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ORIGINAL ARTICLE

Comparative genomics reveals origin of *MIR159A–MIR159B* paralogy, and complexities of PTGS interaction between *miR159* and target GA-MYBs in Brassicaceae

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Abstract

Whole-genome and segmental duplications coupled with sequence and functional diversification are responsible for gene family expansion, and morphological and adaptive diversity. Although broad contours of such processes are understood, detailed investigations on regulatory elements, such as miRNA-transcription factor modules, especially in non-model crop plants with complex genomes, are few. The present study was performed to understand evolutionary history of *MIR159* family, and changes in the miRNA-binding site (MBS) of the targets *MYB33, MYB65*, and *MYB101* that may affect post-transcriptional gene silencing. We established orthology and paralogy between members of *MIR159* family by reconstructing the phylogeny based on 240 precursor sequences sampled across green plants. An unambiguous paralogous relationship between *MIR159A* and *MIR159B* was observed only in Brassicaceae which prompted us to analyze the origin of this paralogy. Comparative micro-synteny of ca. 100 kb genomic segments surrounding *MIR159A, MIR159B*, and *MIR159C* loci across 15 genomes of Brassicaceae revealed segmental duplication that occurred in the common ancestor of Brassicaceae to be responsible for origin of *MIR159A–MIR159B* paralogy; extensive gene loss and rearrangements were also encountered. The impact of polyploidy was revealed when the three sub-genomes—least fractionated (LF), moderately fractionated (MF1), and most fractionated (MF2) sub-genomes of *Brassica* and *Camelina sativa*—were analyzed. Extensive gene loss was observed among sub-genomes of *Brassica*, whereas those in *Camelina* were largely conserved. Analysis of the target MYBs revealed the complete loss of *MYB33* homologs in a *Brassica* lineage-specific manner. Our findings suggest that mature *miR159a/b /c* are capable of targeting *MYB65* across Brassicaceae, *MYB33* in all species except *Brassica*, and *MYB101* only in *Arabidopsis thaliana*. Comparative analysis of the mature miRNA sequence and the miRNA-binding site (MBS) in *MYB33, MYB65*, and *MYB101* showed the complexity of regulatory network that is dependent on strict sequence complementarity potentially leading to regulatory diversity.

Keywords Brassicaceae · *MIR159* · Synteny · MIRNA-binding site (MBS) · *MYB33* · *MYB65* · *MYB101*

Introduction

Segmental duplication and recurrent whole-genome duplications (WGD) have played a major role in plant morphological and adaptive diversity (Jiao et al. 2014; Vanneste et al. 2014; Dodsworth et al. 2015; Song and Chen 2015; Panchy et al. 2016; Cheng et al. 2018). Comparative genomics has now emerged as a powerful approach for understanding evolutionary processes on a genome-wide scale including the impact of polyploidization across various taxonomic hierarchies, and its impact on regulatory genes and elements (Ghircuta and Moret 2014; Chaney et al. 2016). In addition, it permits the study of impact of polyploidy on plant development and adaptation, and clues towards identification of

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orthologs that have implication in plant improvement programs (Peer et al. 2017).

Brassicaceae is a large family with high morphological diversity, and several members are highly economically valued. Six species of *Brassica*—three allo-tetraploids *B. juncea* (AABB), *B. napus* (AACC), and *B. carinata* (BBCC)—have formed as a result of pairwise interspecific hybridization between three diploid parents *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) (Nagaharu 1935; Warwick et al. 2009; Rakow 2004; Cheng et al. 2013). Several members of the family, including ancestral *Brassica* and *Camelina sativa*, are known to have the experienced genome triplication, followed by gene losses resulting in three distinct sub-genomes, designated as least fractionated (LF), moderately fractionated (MF1), and most fractionated (MF2) (Lysak et al. 2005, 2007; Wang et al. 2011a, b; Cheng et al. 2012; Kagale et al. 2014; Liu et al. 2014). Brassicaceae also has known histories of genome duplication as paleo-polyploidy (*A. thaliana*), meso-polyploidy (*B. rapa*/*B. oleracea*), and neo-polyploidy (*B. napus; C. sativa*). Brassicaceae has, thus, also been considered as a model family to analyze the effect of polyploidization and whole-genome duplications.

MicroRNAs are integral parts of regulatory networks involved in development, adaptive responses (Reinhart et al. 2002; Mallory and Vaucheret 2006; Jones-Rhoades et al. 2006; Luo et al. 2013; Comai et al. 2000; Jones-Rhoades et al. 2006), and genomic stability such as mediating responses to genomic shock experienced due to allo-polyploidization as in *Arabidopsis suecica*, [*A. thaliana* × *(A) arenosa*; Ha et al. 2009], and *(B) juncea* (*B. rapa* × *B. nigra;* Ghani et al. 2014).

In plants where sequence-dependent PTGS is the primary mode of interaction between miRNA and the cognate targets, understanding the comparative evolutionary history of microRNA and their targets is important to unravel their conservancy (Comai et al. 2000; Jones-Rhoades et al. 2006; Nozawa et al. 2012).

Previous reports suggest that *MIR159* and *MIR319* are descendants from a common ancestor (Li et al. 2011). Subsequent to their origin, differences in their mature miRNA sequences and expression domains led to functional specialization (Palatnik et al. 2007). It was also shown that mature miR and miR* region from *MIR159* is conserved across land plants, and has more specialized target spectrum than *miR319* in *A. thaliana* (Palatnik et al. 2007; Li et al. 2011). Homologs of *MIR159* have been detected across land plants (Palatnik et al. 2007; Li et al. 2011). In *Arabidopsis thaliana, MIR159* is a three member gene family with their mature products differing by a single nucleotide. The three targets of *miR159–MYB33, MYB65*, and *MYB101*—have been reported to promote floral induction (Achard et al. 2004), vegetative to reproductive transition and anther development (Allen et al. 2007; Millar and Gubler 2005; Alonso-Peral et al. 2012), male-specific cytokinesis (Liu et al. 2017), programmed cell death (PCD), seed germination (Alonso-Peral et al. 2010), leaf morphology, and various abiotic stresses (Li et al. 2016). Similar functions demonstrated in the other species such as *Hordeum vulgare* (Murray et al. 2003), *Oryza sativa* (Aya et al. 2009), *Lolium temulentum* (Woodger et al. 2003), and *Fragaria vesca* (Csukasi et al. 2012) indicate an evolutionarily conserved regulatory role. In spite of the stated importance, the impact of polyploidization on evolution of *MIR159* family and their targets remains unexplored. We, therefore, analyzed in detail the evolutionary history of *MIR159* family, and evaluated the impact on the components of the regulatory module *MYB33, MYB65*, and *MYB101*.

In the present endeavor, we employed comparative genomics to trace the origin of paralogy of *MIR159A–MIR159B*, investigated the impact of polyploidy, and co-evolution of miRNA-MBS in target based on strict sequence complementarity that has the potential to alter regulatory network leading to regulatory diversity. We began by estimating and reconstructing the phylogenetic relationship among homologs of *MIR159* based on the precursor sequences across entire green plants to establish orthology and paralogy. The analysis led to the identification of Brassicaceae specific paralogy of *MIR159A–MIR159B*. The origin of *MIR159A–MIR159B* paralogy in Brassicaceae was an outcome of segmental duplication which was established through synteny-based comparative genomics between homologous and homoeologous segments harboring *MIR159A, MIR159B* and *MIR159C*. Impact of polyploidization was fully revealed when genome fractionation analysis was performed. Comparative analysis of mature miRNA of *MIR159A, MIR159B, MIR159C*, and the microRNA-binding site (MBS) in the putative targets—*MYB33, MYB65*, and *MYB101*—demonstrated that the target spectrum and the MBS in the targets known thus far are variously altered and revealed the intricacies of sequence based PTGS interaction between *miR159* and target MYBs—*MYB33, MYB65*, and *MYB101* in Brassicaceae. In conclusion, our study demonstrates the utility of comparative genomics to understand that polyploidy can impact regulatory interactions that are dependent on strict Watson–Crick pairing as in the case of miRNA-transcription factors, with a potential to generate regulatory diversity.

Materials and methods

Identification of homologues

Homologues from green plants were identified through BLASTN using precursor sequences of the three members of *MIR159—MIR159A, MIR159B*, and *MIR159C*

from *A. thaliana* genome *MIR159A*/*At1g73687* (184 bp); *MIR159B*/*At1g18075* (196 bp); *MIR159C*/At2g46255 (225 bp) retrieved from miRBase (http://www.mirbase.org; Griffiths-Jones et al. 2006, 2007); Kozomara and Griffiths-Jones 2010, 2013) and used as query. BLASTN was performed at BRAD (http://brassicadb.org; Cheng et al. 2011) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html#; Goodstein et al. 2011) databases using the default sets of parameters (program = BLASTN; expect = 10; description = 100; alignment = 50). Database search across miRBase was used for the complete retrieval of sequences across Viridiplantae. The data set reported by Li et al. (2011) on *miR159*/*319* evolution was utilized to retrieve the sequences of *miR159* of some of the species. Homologs of *MYB33* (*At5g06100*), *MYB65* (*At3g11440*), and *MYB101* (*At2g32460*) across Brassicaceae were identified using *A. thaliana* CDS from TAIR Database as query to perform BLASTN at BRAD using the default parameters as described above. Mature miRNA and target-binding site sequences were identified based on miRBase (v21) and published literature and compared.

Phylogenetic reconstruction

Phylogenetic relationships among homologs of *MIR159A*/*MIR159B*/*MIR159C* were estimated using stem-loop precursor sequences from green plants; and using CDS for homologs of *MYB33*/*MYB65*/*MYB101* from Brassicaceae. Sequences were aligned using Multiple Sequence Alignment using the default settings on MAFFT (http://www.ebi.ac.uk/Tools/msa/mafft/; Katoh and Standley 2013; Katoh et al. 2017). Alignments were saved in .NEXUS format and then subjected to BEAUti for 1,000,000 generations using GTR substitution model (Base frequencies = Estimated; Site Heterogeneity model = Gamma; no. of Gamma categories = 4, Yang96 model, and Yule process) to generate .xml file (Drummond et al. 2012; Bouckaert et al. 2014). This .xml file was then subjected to BEAST v1.8.4. The .tre file generated by BEAST was then annotated through TreeAnnotator using tree cut-off as 250 (Drummond et al. 2012; Bouckaert et al. 2014). The "TREE" file was visualized and manually edited using FigTree v4.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Synteny across chromosomal segments and sub-genome fractionation across Brassicaceae

100 kb segments (flanking 50 kb upstream and downstream) harboring *MIR159A, MIR159B*, and *MIR159C* were retrieved from 14 genomes of Brassicaceae. Data set of *B. rapa, B. nigra, B. oleracea, B. juncea, B. napus, Arabidopsis lyrata, Capsella rubella, Sisymbrium irio, Thellungiella halophila, Thellungiella salsuginea, Aethionema arabicum*, and *C. sativa* was retrieved from BRAD; data sets for *Capsella grandiflora* and *Boechera stricta* were obtained from Phytozome. To perform genome fractionation, genomic segments of *Brassica* and *Camelina* genome that have lost homologs of *MIR159A, MIR159B*, and *MIR159C* were retrieved using *A. thaliana* homologs of protein-coding genes flanking the *MIRNA* genes on either side, and selecting the list from "Syntenic Gene" portal (http://brassicadb.org/brad/searchSyntenytPCK.php) of BRAD including all the sub-genomes. As the BRAD portal accepts only protein-coding genes as query, we employed *At1g73680* (upstream of *MIR159A*), *At1g18070* (upstream of *MIR159B*), and *At2g46250* (upstream of *MIR159C*) as query.

Genomic segments were used as input for global alignment using AVID and gVISTA tool (http://genome.lbl.gov/cgi-bin/GenomeVista) against the *A. thaliana* genome (March 2004/2009 release) using default settings (rank VISTA threshold = 0.5) and conserved regions were visualized using VISTA (Bray et al. 2003; Frazer et al. 2004). The 100 kb segments from each species were then subjected to *ab initio* gene prediction through FGENESH (http://www.softberry.com) using gene model of *A. thaliana* (for *(A) lyrata* and *Capsella* species) and *(B) rapa* (rest of the species) as template using default gene finding parameters. Protein sequences obtained by FGENESH prediction tool were used for the BLASTP analysis using BLAST2GO against the nr database at NCBI using *E*-value = 1.0E−3 and number of BLAST hits = 20, word size = 6; low complexity filter = on; HSP length cut-off = 33; blast description annotator = on. A comparative list was prepared based on the identification of orthologs of *A. thaliana*, and annotated genes from BLAST2GO analysis. Synteny diagrams were manually constructed to depict gene conservation, loss, and duplications.

Results

Identification of homologues and phylogeny of *MIR159*

A total of 240 homologs of *MIR159A, B*, and *C* were identified from 84 species of green plants (1 family, 1 species of Bryophyte; 1 family, 1 species of Pteridophyte; 2 families, 6 species of Gymnosperms; 15 families, 76 species of Angiosperm) using *A. thaliana* precursor as query (Supplementary Table 1). We estimated the phylogenetic relationship between these homologs using GTR and doublet models of Bayesian method (Fig. 1). Phylogenetic reconstruction across plants shows two major clades—clade I comprising of *MIR159C* homologs and clade II with a mix of *MIR159A*/B homologs. Within both clade I and II, sequences from monocots and dicots form family-specific sub-clades. Clear distinction between *MIR159A* and *MIR159B* was visible only in

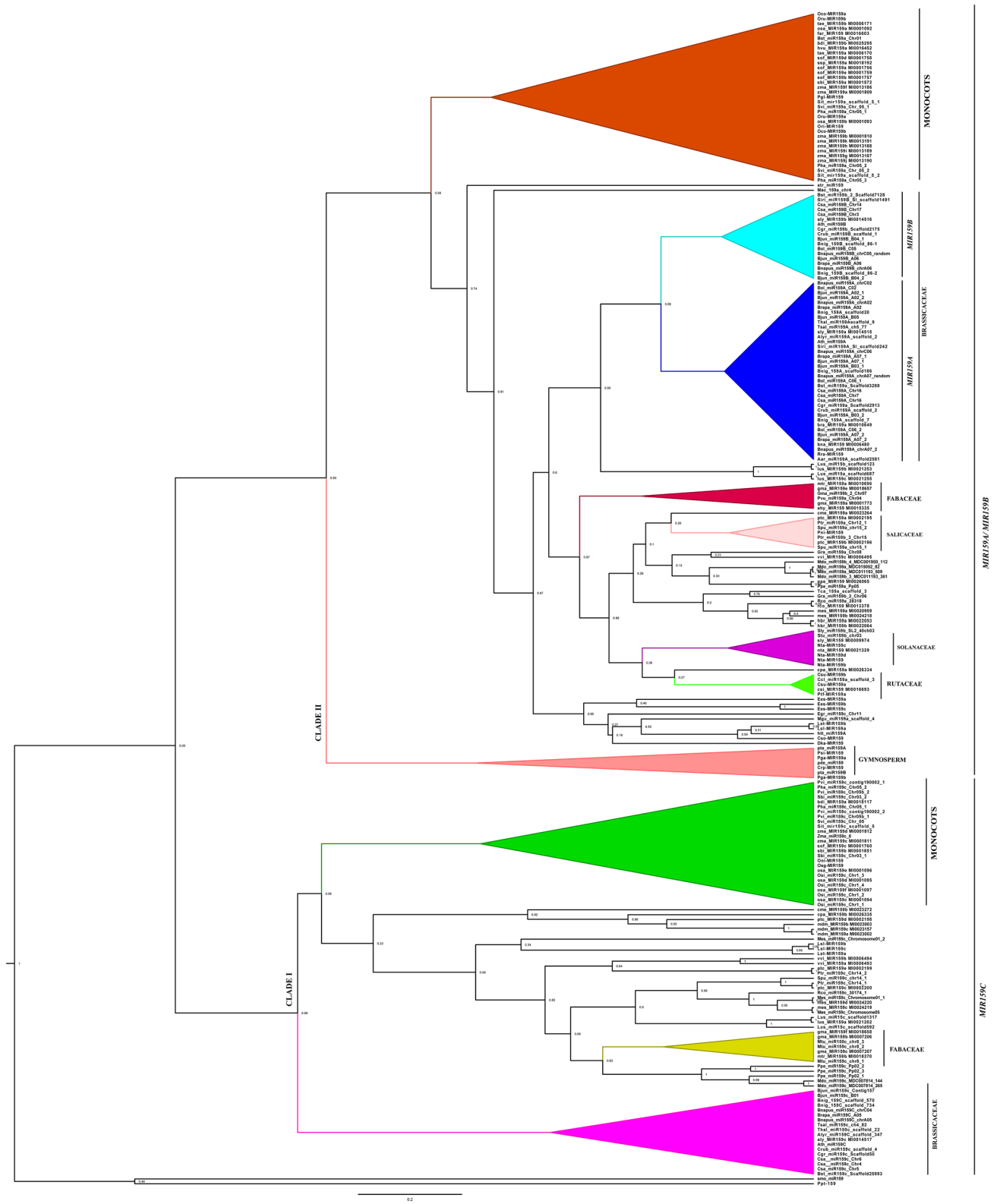


Fig. 1 Phylogenetic reconstruction of *MIR159A*, *MIR159B*, and *MIR159C* across green plants using GTR and doublet model of Bayesian method shows two major clades—clade I consisting of *MIR159C* homologs and clade II of *MIR159A*/*MIR159B* homologs. Within each clade, the majority of the homologs formed family-specific clusters. *MIR159A*/*MIR159B* were found to be mixed in angiosperms other than Brassicaceae, where a duplication causes origin of *MIR159A*–*MIR159B* paralogy (*MIR159A*—Blue; *MIR159B*—sea green). (Color figure online)

the members of Brassicaceae. For the rest of the taxonomic groups, clusters with a mix of *MIR159A* + *B* were observed; within clade II, gymnosperms formed a separate distinct clade, and monocots formed a separate distinct group with a basal angiosperm—*Amborella trichopoda*. In core eudicots, the representative families (Fabaceae, Salicaceae, Solanaceae, and Rutaceae) formed independent and family-specific clusters of *MIR159A*/*B*.

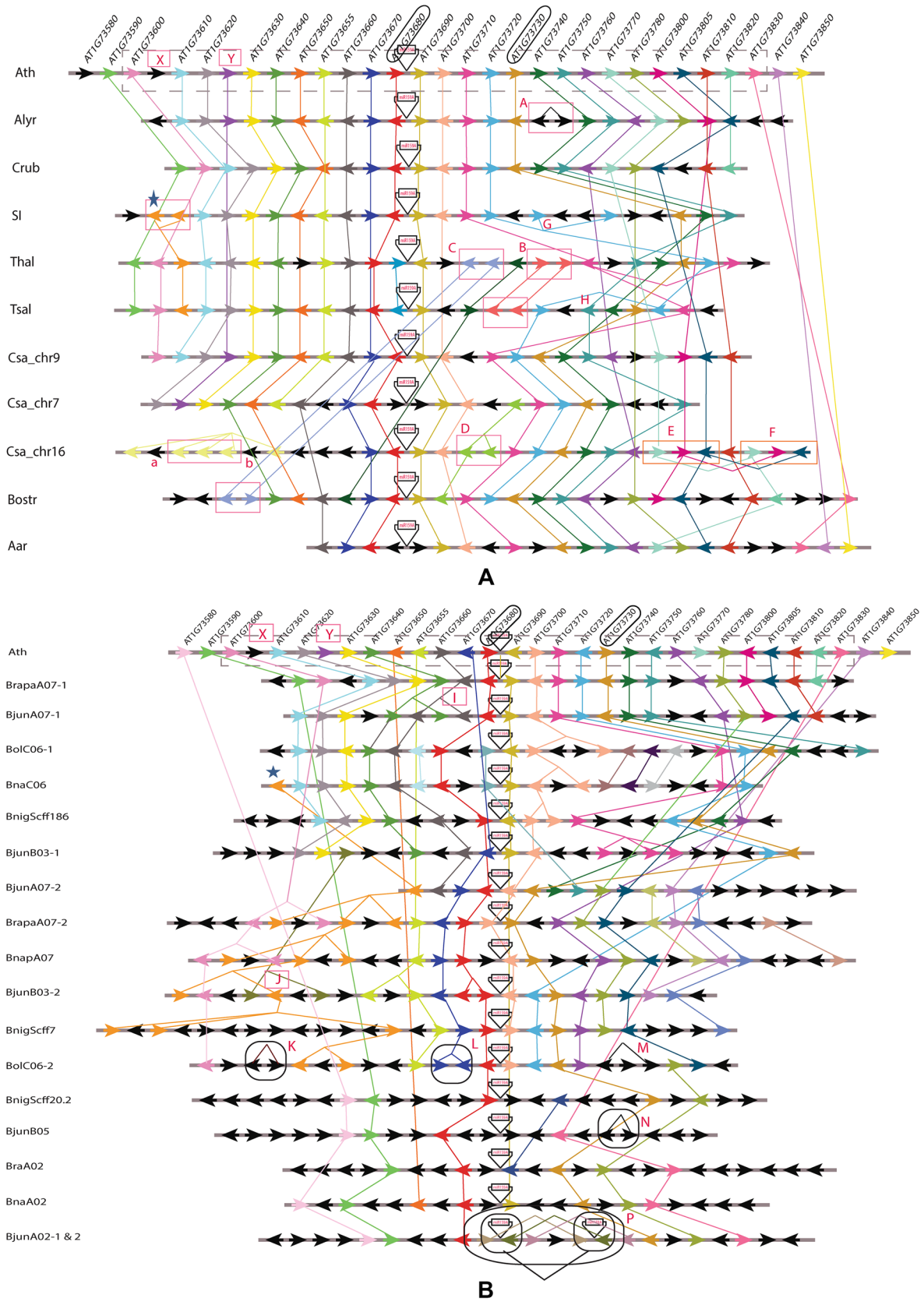
A total of 33 homologues of *MIR159A*, 18 of *MIR159B*, and 18 of *MIR159C* were identified across 15 Brassicaceae genomes (Table 1), and their orthology was confirmed through AVID/VISTA tool (data not shown) following Singh et al. (2017) before undertaking phylogeny, genome organization, and detailed synteny analysis. A separate phylogeny was reconstructed for members of Brassicaceae to understand the history of the *MIR159* family in which also *MIR159C* formed a separate branch, and *MIR159A* and *MIR159B* form a distinct group with paralogous relationship (Supplementary Fig. 2). Within each group, clustering was based on genomic and sub-genomic affiliations–A genome homologs of *Brapa*_159a_A07-1 with homologs from A07-1 copies of *B. napus* (A genome counterpart from AC genome) and *B. juncea* (A genome counterpart from AB genome); B-genome homologs from B03-1 of *B. juncea* (B genome counterpart from AB genome) with scaffold 186 of *B. nigra* (B genome); and C genome homolog from C06-1 *B. oleracea* (C genome) with *B. napus* (C genome counterpart from AC genome).

Organization and synteny analysis of genomic segments encompassing *MIR159A, MIR159B* and *MIR159C* across Brassicaceae

To unravel the cause of paralogy, analyze the genomic organization of segment containing *MIR159*, and to understand the relationship between genome organization and

Table 1 List of *MIR159* homologs and homoeologous across Brassicaceae

Genome	Source	*MIR159A*	Chromosome/scaffold	*MIR159B*	Chromosome/scaffold	*MIR159C*	Chromosome/scaffold
Arabidopsis thaliana	miRBase	1	Chr 01	1	Chr 01	1	Chr 02
Arabidopsis lyrata	BRAD	1	scaffold 2	1	scaffold 1	1	scaffold 2
Aethionema arabicum scaffold v1.0	BRAD	1	scaffold 2581	NA	NA	NA	NA
Brassica rapa chromosome v1.5 (A genome)	BRAD	3	A02, A07-1, A07-2	1	A06	1	A05
Brassica nigra scaffold (B genome)	BRAD	3	scaffold 20, scaffold 186, scaffold 7	2	scaffold 86-1, scaffold 86-2	2	scaffold 570, scaffold 734
Brassica oleracea chromosome v1.0 (C genome)	BRAD	3	C02, C06-1, C06-2	1	C05	NA	NA
Brassica juncea chromosome v1.5 (AABB)	BRAD	7	A02-1, A02-2, A07-1, A07-2, B03-1, B03-2, B05	3	A06, B04-1, B04-2	2	B01, Contig157
Brassica napus chromosome v1.0 (AACC)	BRAD	5	A07-1, A07-2, A02, C06, C02	2	A06, C05	2	A05, C04
Boechera stricta scaffold v1.2	Phytozome	1	scaffold 3288	1	scaffold 712	1	scaffold 25993
Capsella rubella scaffold v1.0	BRAD	1	scaffold 2	1	scaffold 1	1	scaffold 4
Capsella grandiflora scaffold v1.1	Phytozome	1	scaffold 2913	1	scaffold 2175	1	scaffold 50
Camelina sativa chromosome v1.0	BRAD	3	Chr7, Chr9, Chr16	3	Chr3, Chr14, Chr17	3	Chr4, Chr5, Chr6
Sisymbrium irio scaffold v1.0	BRAD	1	scaffold 242	1	scaffold 1491	1	scaffold_717
Thellungiella halophila scaffold v1.0	BRAD	1	scaffold 9	NA	NA	1	scaffold 22
Thellungiella salsuginea scaffold v1.0	BRAD	1	Chr5-77	NA	NA	1	Chr4-82
Total		33		18		18	


AT1G73580
AT1G73590
AT1G73600
AT1G73610
AT1G73620
AT1G73630
AT1G73640
AT1G73650
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AT1G73805
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AT1G73820
AT1G73830
AT1G73840
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Ath
Alyr
Crub
Sl
Thal
Tsal
Csa_chr9
Csa_chr7
Csa_chr16
Bostr
Aar
A
BrapaA07-1
BjunA07-1
BolC06-1
BnaC06
BnigScff186
BjunB03-1
BjunA07-2
BrapaA07-2
BnapA07
BjunB03-2
BnigScff7
BolC06-2
BnigScff20.2
BjunB05
BraA02
BnaA02
BjunA02-1 & 2
B

◂**Fig. 2** Micro-synteny analysis across different genomes of Brassicaceae. Synteny analysis across genomic regions (~ 100 kb) flanking *MIR159A* showing duplication, losses, and rearrangement in Non-Brassica (**a**) and *Brassica* species (**b**). Protein-coding genes, and *MIRNA* in *A. thaliana* and their homologs are represented by different colors, and connected. Genes unique to a particular genomic segment are marked by black arrows; oval region marked as P in Brassica lineage (II) show *Brassica juncea* specific duplication of *miR159A* on Chromosome A02. Details of genes in each genome and alphabetical letters marking specific duplications and insertion are discussed in detail in text, and in Supplementary Table 2. (Color figure online)

evolutionary history, we identified homologs of *A. thaliana MIR159A, MIR159B*, and *MIR159C* from 14 genomes of Brassicaceae based on sequence similarity of the precursor region and retrieved a total of ca. 100 kb genomic segment, flanking 50 kb on either side of the precursor. Their homology was further validated using global alignment tool AVID/VISTA as previously reported (data not shown, available on request; Singh et al. 2017; Jain and Das 2016).

Each of the genomic segments of ca. 100 kb was subjected to FGENESH analysis followed by gene annotation using BLAST2GO, and the gene content was manually represented (Fig. 2, Supplementary Fig. 2A, B). We also computed overall conservation of *A. thaliana* homologs present in genomes and sub-genomes (Fig. 3), conservation of genes in each of the genomes and sub-genomes (Fig. 4; Tables 2, 3), and gene density (Supplementary Fig. 4). A comparison of the rate of gene conservation across genomes between the genomic segment harboring *MIR159A, MIR159B*, and *MIR159C* revealed that the 100 kb segment containing *MIR159C* was most conserved with as many as 11 genes conserved in more than 80% of the genomes. *MIR159A* and *MIR159B* homologous segments have only 3 and 5 genes, respectively, in more than 80% of the genomes *MIR159A*/*MIR159B*.

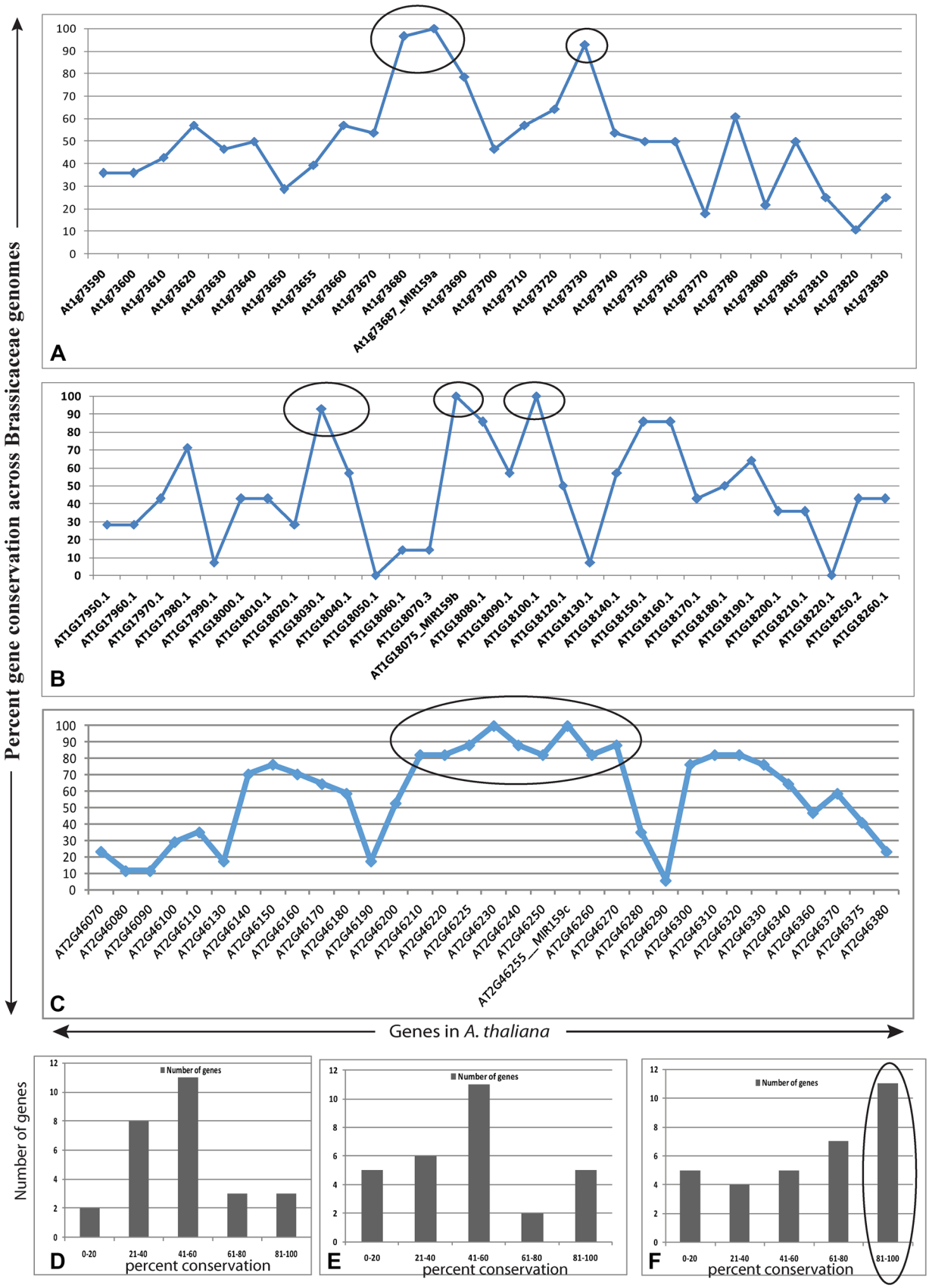
Synteny across genomic segments harboring *MIR159A*

We detected one homologue each of *MIR159A* in *A. lyrata, (A) arabicum, (B) stricta, (C) rubella, C. grandiflora, S. irio, T. halophila*, and *T. salsuginea*; in *C. sativa*, three copies were detected on chromosome 7, 9, and 16. Among the various *Brassica* genomes, three copies each of *MIR159A* were detected each in *B. rapa* (A genome), *B. nigra* (B genome), and *B. oleracea* (C genome); five copies in *B. napus* (AACC genome) and seven copies in *B. juncea* (AABB genome). Two copies of *MIR159A* were located on the same chromosome in *B. juncea* (ChrA02 within a distance of 11.096 kb; Fig. 2 marked P). A critical analysis of the genomic segments revealed that the 100 kb segments of *Bol*-C02 and *Bnap*-A07-1 are not fully sequenced and are represented by N's; similarly, the genome sequence of *Capsella grandiflora* is not yet fully assembled, and the 100 kb genomic segment is present on two different scaffolds. To avoid ambiguity and prevent mis-interpretation, we omitted these three genomes for further synteny analysis leaving us with 29 genomic segments. For ease of representation, the output is divided into two figures, with 5 *Brassica* species (with 17 homoeologous segments representing 18 copies) and 9 non-*Brassica* species (representing 11 genomes) separately, with the *A. thaliana* genomic segment being common to both (Fig. 2a, b; Supplementary Table 2).

The 100 kb segment surrounding *MIR159A* locus in *A. thaliana* is known to contain 27 protein-coding genes (Fig. 2a, b). Gene prediction based on homology to *(A) thaliana/(B) rapa* through FGENESH followed by annotation using BLAST2GO revealed that the number of genes ranged from a low of 19 in *Bjun*_A07-2 (gene density of 1 gene/5.26 kb) to as high as 29 in *B. stricta* and *(C) sativa* Chromosome 16 (1 gene/3.44 kb; Supplementary Fig. 4).

Comparative genomic analysis spanning the 100 kb region across all the 29 genomic segments revealed none of the protein-coding genes to be conserved in all the genomes. *Alpha-dioxygenase* (*AT1g73680*) was found to be present in all the genomes, except *Bjun*_B03-1 (27 out of 28 genomes analyzed; Fig. 3a); *ETHYLENE INSENSITIVE 3-like 3* (*AT1g73730*) was present in all the genomes except *Bnap*_C06 and *Bjun*_B05 (Fig. 2b; encircled; 93%; Fig. 3a); homologs of *AT1G73820* were detected in only 3 out of 28 genomes (10.7%). Homolog of *AT1G73770* was deleted from all the genomes and sub-genomes in a *Brassica*-lineage-specific manner. Two genes in the 100 kb segment surrounding *MIR159A* in *A. thaliana* were predicted by FGENESH that were found to be unannotated in the *A. thaliana* genome release version 10 (TAIR10), and were identified as unknown and *Cation transporter* (Fig. 2a, b; marked X and Y, respectively). The *Cation transporter* locus was found to be present in *A. lyrata, Csa*_chr9, and *Csa*_chr7.

Analysis of gene content and order revealed gene duplications of either dispersed or tandem class, and specific to a particular genome (such as *Prephanate dehydratase* family gene in *(A) lyrata* genome; marked as square box with letter A), *Phosphoethanolamine N-methyltransferase 3* (*AT1G73600*; 99 bp and 506 bp) in *(B) rapa*_A07-2.; or shared across multiple genomes such as *Cysteine-rich receptor kinase 10* across *T. halophila* and *T. salsuginea; Retrovirus-related Pol Poly* from transposon *TNT 1–94* in *T. halophila*, and *B. stricta* (Fig. 2a; square boxes-B and C, respectively). *MIR159A* itself was found to be duplicated (dispersed type) in *B. juncea* on chromosome A02 (named as A02-1 and A02-2). In several cases, the orientation of transcription of the duplicated genes was found to be different from each other (e.g., *At1g73680* in Fig. 2a, b; red arrow and line). In *Csa*_Chr16, homologs of genes present upstream of *At1g73670* in *A. thaliana* are completely missing implying


Percent gene conservation across Brassicaceae genomes
A
B
C
Genes in A. thaliana
Number of genes
D
E
F
percent conservation
0-20
21-40
41-60
61-80
81-100

◂**Fig. 3** Graphical representation showing percent conservation of *A. thaliana* homologs across different genomes of Brassicaceae. Only a few genes (marked by oval) across *MIR159A* (**a, d**), *MIR159B* (**b, e**) genomic segments are conserved in more than 80% of the genomes, except in *MIR159C* (**c, f**) genomic segment which shows the highest conservation among the three segments across Brassicaceae. (Color figure online)

a large segmental deletion or rearrangement specific to *(C) sativa* chromosome 16. 100 kb segment of *Bjun*_A02 also exhibits the duplication of uncharacterized protein LOC103853400, and triplication of *F-box kelch-repeat At4g39560*-like. Some more examples of duplication and deletion have been summarized in Table 2.

Synteny across genomic segments harboring *MIR159B*

BLASTN analysis identified at least one homolog each of *MIR59B* in all the genomes, except in allopolyploids *B. juncea* and *B. napus*, where two copies of *MIR159B* were identified; no homologs of *MIR159B* were identified in *T. halophila* and *T. salsuginea*. Global sequence alignment revealed that the 100 kb genomic segment of *B. napus* chromosome C05 (*Bnap*_C05) does not contain any additional genes homologous to the genomic segment of *A. thaliana* and was thus not included for synteny analysis. The number of genes predicted ranged from 32 in *(A) thaliana* (density of one gene/3.125 kb), 33 in *(B) stricta* (one gene/3.03 kb) to as low as 19 in *(C) sativa* MF sub-genome (gene density of one gene/5.26 kb; Supplementary Fig. 4B; Supplementary Table 3). Among the 15 genomes and sub-genomes that were analyzed for synteny, we found only *MOTHER of FT* and *TFL1* (*AT1G18100*), and *MIR159B* that were shared across all (Supplementary Fig. 2A). Synteny was disrupted on the account of several duplication events including one involving *MIR159B* in B-genome-specific manner on Chromosome B04 in *B. juncea* and scaffold 86 in *B. nigra* (Supplementary Fig. 2A; oval). *UNC93-1* and *FMN-linked oxidoreductases* superfamily genes are deleted in *Brassica* lineage-specific manner (Supplementary Fig. 2A). Some more examples of duplication and deletion are summarized in Table 2.

Synteny across genomic segments harboring *MIR159C*

MIR159C is annotated as *AT2G46255* in *(A) thaliana*. At least one homolog of *MIR159C* was identified in majority of the genomes analyzed; two copies of *MIR159C* were identified each in *(B) juncea* genome (*Bjun*_Contig157 and *Bjun*_B01), *B. napus* (*Bna*_A05 and *Bna*_C04), and *B. nigra* (*Bnig*_scff570 and *Bnig*_Scff734); and three copies in *(C) sativa*. In the 100 kb genomic segment that was analyzed across the 18 genomes, the gene density ranged from one gene per 2.77 kb in *(A) thaliana* (36 genes in 100 kb) to as low as one gene per 5.26 kb in *(B) nigra* scaffold 570 (19 genes in 100 kb) (Supplementary Fig. 4). The conservation of *A. thaliana* homologs ranged from genes present in only 5.9% of the genomes (*At4g46290*, present only in *A. thaliana* and *(C) rubella*), to all the genomes surveyed (100%; *At4g46230* and *At4g46255*-MIR159C; Supplementary Fig. 4). Examples of duplication and deletion in the segments harboring *MIR159C* are summarized in Table 2.

Genome fractionation

A consequence of polyploidization is the creation of multiple copies of the genome that over the course of evolution experiences gene loss creating distinct sub-genomes and homologous copies. The genomes of *Brassica* and *C. sativa* within Brassicaceae are known to have experienced triplication and composed of three distinct sub-genomes annotated as least fractionated (LF), moderately fractionated (MF1), and most fractionated (MF2). Given the triplicated status of the *Brassica* and *C. sativa* genomes, at least three copies of the MIRNA genomic segments are expected in *B. rapa, B. nigra*, and *B. oleracea*, and *C. sativa*; and six copies in *B. napus* and *B. juncea*. We analyzed the impact of genome fractionation across the genomic segments containing *MIR159A, MIR159B*, and *MIR159C* in *Brassica* and *C. sativa* (Supplementary Tables 5, 6).

MIR159A

MIR159A was found to be present in all the three sub-genomic fractions of *B. rapa, B. napus* C and *C. sativa*; in *B. oleracea* (CC) and *B. napus* A, it is presented only in MF1 and MF2 fractions, indicating LF fraction-specific deletion in *B. oleracea* (CC). In *B. napus* C (AACC) genome, the *MIR159A* was lost from LF of A genome after natural hybridization between *B. rapa* (AA) and *B. oleracea* (CC).

In *B. rapa*, gene prediction and annotation revealed the presence of 30, 17, and 20 genes on A07-1 (LF), A02 (MF1), and A07-2 (MF2), respectively. Only two genes, i.e., *Alpha-dioxygenase 2-like* (Supplementary Fig. 5I; marked as A) and *Ethylene insensitive 3-like 3* (Fig. 8I; marked B), are shared among all three genome fractions; four genes are shared among LF and MF1, whereas six genes are shared among LF and MF2 genome fractions, of which *probable inactive serine–threonine-kinase fnkc* (Supplementary Fig. 5I; marked C) gene is present in three copies in MF2 fraction.

In *B. oleracea*, genomic segments on LF (C06-1), MF1 (C02), and MF2 (C06-2) contain 26, 68, and 23 genes, respectively, with most of the genes be unique to each sub-genomes. Only two genes *alpha-dioxygenase 2* (Fig. 5II; marked A) and *ethylene insensitive 3-like 3* (Supplementary

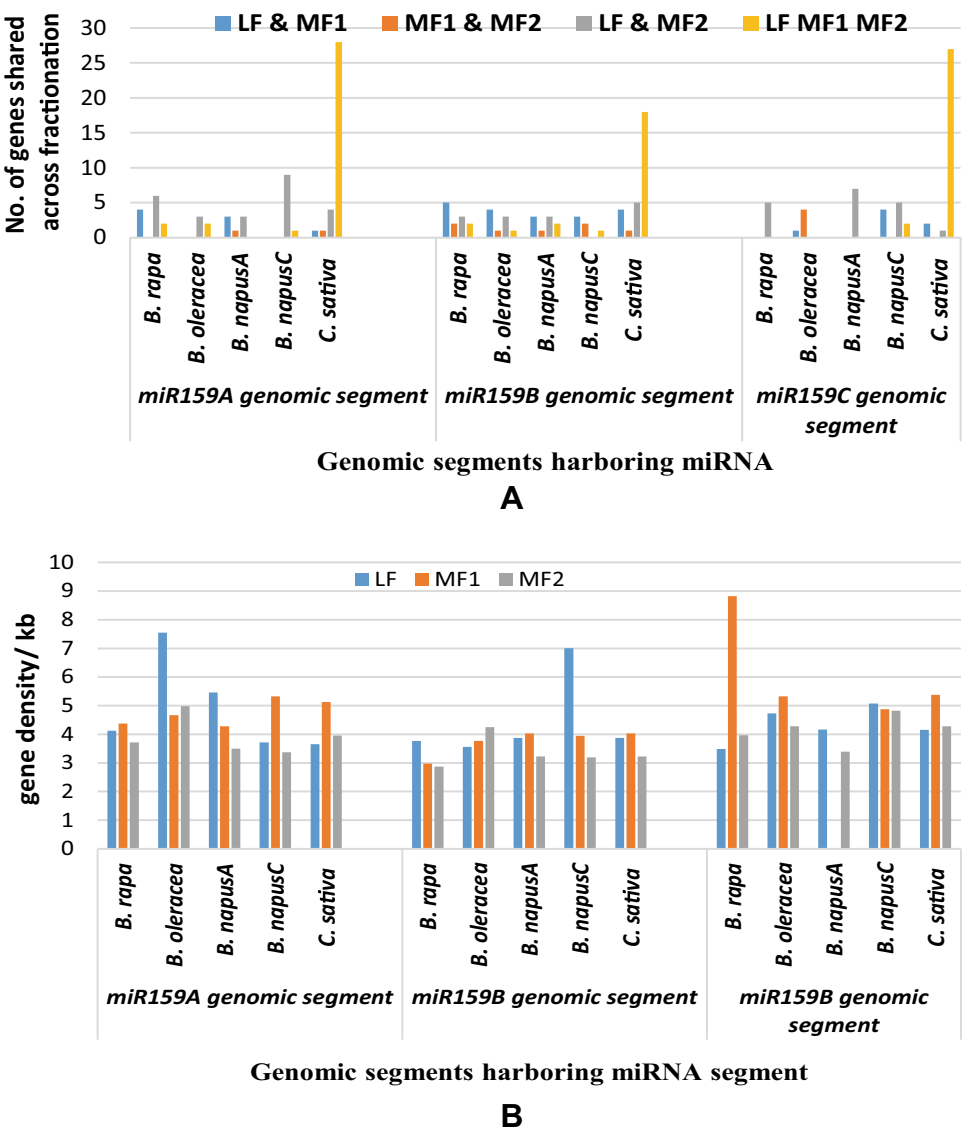


Fig. 4 **a** Graphical representation of number of genes shared across any two or all three sub-genomes (LF and MF1, MF1 and MF2, LF and MF2; LF, MF1, and MF2) harboring *MIR159A, MIR159B*, and *MIR159C*. The three sub-genomes of *Camelina sativa* share the maximum number of genes (orange bar) across all *MIR159A, MIR159B*, and *MIR159C* genomic segments. **b** Gene density (one gene per × kb) across different sub-genomes (LF, MF1, and MF2) harboring *MIR159A, MIR159B* and *MIR159C* of *B. rapa, B. oleracea, B. napus* A, *B. napus* C, and *C. sativa* (**b**). (Color figure online)

Fig. 5II; marked B) are shared among all three sub-genomes; and *Detoxification 17* (Supplementary Fig. 5II; marked as D) and *Suppressor of mec-8 and unc-52 homolog 1* (Supplementary Fig. 5II; marked E) were shared among LF and MF2 sub-genome.

B. napus, an allotetraploid (AACC) of *B. rapa* (AA) and *B. oleracea*, (CC) shows least retention of genes between sub-genomes. Analysis of genomic segment encompassing *MIR159A* revealed 14, 21, and 24 genes on A07-1 (LF), A02 (MF1), and A07-2 (MF2), respectively, with no gene shared among all three sub-genomes. LF and MF2 share three genes of which *Probable inactive serine–threonine-kinase fnkc gene* (Supplementary Fig. 5III; marked as C) is presented in three copies in MF2 fraction. The three sub-genomic segments corresponding to C genome of *B. napus* have 43, 32, and 26 genes on C06-1 (LF), C02 (MF1), and C06-2 (MF2), respectively. LF and MF1 fractions do not share any gene, while LF and MF2 fractions share nine genes of which *Detoxification 17* has two copies in LF fraction (Supplementary Fig. 5IV; marked I).

In *C. sativa*, three genomic segments LF (Chr16), MF1 (Chr07), and MF2 (Chr09) contain 67, 42, and 41 genes of which as many as 28 genes are shared among all the three sub-genomes (Supplementary Fig. 5V; Table 3). Several genes on MF1 such as *Bifunctional inhibitor lipid-transfer seed storage 2S albumin* superfamily (marked J), *ERAD-associated E3 ubiquitin- ligase component HRD3A-like* (marked K), *Detoxification 16-like isoform X1* (marked L), *Ssu72-like* family (marked M), *Receptor kinase At4g00960* (marked N), and *DNA ligase* (marked O) are duplicated on LF and/or MF2 segments; *Thaumatin isoform X1* (marked P) present as two copies on MF1 fraction is shared among the rest two genomic segments. A total of four genes—*Coiled-coil domain-containing 1-like* (marked S), *Non-specific*

Table 2 Duplication, deletion, or insertion of protein-coding genes in 100 kb syntenic fragments of three members of *MIR159* across Brassicaceae

Fragments		Copies	Status	Figure
MIR159A				
*Csa*_Chr16	*Lectin gene (AT1g53080 and AT1g53070)*	5	Tandemly triplicated and flanked on either side by another copy each disrupted by Uncharacterized mitochondrial g00810-like gene and an unannotated gene	Figure 2a; marked I and J, respectively
*Csa*_Chr16	*Detoxification 16-like gene*	2	Duplication	Figure 2a; in tandem; 1500 bp and 354 bp, marked as letter D
*Csa*_Chr16	*Coiled-coil domain-containing 1-like, 3-interacting 1 and SAR DEFECIENT 1-like (dispersed type)*	2	Segmental duplication interrupted by DNA-binding gene	Figure 2; square boxes—e and f
S. irio	*Suppressor of mec-8 and unc-52 homolog (At1g73720)*	3	Triplication	Figure 2a, marked G
T. halophila	*Suppressor of mec-8 and unc-52 homolog (At1g73720)*	2	Duplication	Figure 2a, marked H
*Bol*_C06-1	*DETOXIFICATION 17 (At1g73700)*	2	Duplication	Figure 2e
*Bna*_C06	*DETOXIFICATION 17 (At1g73700)*	2	Duplication	Figure 2e
*Bnig*_scaffold186	*DETOXIFICATION 17 (At1g73700)*	2	Tandem duplication	Figure 2e
T. halophila	*Pentatricopeptide repeat-containing (At1g73710)*	2	Duplication	Figure 2a
*Bjun*_B03-1	*Pentatricopeptide repeat-containing (At1g73710)*	3	Tandem triplication is disrupted by insertion of BnaC01g30690D homolog	Figure 2b
*Bjun*_A07-1	*ras-related RABA6a (At1g73640) and serine–threonine–kinase EDR1-like/CTR1-like kinase (At1g73660)*	2	Dispersed duplication	Figure 2b, marked I
*Bjun*_B03	*Glutathione S-transferase T3-like isoform X2*	2	Specific to B genome of *B. juncea*	Figure 2b, marked J
*Bjun*_B03-2	*Peptidyl-prolylcis-transisomerase FKBP17-chloroplastic (At1g73655)*	2	Dispersed duplication	Figure 2b
*Bjun*_B03-2	*Alpha-dioxygenase 2 (At1g73680)*	2	Tandem duplication	Figure 2b
*Bol*_C06-2	*Replication A 70* kDa *DNA-binding subunit C-like*	2	Tandem duplication	Figure 2b, marked K
*Bol*_C06-2	*Mitogen-activated kinase 15*	2	Tandem duplication	Figure 2b, marked L
*Bol*_C06-2	*Ulp protease*	2	Dispersed duplication	Figure 2b, marked M
*Bjun*_B05	*STRICTOSIDINE SYNTHASE-LIKE 12-like*	2	Tandem duplication (987 bp and 981 bp)	Figure 2b; marked N
MIR159B				
*Csa*_Chr17	*Threonyl-tRNAsynthetase (AT1G17960)*	2	Duplication	Supplementary Fig. 2A
*Bjun*_A06	*MOTHER of FT and TFL1 (AT1G18100)*	2	Duplication	Supplementary Fig. 2A
*C.gra*_Scff2175 & *Bjun*_B04	*Probable phosphatase 2C 8 (AT1G18030.1)*	2	Duplication	Supplementary Fig. 2A

Table 2 (continued)

Fragments		Copies	Status	Figure
*C.gra*_scaffold2175	*UNC93 1, Probable phosphatase 2C 8, Cyclin-dependent kinase D-3 and Translation elongation factor EF1A/initiation factor IF2gamma family protein*	2	Segmental duplication with the entire segment being tandemly duplicated	Supplementary Fig. 2A; boxed and marked a, b
*Csa*_Chr3	*Nuclear poly (A) polymerase 1 (AT1G17980.1)*	2	Tandem duplication	Supplementary Fig. 2A
*Csa*_Chr17	*Laccase-1 (AT1G18140.1)*	2	Tandem duplication	Supplementary Fig. 2A
*Csa*_Chr17, *S. irio*_scff491, *A. lyrata*_scff1, and *Crub*_Scff1	*UNC93 1*	2	Tandem duplication of UNC93 1 AT1G18000.1 and AT1G18010.1; tandemly duplicated in *A. thaliana*	Supplementary Fig. 2A
A. thaliana and *Alyr*_Scff1	*FMN-linked oxidoreductases superfamily (At1g17990.1 and At1g18020.1)*	2	Duplicated in *Arabidopsis*-specific manner in *A. thaliana* and *Alyr*_Scff1	Supplementary Fig. 2A; marked as oval with asterisk
MIR159C				
A. lyrata	*TGF-beta receptor interacting protein 1/eukaryotic translation initiation factor 3 subunit*	2	Duplicated in lineage-specific manner	Supplementary Fig. 2B
S. irio	*BAG6/BCL-2-associated athanogene 6*	2	Duplicated in lineage-specific manner	Supplementary Fig. 2B
*Csa*_Chr5 & *Csa*_Chr6	*Myosin heavy chain-related*	2	Duplicated in lineage-specific manner	Supplementary Fig. 2B
*Bna*_C04	*Unknown protein*	2	Duplicated in lineage-specific manner	Supplementary Fig. 2B
*Bjun*_B03	*PIN domain-like family protein*	2	Duplicated in lineage-specific manner	Supplementary Fig. 2B

Table 3 Duplication, deletion, or retention of protein-coding genes in genome fractionation (LF, MF1, and MF2) harboring three members of *MIR159* across Brassicaceae

Fragments		Presence				Figure	Remarks
		LF, MF1 and MF2	LF and MF1	MF1 and MF2	LF and MF2		
MIR159A							
B. rapa	*Alpha-dioxygenase 2-like and ethylene insensitive 3-like 3*	Yes				Supplementary Fig. 5I; marked A and B	
B. oleracea	*Alpha-dioxygenase 2-like and ethylene insensitive 3-like 3*	Yes				Supplementary Fig. 5II; marked A and B	
B. oleracea	*Detoxification 17 and Suppressor of mec-8 and unc-52 homolog 1*	No			Yes	Supplementary Fig. 5II; marked D and E	
B. oleracea	*Nudix hydrolase chloroplastic-like, Lipid-transfer vas, Auxin efflux carrier component 1-like*	No	Yes			Supplementary Fig. 5III; marked F, G and H	
B. napus	*Probable inactive serine–threonine-kinase fnkc gene*	No			Yes	Supplementary Fig. 5III; marked C	
B. napus	*Alpha-dioxygenase 2-like*	Yes					
C. sativa	*RNA-binding (RRM RBD RNP motifs) family gene*	No	Yes			Supplementary Fig. 5V; marked C	
C. sativa	*Unknown protein*			Yes			
MIR159B							
B. rapa	*Nuclear poly(A) polymerase1*	Yes				Supplementary Fig. 6I; marked A	Two copies in LF segment
B. rapa	*Guanine nucleotide-binding beta subunit*	Yes				Supplementary Fig. 6I; marked B	
B. oleracea	*Nuclear poly (A) polymerase 1*	Yes				Supplementary Fig. 6II; marked A	
B. oleracea	*Homeobox-leucine zipper HDG12-like*		Yes			Supplementary Fig. 6II; marked C	
B. oleracea	*Eukaryotic peptide chain release factor GTP-binding subunit ERF3A-like*		Yes			Supplementary Fig. 6II; marked D	
B. oleracea	*Small ubiquitin-related modifier 5-like*		Yes			Supplementary Fig. 6II; marked E	
B. oleracea	*Probable calcium-binding CML27*		Yes			Supplementary Fig. 6II; marked F	
B. oleracea	*Guanine nucleotide-binding subunit beta*				Yes	Supplementary Fig. 6II; marked B	
B. oleracea	*MOTHER of FT and TFL1*				Yes	Supplementary Fig. 6II; marked G	
B. oleracea	*Mitogen-activated kinase 8 isoform X1*				Yes	Supplementary Fig. 6II; marked H	
B. oleracea	*Cyclin-dependent kinase D-3*			Yes		Supplementary Fig. 6II; marked I	
*B. napus*A	*Transcription factor BTF3 homolog 4*	Yes				Supplementary Fig. 6III; marked J	

Table 3 (continued)

Fragments		Presence				Figure	Remarks
		LF, MF1 and MF2	LF and MF1	MF1 and MF2	LF and MF2		
*B. napus*A	*Nuclear poly(A) polymerase 1-like*	Yes				Supplementary Fig. 6III; marked A	Two copies in LF segment
*B. napus*A	*Homeobox-leucine zipper protein HDG12-like*		Yes			Supplementary Fig. 6III; marked C	
*B. napus*A	*Eukaryotic peptide chain release factor GTP-binding subunit ERF3A-like*		Yes			Supplementary Fig. 6III; marked D	
*B. napus*A	*Golgin candidate 2*		Yes			Supplementary Fig. 6III; marked K	
*B. napus*A	*Cyclin-dependent kinase D-3*			Yes		Supplementary Fig. 6III; marked I	
*B. napus*A	*Guanine nucleotide-binding subunit beta*				Yes	Supplementary Fig. 6III; marked A	
*B. napus*A	*MOTHER of FT and TFL1*				Yes	Supplementary Fig. 6III; marked G	
*B. napus*A	*Mitogen-activated kinase 8-like isoform X1*				Yes	Supplementary Fig. 6III; marked H	
C. sativa	*Serine–threonine-kinase EDR1-like*	Yes				Supplementary Fig. 6V; marked M	Two copies in MF1
C. sativa	*Aminotransferase-plant mobile domain family*	Yes				Supplementary Fig. 6V; marked N	Two copies in MF2
C. sativa	*Golgin candidate 2 isoform X1*	Yes				Supplementary Fig. 6V; marked M	
MIR159C							
C. sativa	*PREDICTED: uncharacterized protein LOC104734395*		Yes			Supplementary Fig. 7V; marked E	
C. sativa	*Glycosyl hydrolase family*		Yes			Supplementary Fig. 7V; marked F	
C. sativa	*PREDICTED: uncharacterized protein LOC104784679*				Yes	Supplementary Fig. 7V; marked G	

lipid-transfer 2-like (marked T), *SAR-Deficient 1* (marked U), *Core-2 I-branching beta-1,6-N-acetylglucosaminyltransferase* family (marked V), are shared between LF and MF2 fractions of which *Core-2 I-branching beta-1,6-N-acetylglucosaminyltransferase* family has three copies in LF fraction. Some more examples of duplication and deletion are summarized in Table 3.

MIR159B

Analysis of homologous fragments corresponding to sub-genomes revealed loss of *MIR159B* in several genomes as a result of genome fractionation as it was detected in only LF fractions of *B. rapa, B. oleracea*, and *B. napus*A, and completely deleted from all three sub-genomes of C genome in *B. napus*. *MIR159B* was found to be present on all the sub-genomic fractions of *C. sativa*.

In *B. rapa*, the three sub-genomic fragments have variable number of genes with LF-A06 (29 genes) fraction genomic segment having the most number of genes (MF1-A08-21 genes and MF2-A09-17 genes). Most of the genes are unique to respective genomic fragments, and only 5, 2, and 3 genes are shared among LF-MF1, LF-MF2, and MF1-MF2 genomic fragments, respectively (Table 3).

In *B. oleracea*, sub-genomic segments on C06-1 (LF), C02 (MF1), and on C06-2 (MF2) contain 60, 31, and 17 genes, respectively, with most of the genes unique to sub-genomes. A single gene—*Nuclear poly (A) polymerase*

1 (Supplementary Fig. 6II; marked A)—was found to be shared by all three sub-genomes; only four genes shared between LF and MF1 sub-genomes; three genes between LF and MF2; and only a single gene, *Cyclin-dependent kinase D-3* (Supplementary Fig. 6II; marked I) shared among MF1 and MF2 (Table 3).

The sub-genomes of the A genome of *B. napus* contain highly variable number of genes, 43 in LF (A06), 17 in MF1 (A08), and 15 in MF2 (A09). Most of the genes from LF have no homologs on MF1 and MF2. *Eukaryotic peptide chain release factor G5TP- binding subunit ERF3A* gene (Supplementary Fig. 6IV; marked as P) was found to be shared across all the sub-genomic fractions of *B. napus* C, indicating that deletion of *MIR159B* is not because of the deletion of the whole segment (Table 3; Fig. 4; Supplementary Fig. 6IV).

In *C. sativa*, LF and MF1 contain 38 genes each, and MF2 contains 34 genes. Four genes are shared only between LF and MF1, and five between only LF and MF2. A single gene *Peptidyl-prolyl cis-trans isomerase FKBP17-chloroplastic-like* (Supplementary Fig. 6V; marked as O) is shared between only MF1 and MF2. Most of the other genes are shared among all the three segments (Supplementary Fig. 6V; Laccase 1-marked L; 2 copies in LF).

MIR159C

Genome fractionation analysis in *B. rapa* revealed that the three sub-genomic segments harbor gene numbers ranging from only 3 genes (MF1), 11 (MF2), and 38 in LF (Supplementary Fig. 7I), with no shared genes among all the three sub-genomes. Only five genes were found to be shared between LF and MF2 fraction segments.

The MF1 sub-genomic segment of A-genome in *B. napus* was found to contain several incomplete sequence stretches, and was thus excluded from analysis. Seven genes are shared between LF (total 33 genes) and MF2 (total 13 genes) sub-genomic segment. The sub-genomic segments—LF, MF1, and MF2 of C genome in *B. napus*—contain 44, 8, and 16 genes, respectively. Two genes (Supplementary Fig. 7IV; marked A and B) are shared among all three fractions; LF and MF1 share four genes, while LF and MF2 share five (Fig. 4a; Table 3).

In *C. sativa*, three sub-genomic segments LF (Chr04), MF1 (Chr06), MF2 (Chr05) contain 35, 39, and 34 genes of which 27 genes are common to all. Two genes—*Myosin heavy chain* and *Eukaryotic translation initiation factor 3 subunit I isoform X1*—from LF sub-genome are duplicated on MF1 and/or MF2 segments (Supplementary Fig. 7V; marked C and D).

Comparison of synteny across the sub-genomic fractions for *MIR159A* (Supplementary Fig. 5), *MIR159B* (Supplementary Fig. 6), and *MIR159C* (Supplementary Fig. 7) revealed that among all the genomic segments, *C. sativa* displayed most number of genes to be conserved in all the fractions (Fig. 4). Gene density was found to be as high as one gene/3.37 kb (*Bnapus*CMF2) to as low as one gene/7.53 kb (*Bol*_LF) in *MIR159A* genomic segments; from one gene/2.87 kb (*Brapa* MF2) to one gene/7.0125 kb (*Bnapus*C LF) in *MIR159B* segments; and from one gene/3.39 kb (*Bnapus*A MF2) to as low as one gene/8.82 kb (*Brapa* MF1) in *MIR159C* segments (Fig. 4b).

Segmental duplication

Phylogenetic reconstruction revealed that the paralogous relationship between *MIR159A* and *MIR159B* is specific to Brassicaceae. Whether this paralogy arose as a result of local duplication or as a part of segmental duplication was analyzed by performing pairwise synteny analysis between paralogs, i.e., between genomic segments harboring *MIR159A* and *MIR159B*, across genomes of Brassicaceae. Eight genes apart from *MIR159A–MIR159B* were paralogous and syntenic in *S. irio*, six genes in *A. thaliana* (Fig. 5a) and *C. rubella*, five in *(A) lyrata*, four in *(B) stricta*, between one and three genes in *(C) sativa*, and a single gene in *B.rapa*—MF2 and *B. oleracea*—LF (Fig. 5). The *Serine–threonine-kinase EdR-like* was found to be retained across paralogous blocks in majority of the genomes (Fig. 5; brown box).

Phylogeny of MYB33, MYB65, and MYB101

We estimated the phylogenetic relationship between 67 homologs of selected members of MYB family—*MYB33, MYB65*, and *MYB101*—from Brassicaceae using the CDS data set through GTR and doublet models implemented under the Bayesian method. These are reported to be PTGS targets of *miR159* in *A. thaliana*. We also compared the conservation and divergence in the microRNA-binding site (MBS), along with the conservation of the mature 21-nt miRNA sequence along the phylogenetic cluster.

Homologs of *MYB33* were not detected in any of the *Brassica* species. We observed three distinct clusters for the three MYBs with *MYB33* and *MYB65* sharing a recent common ancestor (Fig. 6). Within each of the clades, the tree topology reflected the clustering of homologs of base genome, and homoeologs from sub-genomes together. For instance, homologs from *Arabidopsis* species, *Capsella* species, *Thellungiella* species grouped with each other; similarly, homoeologs from LF1 of A genome, B genome, and C genome grouped together (and so on for MF1 and MF2). We further located the MBS within the sequences, and analyzed sequence and length polymorphisms in Brassicaceae (Supplementary Fig. 8A, B; Fig. 7). The mature *miR159* sequence was found to be conserved across the Brassicaceae except in *MIR159B* of *Brassica* species, and in the *MIR159C* derived from B genome

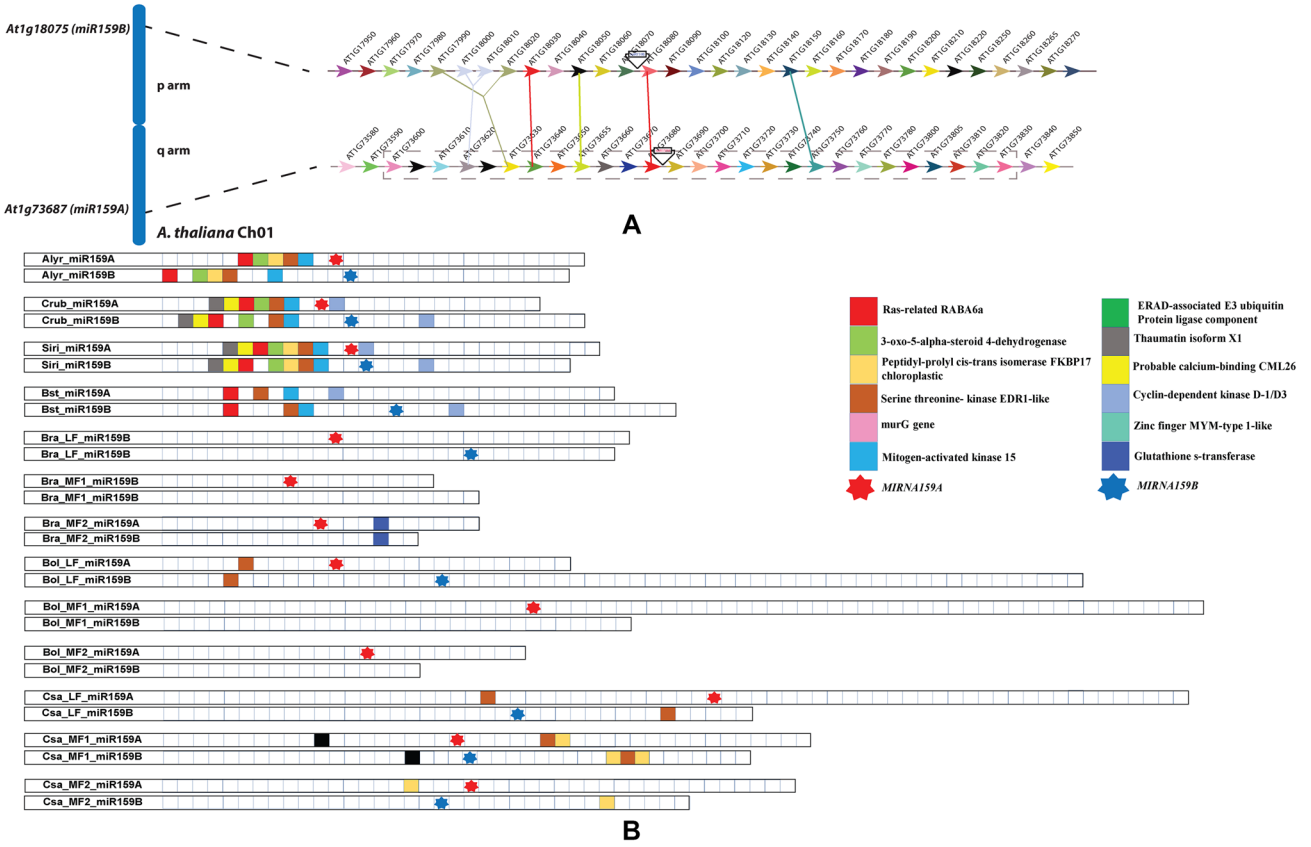


Fig. 5 Analysis of synteny between genomic segments harboring *MIR159B* (*At1g18075*) and *MIR159A* (*At1g73687*) to establish segmental duplication as a cause of paralogy. **a** Diagrammatic representation of genomic segment containing *MIR159B* and *MIR159A* present on the p and q arms, respectively, on chromosome 1 of *A. thaliana*, shows the presence of six common genes. **b** Diagrammatic representation of comparison of genes present in genomic segment containing *MIR159B* and *MIR159A* for 11 genomes (Ath—*A. thaliana*, Alyr—*(A) lyrata*, Crub—*C. rubella*, Siri—*S. irio*, and genomic fractions of Csa—*C. sativa*, Bol—*(B) oleracea*, and Bra—*B. rapa*). Colored blocks represent the genes in the each genomic segment. (Color figure online)

(*B. nigra* and *B. juncea*). Analysis of the MBS showed that the miRNA-binding region was entirely missing in *MYB65* from A genome in *B. napus* (Fig. 7; Supplementary Fig. 8A, B). The MBS, especially the target cleavage site (at 9/10 position, 5′-TTCA-3′), was found to be conserved within the homologs of *MYB65* and *MYB33* across members of Brassicaceae (Fig. 7a). The MBS in *MYB101*, however, showed variation. In *A. thaliana*, the MBS was similar/identical to that found in *MYB65/33*, especially with reference to the nucleotide composition at the putative cleavage site at 9/10 position (TTCA/TTCT) (Fig. 7b). In all the other members of Brassicaceae, the putative MBS along with the cleavage site revealed sequence polymorphisms—ACCG in *Arabidopsis lyrata* and TGCG in the rest of the species (Fig. 7; Supplementary Fig. 8).

Discussion

Understanding the genome organization, evolutionary history, and genomics of regulatory elements, especially of those in polyploid genomes, and in crop species remains a major challenge and is an important area of research. To the best of our knowledge, this is the first detailed analysis of the evolutionary history of *MIR159* family and co-evolution of miRNA-binding sites (MBS) in PTGS targets. Phylogenetic reconstruction across green plants revealed paralogy between *MIR159A–MIR159B* only in Brassicaceae. Analysis and comparison of phylogeny and synteny both across orthologous and paralogous segments

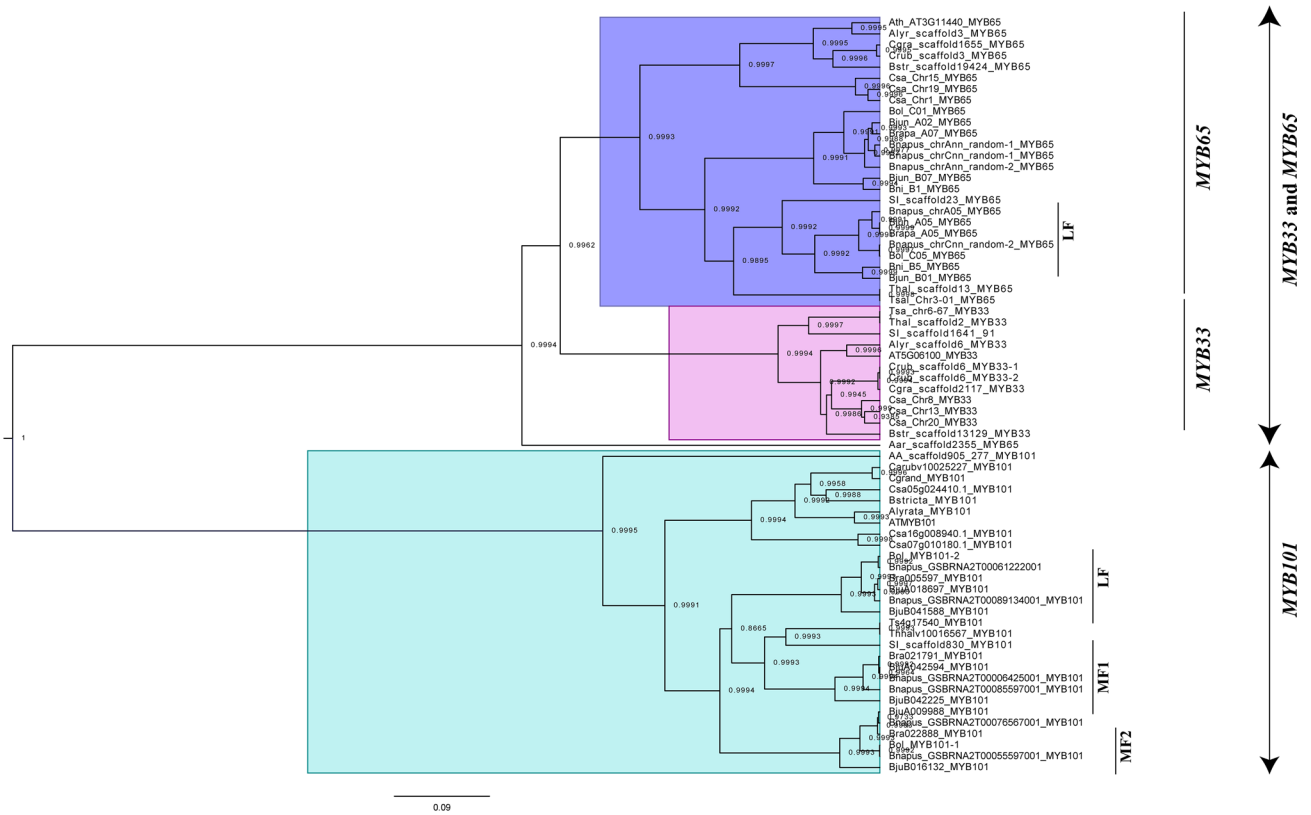


Fig. 6 Phylogenetic reconstruction of homologs of *MYB33, MYB65*, and *MYB101* from Brassicaceae using GTR and doublet model of Bayesian method. *MYB33* (Magenta) and *MYB65* (Blue) form sister clade. *MYB33* was found to be completely absent from *Brassica*. (Color figure online)

across Brassicaceae implicate a segmental duplication in ancestral Brassicaceae being responsible for origin of *MIR159A–MIR159B* paralogy. The impact of polyploidy including genome fractionation was evident when homoeologous segments were analyzed with respect to gene content and conservation status. Homology search indicated that *MYB33* is completely lost in *Brassica* species, but retained in rest of the members of Brassicaceae. A comparison of the mature miRNA from *miR159a, miR159b*, and *miR159c*, and the miRNA-binding site (MBS) of the PTGS targets showed that the mature miRNA isoforms are capable of targeting *MYB65* across Brassicaceae, *MYB33* in all species except *Brassica*, and *MYB101* only in *A. thaliana*. Results from the present study, thus, reveal novel insights into the genomics and evolution of *MIR159* family, and comparative analysis of the regulatory pair of *miR159—MYB33/MYB65/MYB101* unraveled intricacies between components of regulatory module that are involved in sequence-dependent interactions, such as in post-transcriptional gene silencing (PTGS). The full impact of polyploidization, genome fractionation, and sequence polymorphism in the mature miRNA and the cognate targets on functional diversification including neo- and sub-functionalization can only be quantified when functional analysis of the regulatory modules vis-à-vis their role in various developmental and adaptive processes are undertaken in future.

A combinatorial analysis that investigates genome organization and structure in a phylogenetic context is useful to gain insights into evolutionary and functional aspects of modules of regulatory elements that act in pairs. An example is miRNA–PTGS target as module pairs in plants that largely function through post-transcriptional gene silencing (PTGS) based on strict criteria of perfect or near-perfect sequence complementarity (Voinnet 2009; Axtell and Bowman 2008). Polyploid genomes with paralogous copies can accumulate polymorphisms and exhibit sequence diversity in either component of the module. Such polymorphisms can lead to disruption of function and possibility of perturbation of existing networks, and may allow the formation of novel regulatory interactions and networks. The present investigation was designed to understand the evolutionary history of *MIR159*, a three member gene family in *A. thaliana* with mature 21-nt isoforms that differ by one nucleotide (Allen et al. 2010); they regulate several developmental processes such as transition to flowering time (Achard et al. 2004; Millar and Gubler 2005), anther development (Allen et al. 2007), and biotic (Du et al. 2014) and abiotic (Li et al.

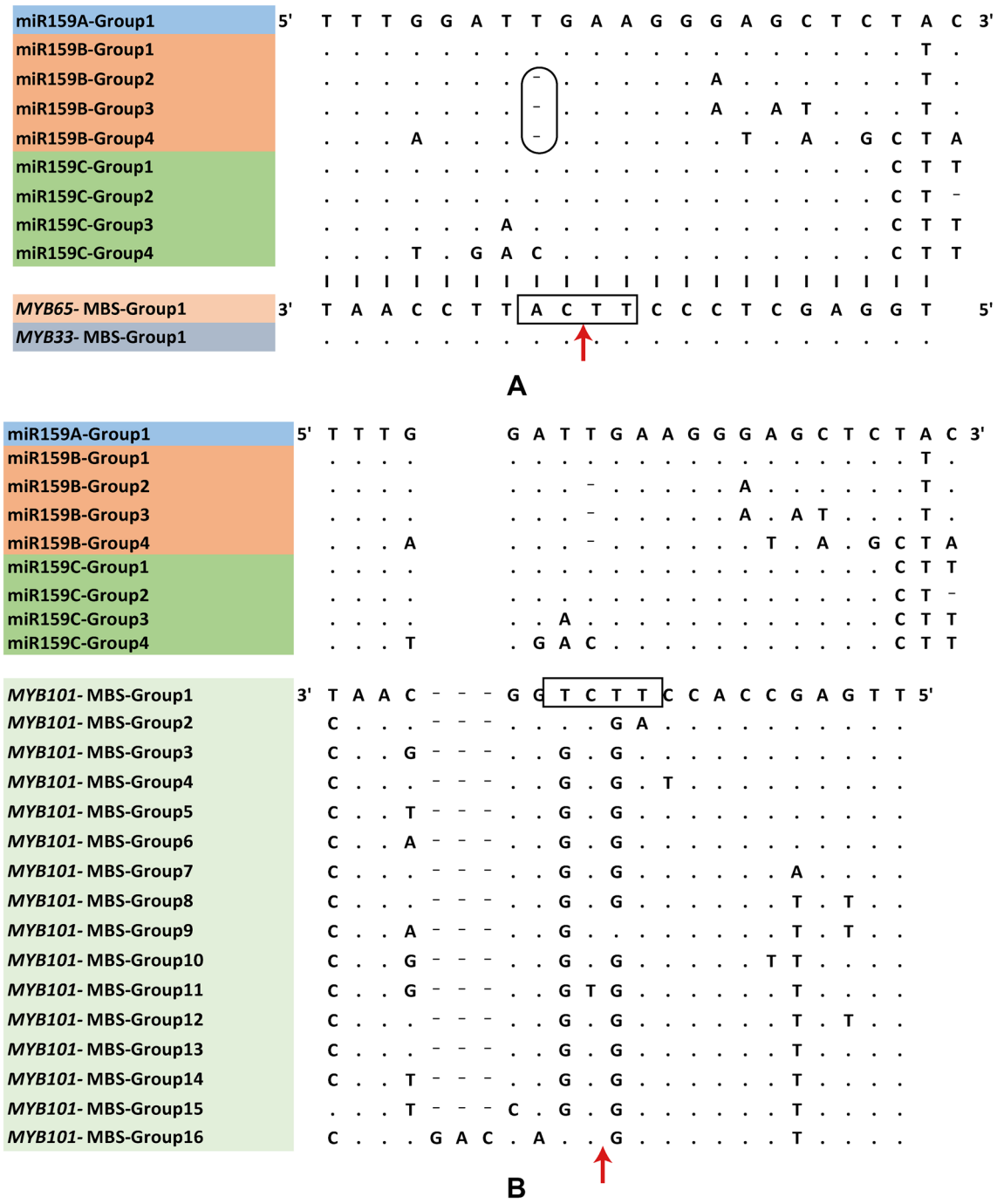


Fig. 7 Sequence comparison of mature 21-nt *miR159A, miR159B*, and *miR159C* to the miRNA-binding site (MBS) in the target transcription factors—*MYB33, MYB65*, and *MYB101*—across Brassicaceae to reveal polymorphism and conservation. Sequence alignment revealed sequence divergence and the genomes can be categorized into various groups (details in Supplementary Fig. 8). Group 1 refers to *A. thaliana* homolog in each category. Identical nucleotides are marked by dot; any polymorphism is marked by respective nucleotide; sequence complementarity is marked by vertical line. Boxed region and arrow indicates seed region (9/10) and cleavage site. *Brassica* specific deletion of "T" can be observed in *miR159B* group 2, 3, and 4 (boxed). Maximum conservation in miRNA-binding site (MBS) and mature miRNA was detected in *MYB33* and *MYB65* (**a**). *MYB101* shows extensive sequence polymorphism at MBS across Brassicaceae (**b**). (Color figure online)

2016) stress responses. The regulation of selected member of *MYB*s-*MYB33, MYB65*, and *MYB101* by *miR159a, miR159b*, and *miR159c* through strict complimentary base pairing via PTGS is known (Palatnik et al. 2007; Li et al. 2011). However, evolutionary history, impact of polyploidy on *MIR159* gene family, sequence variation, if any, encountered in the mature 21-nt sequence in *MIR159* family in the other species outside *A. thaliana*, spectrum of potential PTGS targets, and on miRNA-target pairing that is critical for sequence-dependent PTGS remains unexplored.

Our mining of plant databases revealed that, from a single copy in *Physcomitrella patens* and *Selaginella moellendorffii, MIR159* has undergone several events of genome and family-specific expansion, and we could detect as many as 11 copies in *B. juncea* and *Zea mays* and 10 copies in *B. napus* and *O. sativa*. The single copy in *Selaginella moellendorffii* coincides with the lack of evidence of whole-genome duplication (Baniaga et al. 2016; Banks et al. 2011; Jiao et al. 2011). Phylogenetic reconstruction revealed the clustering of homologs in a family-/lineage-specific manner, implying that the expansion in gene family is an outcome of family-/lineage-specific expansion events. Such expansion of gene families along the plant phylogenetic tree has also been demonstrated in KCS gene family (Singh et al. 2018). A clear demarcation and paralogy between *MIR159A* and *MIR159B* was detected only in Brassicaceae. We, thus, analyzed data from members of Brassicaceae, exploiting the availability of a number of complete genome sequences including those after various stages polyploidization such as paleo-polyploidy (*A. thaliana*; Blanc and Wolfe 2004), meso-polyploidy (*B. rapa*/*B.oleracea*; Wang et al. 2011a, b), and neo-polyploidy (*B. napus; C. sativa;* Chalhoub et al. 2014; Kagale et al. 2014). Brassicaceae-specific whole-genome duplication events such as mesoploidy (triplication in *B. rapa* and *B. oleracea*, allo-tetraploidization in B. *napus* and *B. juncea*) and neopolyploidy (hexaploidy in *C. sativa*) resulting in duplication, retention, and losses of several genes, and has been proposed to be responsible for evolution of a group of plants diverse in form and function (Kellogg 2016; Tank et al. 2015). In the present study, synteny across Brassicaceae revealed origin and expansion of genes in genome- or lineage-specific manner (e.g., homolog *Brassica*—lineage-specific deletion of *AT1G73770; Thellungiella*—lineage-specific duplication of Cysteine-rich receptor kinase 10), and disruption of synteny.

Genomes of *Brassica* and *C. sativa* have undergone triplication in past, and thus, the genomes of *B. rapa, B. nigra, B. oleracea*, and *C. sativa* are composed of three distinct sub-genomes annotated as LF, MF1, and MF2, with the pattern of gene retention being LF > MF1 > MF2 (Wang et al. 2011a, b). We expected at least three copies of the MIRNA genomic segments in *B. rapa, B. nigra, B. oleracea*, and *C. sativa*; and six copies in *B. napus* and *B. juncea*. The present study did not show a clear pattern of LF > MF1 > MF2 among the *MIR159* genomic segments. In contrast, the previous comparative genomic analyses have revealed that miRNA-encoding genes are subjected to similar rules of genome and sub-genome fractionation and diversification as protein encoding genes do (Jain and Das 2016).This discordance implies that rules of genome fractionation are not uniform across the entire genome landscape.

When synteny analysis was correlated with results obtained from genome fractionation analysis using homologous segments, the extent and the complexity of gain and loss of genes and genetic elements become evident. For instance, *MIR159B* is retained in only LF fractions of *B. rapa* (A genome), *B. oleracea* (C-genome), and *B. napus* A genome but not in any of the sub-genomes of C genome of *B. napus* (including LF) implying *B. napus* C-genome-specific loss of LF counterpart. In contrast, *Nuclear poly (A) polymerase* and *EF1A* were found to be retained across all the sub-genomes of A and C genomes in *MIR159B* segment. *EF1A* is involved in translation termination in response to termination codons and stimulates the activity of *ERF1* (Valouev et al. 2002). *Nuclear Poly (A) Polymerase* generates 3′-poly (A) tail of mRNAs and also required for endoribonucleocytic cleavage reaction at some polyadenylation sites (Proudfoot 2011). Conservation of such genes across all fractions probably reflects their indispensable role in transcriptional and translational processes. A detailed investigation on the selection pressure operative on the genetic elements flanking *MIR159* family will throw an additional light on the evolutionary trajectory. Loss/retention and copy-number expansion of genes in multiple genomes and sub-genomes are best addressed through character and phylogenetic state reconstruction as was shown recently (Singh et al. 2018). Synteny and genome fractionation analysis revealed extensive gene loss and gain in *Brassica* species as compared to *C. sativa* given the older age of polyploidization in *Brassica* lineage than *Camelina* (Kagale et al. 2014).

Phylogenetic analysis among green plants clearly revealed that *MIR159A* and *MIR159B* share a result of paralogous relationship limited to Brassicaceae. A combination of synteny among the paralogous segments of *MIR159A* and *MIR159B* in a pairwise manner across each genome showed the presence of several other genes other than *MIR159A–MIR159B* raising the probability that the genomic segments harboring *MIR159A* and *MIR159B* have arisen as an outcome of segmental duplication. *MIR159B* and *MIR159A* are present on the top arm and bottom arm of chromosome 1 of *A. thaliana*, which has been shown to be related by a large segmental duplication and also responsible for expansion of *MIR395A–B–C* and *MIR395D–E–F* family and *KCS5–KCS6* paralogy (Rathore et al. 2016; Singh et al. 2018). Taken together data that have been previously published (Rathore et al. 2016; Singh

et al. 2018) and data obtained in the present investigation, we can conclude that *MIR159A–MIR159B* paralogy arose due to a segmental duplication that is shared across Brassicaceae.

Small RNAs such as miRNAs involved in the regulation of their targets through PTGS require precise base pairing. This is in contrast to translational repression where a relaxed pairing criterion is applicable (Carthew and Sontheimer 2009). It is evident that the 21-nt mature miRNA sequence, and the MBS in the target are under a strict selection pressure which does not permit any mutation to accumulate (Zhang et al. 2006). Any mutation in mature miRNA or in the MBS of the target, thus, has the potential to disrupt the RNA:RNA interaction necessary for PTGS, and can lead to formation of novel regulatory interaction (Chen and Rajewsky 2007; Wang and Adams 2015). The probability of such disruptions in interaction is higher in polyploid genomes.

The potential targets of *MIR159—MYB33, MYB65*, and *MYB101*—in *A. thaliana*, are post-transcriptionally silenced through target cleavage (Li et al. 2011; Zheng et al. 2017). Mere presence of miRNA-binding site (MBS) in the target transcript is not sufficient to ensure PTGS through target cleavage; accessibility of the MBS in the target is governed through the formation of secondary structure and polymorphism in the sequences flanking MBS that influences secondary structure of the target has been shown to be responsible for efficiency of cleavage of *miR159* target across plants (Zheng et al. 2017). The MBS for *miR159* was found to be conserved among eight MYB-TFs including *MYB33, MYB65, MYB81, MYB97, MYB101, MYB104, MYB120*, and *DUO1*. However, the flanking sequences—100 bp each on 5′- and 3′- of MBS of only *MYB33* and *MYB65*—was predicted to permit the formation of a RNA stem-loop structure which correlated with highly efficient cleavage of the transcripts by *miR159* (Zheng et al. 2017). We did not detect any homologs of *MYB33* in *Brassica* species leaving only *MYB65* and *MYB101* as being potential targets in *Brassica*. A comparison of sequence complementarity between *mi159a/b/c* isoforms and *MYB33/MYB65/MYB101* revealed that *MYB65* is a universal potential target across Brassicaceae, *MYB33* remains a target in all species examined except *Brassica* species, and *MYB101* acts as a target only in *A. thaliana*. Existence of such sequence polymorphism in MBS of *MYB101* can lead to change in spectrum of target leading to specialization.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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