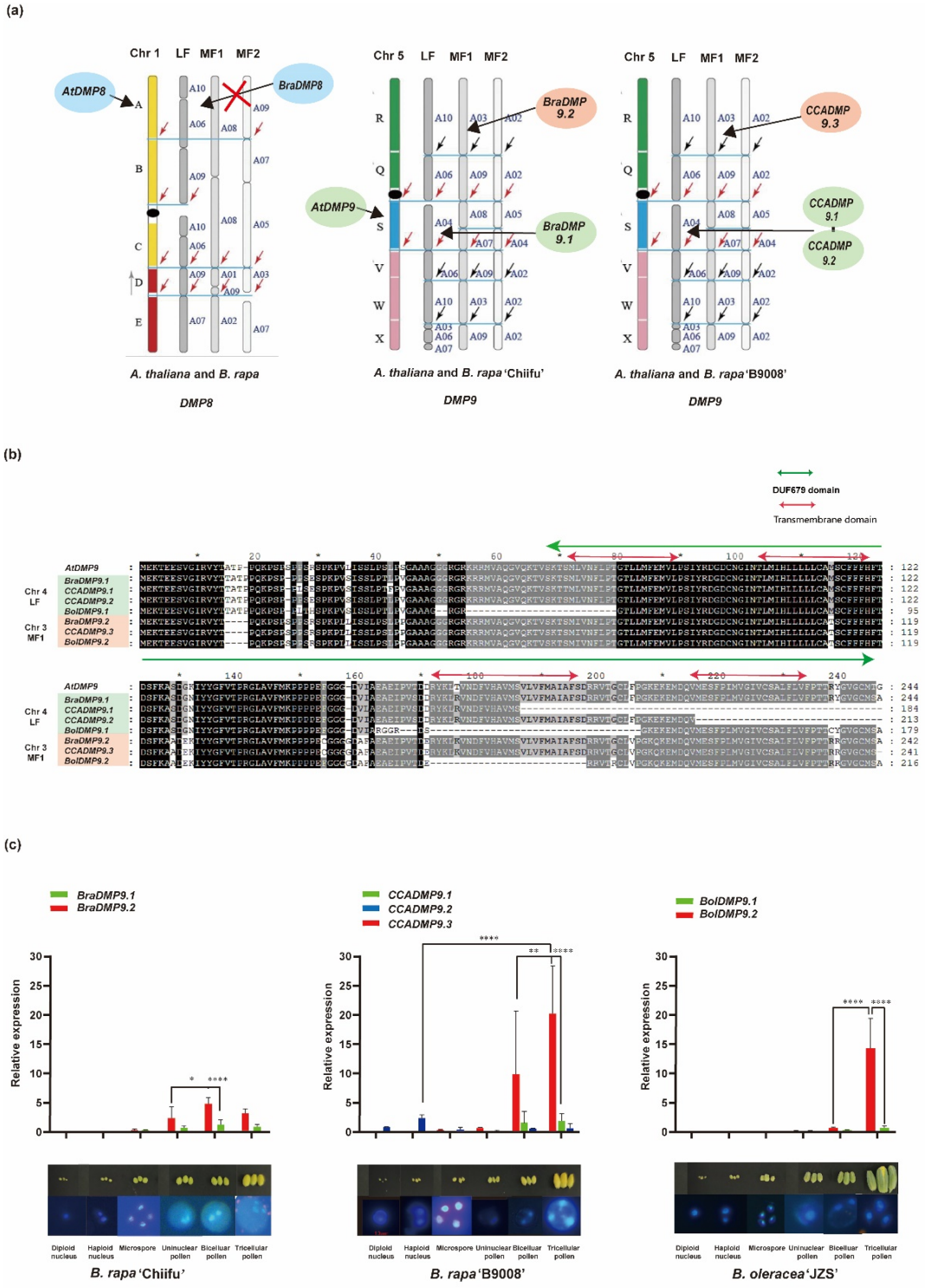

Figure 3. (**a**) The syntenic relationship of *A. thaliana* and *B. rapa* "Chiifu" and "B9008". (**b**) Alignment of amino acid sequences encoded by *DMP9* genes of *B. oleracea* and two *B. rapa* species. The green background represents the *DMP9* homologous genes of the LF subgenome. The red background represents the *DMP9* homologous genes of the MF1 subgenome. (**c**) Relative expression profiles of *DMP9* homologous genes in *B. rapa* "Chiifu", *B. rapa* "B9008", and *B. oleracea* "JZS" at the different pollen developmental stages. The error bars represent the standard deviations of three independent biological repeats. The asterisks represent significant differences via *t*-tests (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Pollen at different developmental stages was observed by DAPI staining.

To further compare the differences of *DMP9* copies between the two *B. rapa* species, we analyzed the corresponding relationship and amino acid sequences with *A. thaliana*. The results showed that although the *DMP9* copies contained the conserved domain, in the third and fourth transmembrane (TM) spans, the proteins encoded by the tandem duplicated copies *CCADMP9.1* and *CCADMP9.2* showed a greater structural divergence than others (Figure 3b).

To explore the expression patterns of *DMP9* homologous genes, we analyzed RNA-seq data generated from different pollen developmental stages, including the diploid nucleus, haploid nucleus, microspore, uninuclear pollen, bicellular pollen, and tricellular pollen (mature pollen) (Figure 3c). An expression analysis of the *DMP9* homologous genes revealed that the *DMP9* homologous genes of *B. rapa* located in the LF (*BraDMP9.1* and *CCADMP9.1*) and MF2 (*BraDMP9.2* and *CCADMP9.3*) subgenomes were highly expressed in mature pollen, but were expressed at much lower levels in immature pollen of different stages, as in *A. thaliana* (Figure 3c) [15]. In addition, the expression level of *DMP9* copies in the MF1 subgenome was higher than that of the genes in the LF subgenome. In contrast, the tandem duplicated *DMP9* homologous gene (*CCADMP9.2*) in *B. rapa* "B9008" was most highly expressed at the haploid nucleus stage, indicating that the *DMP9* gene diversified in expression patterns. In *B. oleracea*, the two *DMP9* copies (*BolDMP9.1* and *BolDMP9.2*) were highly expressed in mature pollen, which was consistent with that observed in *A. thaliana* and *B. rapa*, and the MF1 copy (*BolDMP9.2*) had a higher expression level than the LF copy (*BolDMP9.1*).

4. Discussion

Doubled haploid (DH) technology based on in vivo HI has led to a new approach to crop breeding. Significant breakthroughs in DH production have been made in the last few years by identifying maize genes that induce maternal haploid embryos in vivo. In *A. thaliana*, the loss function of the homologous genes of maize, *ZmDMP*, *AtDMP8*, and *AtDMP9*, could trigger a maternal haploid [10]. Significant breakthroughs in DH production have been made in the last few years by identifying maize genes that induce maternal haploid embryos in vivo.

Recently, more studies have reported that a loss of function of the *DMP8* and *DMP9* homologous genes could trigger HI in *Medicago truncatula*, tomato, *Brassica napus*, *Brassica oleracea*, and *Nicotiana tabacum* [12–14,38]. However, the use of this new HI mechanism is still limited in Brassicaceae species.

The Brassicaceae is a medium-sized family that includes many important economical crops. These crops have undergone WGDs that were followed by gene losses during diploidization. The *Brassica* genus underwent an extra WGT event, resulting in a complex relationship among the duplicated *DMP* genes. Therefore, the diversification of the *DMP8* and *DMP9* genes is central to the HI function in Brassicaceae species. In this study, we took advantage of 26 sequenced Brassicaceae genomes to investigate the evolution and diversification of the *DMP8* and *DMP9* homologous genes. We proposed that the *DMP8* gene was retained in the model plant *A. thaliana* and its congener *A. lyrata*, but that it cannot be found in other Brassicaceae species. Although it is likely that *DMP8* was a unique gene duplication in the common ancestor of *A. thaliana* and *A. lyrata*, we did not exclude the possibility that it may have been lost in other Brassicaceae species. Therefore, the *DMP9* homologous genes should be the major focus when we plan to produce HI in *Brassica* crops. The importance of *DMP9* has been demonstrated by the successful generation of HI in *B. oleracea* [15].

Thereafter, we identified *DMP9* homologous genes in 26 sequenced Brassicaceae species through a BLASTP search. The results showed that *DMP9* copies were diverse. Among the 51 *DMP9* copies identified from the Brassicaceae genomes, four copies were not annotated on the genome. The synteny analysis showed that the *DMP9* homologous genes were divided into syntenic genes and non-syntenic genes. The syntenic *DMP9* genes are located in two genomic blocks: the S block and the R block. Most of the *DMP9* copies were

located in the S block, while only the genus *Brassica*, *R. sativus*, and *C. himaliaca* retained one copy in the R block. We also found that the directions of *DMP9* copies were different in the genomes. In addition to the gene loss and gene fragment duplication of *DMP9* copies, we also observed the tandemly duplicated *DMP9* in the *B. carinata* genome. In summary, the diversification of *DMP9* copies makes it more difficult to obtain the haploid inducer lines in Brassicaceae. Based on the currently sequenced genome data, we analyzed and obtained valuable information. We believe that after the assembly of these genomes has been improved, we can determine the locations of those *DMP* genes that are not allocated.

At present, haploid inducer systems with *DMP9* genes have been reported for *B. napus* and *B. oleracea* in the *Brassica* genus [14,15,38]. For example, in *B. napus*, one genome retained three *DMP9* copies with a gene loss on the LF subgenome [14], while another genome retained four *DMP9* copies [37]. This result is consistent with what we found in the *B. rapa* pan-genome. The copy number variations detected in the pan-genome were all due to *DMP9* gene duplication on the LF subgenome, while the MF subgenome seemed more stable than the LF subgenome. In *B. oleracea*, Zhao et al. [15] revealed that two *DMP9* copies were retained. However, the *DMP9* copy on the LF subgenome lost its function due to a 1 bp deletion in the exon. These results indicated that *DMP9* genes vary in the copy number, and it was necessary to determine the appropriate copy as the target gene in the construction of HI in *Brassica* crops.

Regarding the loss of function of the *DMP9* duplicates in *B. napus* and *B. oleracea*, we examined the *DMP9* duplicate sequences and expression patterns in the *B. rapa* pan-genome. The results showed that the *DMP9* copies on the LF did not contain the deletion of that of *B. oleracea* in 18 *B. rapa* genomes. However, the *B. rapa* genomes retained tandem duplicates. An expression analysis revealed that the tandem duplicates showed expressional divergence, indicating the functional divergence between duplicate genes. Local gene duplication generates tandem duplicated genes and is ubiquitous during genome evolution [39]. In *Brassica* species, although the haploid inducer lines have been obtained in *B. napus* and *B. oleracea*, whether all the varieties or subspecies can obtain the haploid inducer needs further analysis.

In addition to the role in haploid induction, it is reported that *AtDMP8* and *AtDMP9* regulate HAP2/GCS1 trafficking for egg–sperm fusion, and that this function of sperm cell activation is conserved in seed plants [40]. The DMP9 protein is involved in gamete interactions that lead to correct double fertilization in flowering plants [11,41]. The increased number of *DMP9* genes in *Brassica* and their divergence in gene expression indicate their functional differentiation. Therefore, our research on the evolutionary relationship of *DMP8* and *DMP9* genes is of great value in elucidating the gene function of *DMP8* and *DMP9* duplicates.

In this study, the *DMP9* evolution analysis has brought a lot of valuable information regarding the HI line in Brassicaceae species, especially in crops such as *B. rapa*, for which it is difficult to obtain transgenic plants. The target genes can be found through an evolutionary analysis, which can greatly improve the efficiency of obtaining haploid inducer lines, especially for crops with multi-copy genes. Although the genetic transformation of *B. rapa* has not been well developed, we can use other biological methods to further evaluate the gene function of *DMP9* homologous genes, such as in situ reverse transcription polymerase chain reaction (in situ RT-PCR) or insertion–deletion (InDel) markers utilizing high-resolution melting (HRM) curve analysis, which have been exploited and used to detect the expression position and the population genetic analysis [42,43]. On the whole, the results from our present study lay the foundation for establishing the *B. rapa* HI line.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8111095/s1, Table S1: List of *DMP8* and *DMP9* homologous genes in the 26 Brassicaceae species [44–64].

Author Contributions: T.Z. and X.C. analyzed and interpreted the data. T.Z. drafted and revised the manuscript. T.Z. grew plants, collected tissues, and extracted RNA. J.L., L.Z., J.W. and X.W. improved the manuscript. J.W. and X.W. conceived the research, supervised the experiment and data analysis, and modified the manuscript. All authors have read and agreed to the published version of the manuscript.

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