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Research Article

miRNA expression differentiation induced by polyploidization in newly formed triploids of black poplar

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Abstract

During whole genomic duplication (WGD) events, micro RNAs (miRNAs) are involved in stabilization of chromatin and genome and epigenetic regulation of gene expression. In this study, a newly induced triploid group of hybrids between sect. *Tacamahaca* and sect. *Aigeiros* in *Populus,* was characterized for genome-wide miRNA expression after WGD. Seven miRNA libraries (male parent, female parent, group of triploid offspring's, group of diploid offspring, and three triploid individuals) were constructed and variation of miRNA expression from diploid parents to triploid offspring's as well as distinction between triploid and diploid offspring were analyzed. The results showed that a total of 240 miRNAs were predicted including 187 known miRNAs and 53 novel miRNAs. 81.25% of miRNAs in triploid offspring were non-additively expressed in which 52.31% were down-regulated. A novel miRNA with 24nt in length choosing adenine as its first base was found in triploid offspring group suggesting its potential role in regulation of DNA methylation after WGD. A total of 18 novel miRNAs were specifically expressed in the library of triploid group. Targeted genes of different expressed miRNAs in three comparison sets (triploid offspring group vs female parent, male parent, and diploid offspring group) were all enriched in ADP binding (GO: 0043531; FDR < 0.05). KEGG enrichment pathway of all three comparison sets was plant-pathogen interaction. This study revealed an essential role of miRNAs involving in epigenetic regulation after WGD in poplar and provided a good model for further studies of polyploidization advantages in woody plant.

Keywords: miRN; newly formed triploid; poplar polyploidization

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Introduction

Polyploidization has been indicated as one of the major dynamics in plant evolution (*Arabidopsis* Genome Initiative, 2000; Chalhoub *et al*., 2014; Vanneste *et al*., 2013; Wendel *et al*., 2016; Qiao *et al*., 2019; Renny-Byfield and Wendel, 2014; Peer *et al*., 2017; Stull *et al*., 2021) and newly formed polyploid plant will undergo various levels of responses to deal with the "genome shock" brought by whole genomic duplication (WGD) (Jonathan *et al*., 2016). In the first few generations to the formation of a polyploid plant, massive and extensive responses over DNA-level and expression-level to recover stability and fertility were rapidly activated (Lynch and Conery, 2000; Ozkan *et al*., 2001; Soltis *et al*., 2015; Gyorfy *et al*., 2021; Birchler and Yang, 2022). Expression-level responses including sub functionalization and neofunctionalization of gene expression patterns (Hughes *et al*., 2014; Liu *et al*., 2015; Shi *et al*., 2020) were thought to be associated with some epigenetic regulatory mechanism and interactive network such as DNA methylation and small RNAs (sRNAs) downstream regulation especially in the newly formed polyploidy plants (Chen, 2007; Abrouk *et al*., 2012; Ding and Chen, 2018). Small RNAs have suppressive activity and act as modulators and mitigating factors of the "genome shock" effects brought by polyploidization (Chen, 2007; Ha *et al*., 2009).

Micro RNAs (miRNAs), as the most widely-studied subset of sRNAs playing an essential role in epigenetic regulation, are divided into two secondary groups: canonical miRNAs (cmiRNAs) and long miRNAs (lmiRNAs) which have effects on target degradation and DNA methylation, separately (Axtell, 2013). cmiRNAs with 21nt in length, precursors of which are cleaved and processed by Dicer-like 1 (DCL1), are incorporated into Argonaute 1 (AGO1) protein and have silencing effects on their targets in post-transcriptional level through mRNA cleavage (Carrington and Ambros, 2003; Mallory and Vaucheret, 2006; Kim *et al*., 2009). Another kind of miRNAs with the length of 23- to 27-nt which are generated from precursors by DCL3 (Chellappan *et al*., 2010) associate with AGO4 and direct RNA dependent DNA methylation (RdDM) at loci both from where they are produced and *in trans* at their target genes (Wu *et al*., 2010).

Coevolution of miRNAs and their targets over millions of years after WGDs has been proven by many researches. Like protein-coding genes, *MIRNAs* generating precursors of miRNAs are subjected to duplication and subsequent multiple regulatory mechanisms underlying polyploidization (Zhao *et al*., 2015) which causes common emergence of lineage-specific miRNAs (Chávez Montes *et al*., 2014) that is potential indication of sub functionalization and neofunctionalization. Besides, the original balance between miRNAs and their targets is broken in this process of evolution and a new equilibrium is established after the WGD event (Abrouk *et al*., 2012). However, research on rapid responses of miRNA level in a few generations after WGD is limited. How miRNAs changes in newly formed polyploid plants especially in trees is not well studied.

Natural accessions of polyploids have experienced varied and extensive genomic alteration including chromosome rearrangements and genomic diploidization which finally due to an established genome that means massive information of immediate response and process of differential gene expression patterns are lost (Wendel *et al*., 2016; Qiao *et al*., 2019; Zhang *et al*., 2021). Synthetic polyploids, necessaries in study of early genetic and downstream responses and regulations to WGD events, are applied to the research of polyploidization effects in the model plant *Arabidopsis thaliana* (Wang *et al*., 2004) and important crop wheat (Li *et al*., 2014). Material system in this study was developed according our previous work (Xu *et al*., 2019). Poplar triploids were achieved by hybridization of *Populus simonii* Carr × *P. nigra* var. *Italica* (Moench.) Koehne with *P. simonii* × (*P. pyramidalis* + *Salix matsudana*) after embryo sac doubling. We analyzed the sRNA sequencing results and compared the different expression patterns of miRNAs and their targets between the triploid line and their diploid parents and full sibs.

Materials and Methods

Plant materials

Triploids of poplar were achieved by hybridization between *P. simonii* Carr × *P. nigra* var. *italica* (Moench.) Koehne and *P. simonii* × (*P. pyramidalis* + *Salix matsudana*). cv., female parent of which experienced artificial chromosome doubling of embryo sac (Xu *et al*., 2019). Leaf materials for RNA isolation were selected from biennial cuttings of the two parents, seedlings of triploid and diploid offspring, separately.

Total RNA isolation and small RNA library construction

Full expanded leaves at second or third position from morphological apex were collected at 9:00 am and immediately frozen in liquid nitrogen for subsequent experiment. Total RNA isolation was implemented adopting TRIzol reagent (Invitrogen, USA) following the procedure on manufacturer's instructions. Quality control of total RNA was performed before library construction including degradation and contamination detection using 1% agarose gel electrophoresis, RNA purity using value of OD260/OD280 measured by NanoDrop 2000, precise quantification of RNA concentration using Qubit 2.0 fluorimeter (Life Technologies, CA, USA), and RNA integrality assessment using RNA Nano 6000 Assay Kit of Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Small RNA Sample Pre Kit (Illumina, NEB, USA) was used for library construction. A total of seven libraries were constructed for subsequent analysis including female parent (DFP), male parent (DMP), triploid offspring pool with seven genotypes (TOM), diploid offspring pool with seven genotypes (DOM), and three individual genotypes random extracted from triploid offspring group (TO1, TO2, TO3). Libraries were quantified by Qubit 2.0 and diluted to 1ng/μl. Length of inserted fragment was evaluated by Agilent 2100 before precise quantification of library effective concentration (>2nM) using Q-PCR.

Sequencing and data analysis

High-throughput sequencing and bioinformatics analysis were processed by Novogene (Beijing, China). Library preparations were sequenced on an Illumina Hiseq 2500/2000 platform and 50bp single-end reads were generated after cluster generation on cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumia, NEB, USA). Raw data were filtered according common pipeline and small RNA tags were mapped to *Populus trichocarpa* genome (*http://popgenie.org*) by Bowtie (Langmead, 2009).

Mapped small RNA tags were used to obtain known miRNA by referring them to miRBase20.0. The secondary structures were drawn by modified software mirdeep2 (Friedlander *et al*., 2011) and srna-tools-cli. Novel miRNA identification was performed by the available software miREvo (Wen *et al*., 2012) after the hairpin structure of miRNA precursor was predicted. Target genes of miRNAs were predicted by psRobot_tar in psRobot (Wu *et al*., 2012).

Expression levels of miRNAs were normalized to transcript per million (TPM) according to the following criteria (Zhou *et al*., 2010): normalized expression = mapped read count/total reads*1,000,000. Differential expression of two samples was analyzed using DEGseq (2010) R package. qvalue<0.01 and | log2 (foldchange) | > 1 was set as the threshold for significantly differential expression by default.

Gene Ontology (GO) enrichment analysis based on target genes of differentially expressed miRNAs was performed online (*https://david.ncifcrf.gov/home.jsp*). Kyoto Encyclopedia of Gene and Genomes (KEGG) enrichment analysis was implemented online (*http://www.genome.jp/kegg/*). KOBAS software (Mao *et al*., 2005) was applied for statistical enrichment verification of target genes in KEGG pathways.

Results

Quality assessment and data filtering

A total number of seven libraries were sequenced on an Illumina Hiseq 2500/2000 platform that produced 10583302, 12546572, 9979983, 17639134, 11267517, 10309069 and 10607370 clean reads after filtering low quality reads and empty adaptor sequences, respectively (Table 1).

Table 1. Data filtration of triploid offspring group and its parents, diploid full sib group, and three individual triploids

Sample	Total reads	Clean reads
DFP (diploid female parent)	11,019,610	10,583,302 (96.04%)
DMP (diploid male parent)	13,034,030	12,546,572 (96.26%)
TOM (triploid offspring mixture)	10,416,344	9,979,983 (95.81%)
DOM (diploid offspring mixture)	18,165,987	17,639,134 (97.10%)
TO1 (triploid offspring 1)	11,970,059	11,267,517 (94.13%)
TO2 (triploid offspring 2)	10,991,861	10,309,069 (93.79%)
TO3 (triploid offspring 3)	11,208,482	10,607,370 (94.64%)

Overview of the sRNAomes

The clean reads represented around 1550525, 2783846, 1530452, 2530282, 1628459, 1379552 and 1467659 uniq sRNA sequences in the seven libraries, respectively. About 66.38, 61.65, 64.32, 75.79, 62.86, 71.91, 63.28% of clean reads with 18-30nt in length, respectively, were mapped to the reference genome (Table 2). Quite a number of unmapped sRNAs suggested that specific sRNAs were existed in the hybrids compared to *P. trichocarpa*.

Table 2. Summary of mapped results in triploid offspring group and its parents, diploid full sib group, and three individual triploids

Samples	Total sRNAs	Uniq sRNAs	Mapped sRNAs
DFP (diploid female parent)	7,806,426	1,550,525	5,182,277 (66.38%)
DMP (diploid male parent)	8,484,477	2,783,846	5,230,575 (61.65%)
TOM (triploid offspring mixture)	6,236,398	1,530,452	4,011,331 (64.32%)
DOM (diploid offspring mixture)	13,471,976	2,530,282	10,210,026 (75.79%)
TO1 (triploid offspring 1)	7,366,879	1,628,459	4,630,997 (62.86%)
TO2 (triploid offspring 2)	7,355,918	1,379,552	5,289,373 (71.91%)
TO3 (triploid offspring 3)	7,327,818	1,467,659	4,637,180 (63.28%)

Length distribution of sRNAs from 18 to 30nt showed interesting phenomena that was different from previous studies in *Populus* (Barakat *et al*., 2007; Ren *et al*., 2012). The 24nt class was most abundant in the two parents (Figure 1A, B) and both triploid and diploid offspring groups presented a similar trend to their parents (Figure 1C, D). However, ratios of 21nt-class sRNAs had a noticeable rise in the three libraries of individual triploid genotypes (Figure 1E-G), in two of which 21nt class showed the highest degree of redundancy among sRNAs with lengths between 18 and 30nt.

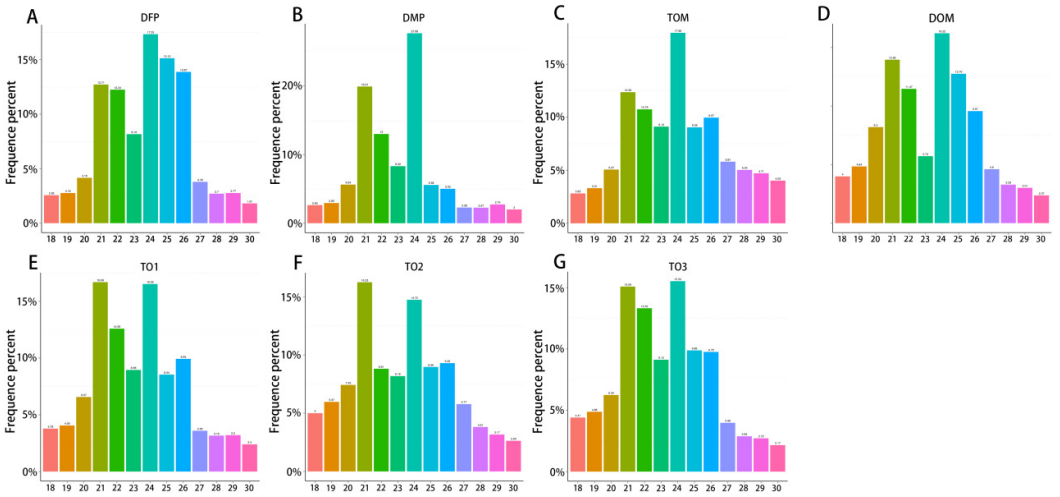

Figure 1. Length distribution of sRNAs from 18 to 30nt in seven libraries. (A) DFP, diploid female parent. (B) DMP, diploid male parent. (C) TOM, triploid offspring mixture. (D) DOM, diploid offspring mixture. (E) TO1, triploid offspring 1. (F) TO2, triploid offspring 2. (G) TO3, triploid offspring 3.

First base bias and length distribution of known and novel miRNAs in seven libraries

Summary of predicted miRNAs was seen in Table 3. A total of 240 miRNAs generating from 257 precursors in all seven libraries including 187 known miRNAs from 191 precursors and 53 novel miRNAs from 66 precursors, separately.

Table 3. Summary of known and novel miRNAs in triploid offspring group and its parents, diploid full sib group, and three individual triploids

Types	Mapped mature miRNA		Mapped precursor		Mapped uniq sRNA		Mapped total sRNA	
	Known	Novel	Known	Novel	Known	Novel	Known	Novel
DFP (2×)	146	43	159	54	1,739	238	372,120	3,153
DMP (2×)	137	38	152	49	1,927	269	767,416	4,219
TOM (3×)	143	39	157	49	1,360	210	214,473	2,202
DOM (2×)	157	49	166	61	2,006	316	586,713	6,689
TO1 (3×)	153	44	165	52	1,881	223	412,949	3,767
TO2 (3×)	146	36	156	52	1,541	196	310,583	4,198
TO3 (3×)	138	40	160	52	1,555	216	342,129	3,434
Total	187	53	191	66	12,009	1,668	3,006,383	27,662

Nucleotide bias at first position of both known and novel miRNAs in all libraries was investigated. The results in known miRNAs showed a strong bias to uridine at the first position by the ratios over 90% in all seven libraries (Figure 2A). However, nucleotide bias at first position in novel miRNAs presented an obvious increasing ratio of adenosine compared to that of known miRNAs. Most of our libraries including four triploid libraries (one mixed library and three individual genotype libraries) had adenosine replacing uridine as the dominant nucleotide at first position of novel miRNAs (Figure 2B).

There was obvious difference in length distribution between known and novel miRNAs. 21nt of length dominated in known miRNA group (72.73%) while length distribution of novel miRNAs had a more diversified result (Figure 2C). 24nt (43.4%) instead of 21nt became the most miRNAs in novel group (Figure

2D). Besides, 21nt (26.42%) and 22nt (20.75%) were the second and third most sets of novel miRNAs, separately (Figure 2D).

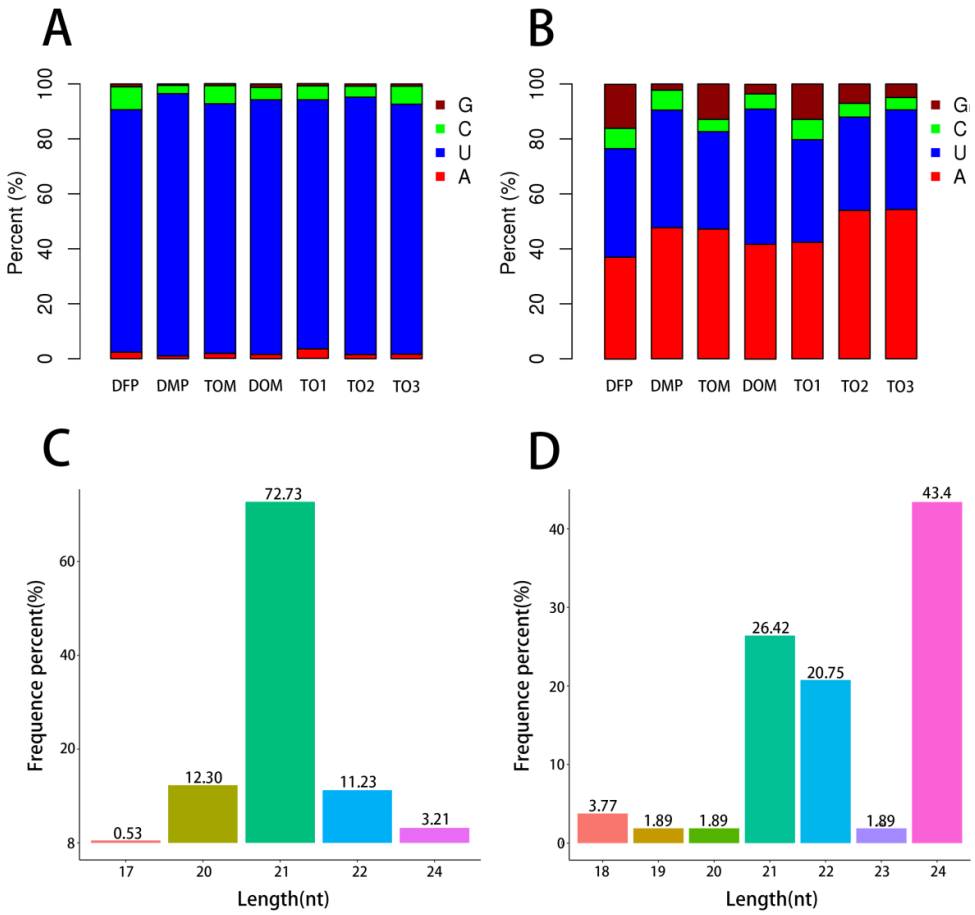

Figure 2. First base bias and length distribution of known and novel miRNAs in triploid offspring group and its parents, diploid full sib group, and three individual triploids. **(A)** First bias of known miRNAs; showed a strong bias to uridine at the first position. G: miRNAs with guanosine at first position; C: miRNAs with cytidine at first position; U: miRNAs with uridine at first position; A: miRNAs with adenosine at first position. **(B)** First bias of novel miRNAs; showed an increasing ratio of adenosine at the first position in novel miRNAs. Caption of 'G, C, U, A' was consistent as in (A). **(C)** Length distribution of known miRNAs; with 21nt as the dominant length. **(D)** Length distribution of novel miRNAs; with 24nt as the dominant length

Expression patterns of different miRNA families in seven libraries

The result of expression analysis in miRNA families with more than five members indicated different expression patterns between high expression families and low expression families. In high expression miRNA families, dominance of one or two members to others in the same family had a similar trend in all seven libraries (Figure 3A-E). In contrast, expression of different members in the same family had no obvious regularity in low expression miRNA family (Figure 3F). For example, in miR482 family, the expressions of ptc-miR1448 and ptc-miR482a.2 were dominant over all seven libraries (Figure 3A). While referring to miR169 family with low expression levels, different members expressed predominantly in different libraries (Figure 3F). These results indicated that high expression miRNA families had stable expression patterns in parents and offsprings with

different ploidy while miRNA family with low expression had differential expression patterns in different individuals and groups.

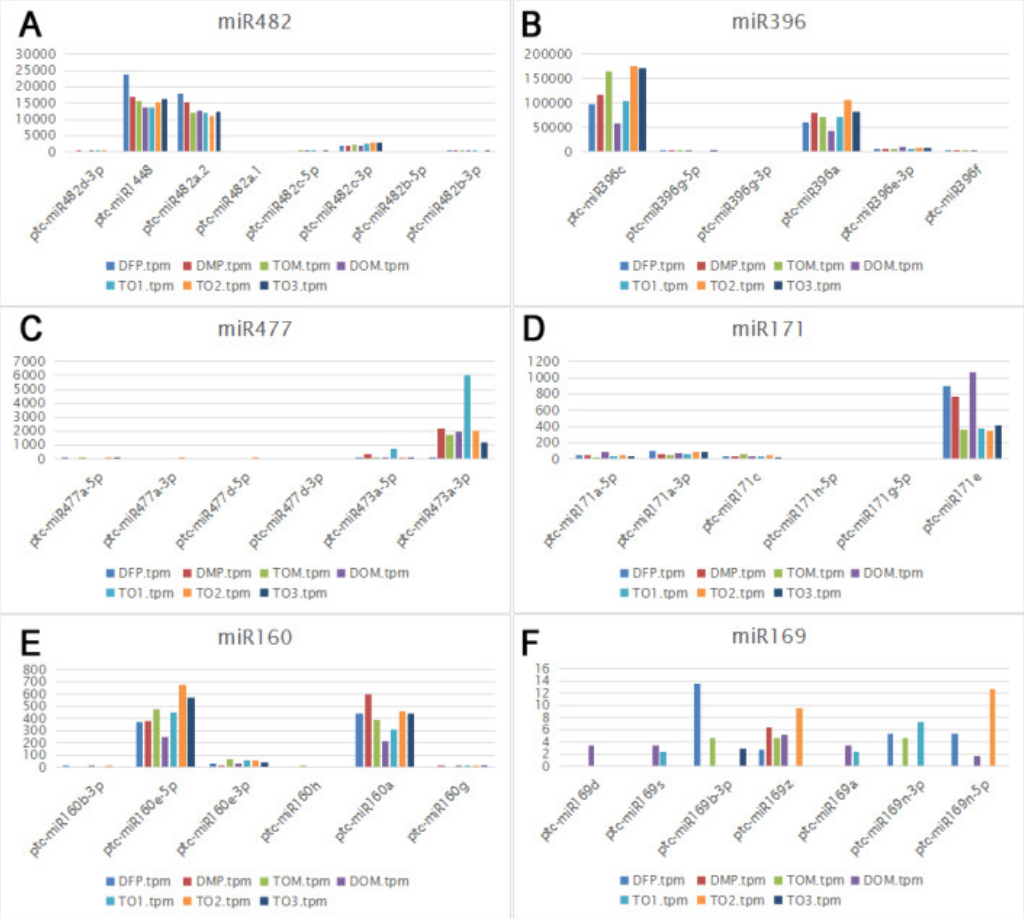

Figure 3. Expression patterns of different miRNA families in triploid offspring group and its parents, diploid full sib group, and three individual triploids. **(A)** miR482 family; with ptc-miR1448 and ptc-miR482a.2 as the dominating expressed members. **(B)** miR396 family; with ptc-miR396c and ptc-miR396a as the dominating expressed members. **(C)** miR477 family; with ptc-miR473a-3p as the dominating expressed member. **(D)** miR171 family; with ptc-miR171e as the dominating expressed member. **(E)** miR160 family; with ptc-miR160e-5p and ptc-miR160a as the dominating expressed members. **(F)** miR169 family; had no obvious regularity in seven libraries

Specific expression of miRNAs in triploid group

Most miRNAs were non additively expressed in both triploid and diploid offspring groups compared to their parents. In triploid offspring group, a total of 195 (81.25% of total 240 miRNAs) miRNAs were non additively expressed including 93 (47.69% of nonadditive miRNAs) up-regulated ones and 102 (52.31% of nonadditive miRNAs) down-regulated ones. In diploid offspring group, a total of 190 (79.17% of total 240 miRNAs) miRNAs were non additively expressed including 98 (51.58% of nonadditive miRNAs) up-regulated ones and 92 (48.42% of nonadditive miRNAs) down-regulated ones. These results indicated that both polyploidization and hybridization could lead to nonadditive expression of miRNAs. While polyploidization in this study had a more obvious effect on down-regulated expression of miRNAs compared to hybridization.

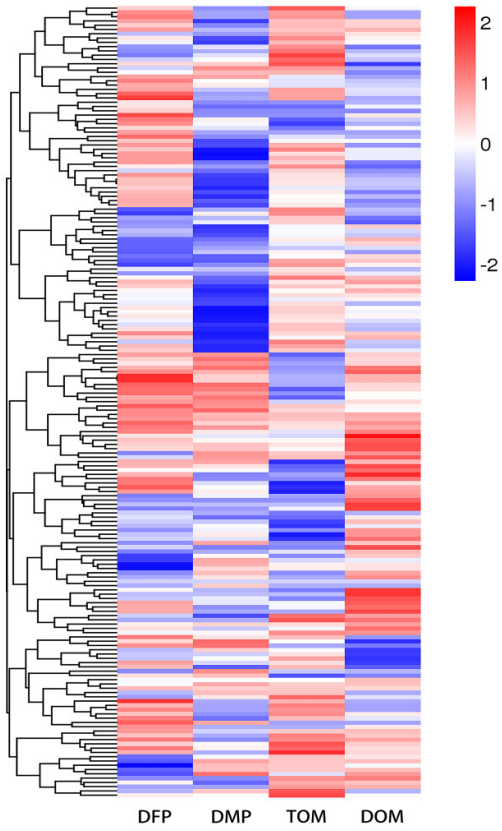

Figure 4. Cluster analysis of differentially expressed miRNAs in diploid female parent, diploid male parent, triploid offspring mixture, and diploid offspring mixture

According to the cluster analysis of differentially expressed miRNAs, miRNAs in triploid group exhibited a unique expression pattern compared to its diploid full sib group inherited from their two parents (Figure 4). A total number of 18 miRNAs including 6 novel miRNAs and 12 known miRNAs specific expressed in triploid group were identified (Table 4). Among these miRNAs, 8 miRNAs (novel_3, ptc-miR171a-5p, ptc-miR171e, ptc-miR396g-3p, ptc-miR478h, ptc-miR482a.1, ptc-miR6438b, and ptc-miR6459a-5p) were down-regulated and 5 miRNAs (novel_44, novel_69, ptc-miR169q, ptc-miR3627b, and ptc-miR482c-5p) were up-regulated compared with the two parents and diploid full sib group. Furthermore, novel_124, expressing extremely lower in triploid group than in the male parent and diploid full sib group, had characters (with a 5' terminal adenosine and was 24nt in length) that could recognized by AGO4 suggesting the potential possibility of involving in RdDM regulation.

Table 4. Specific expressed miRNAs in triploid offspring group

miRNA	Sequence	Expression (TPM)			
		DFP (2×)	DMP (2×)	TOM (3×)	DOM (2×)
novel_124	acuuugagacuggacacucuuggc	0.00	1548.54	316.56	1852.88
novel_125	ugucguuggauucaucuugucgag	97.53	0.00	41.90	170.61
novel_3	ucggaccaggcuucauucccc	197.76	186.29	60.52	126.25
novel_44	cugaaguguuuggggaacuc	2.71	0.00	9.31	1.71
novel_69	uuugcucuucguuuucucaug	130.03	0.00	297.94	88.72
novel_94	ugacaggcucuucucucucaug	165.25	0.00	60.52	27.30
ptc-miR169q	uagccaaggacgacuugccug	2.71	0.00	9.31	1.71
ptc-miR171a-5p	ggauauugguacgguucaauc	56.89	47.87	23.28	87.01
ptc-miR171e	ugauugagccgugccaauauc	899.40	765.86	363.11	1061.22
ptc-miR3627b	ugucgcaggagagauggcgcua	0.00	1.29	13.97	1.71
ptc-miR396g-3p	cucaagaaagccgugggaaaa	224.85	141.01	46.55	114.31
ptc-miR397a	ucauugagugcagcguugaug	975.26	11.64	311.90	54.60
ptc-miR478h	uaacgugucuccuauuuuuaggga	78.56	21.99	4.66	35.83
ptc-miR482a.1	ccuacuccucccauucc	24.38	37.52	9.31	61.42
ptc-miR482c-5p	uaugggagaggcgggaaugacu	86.69	100.91	279.32	105.78
ptc-miR6438b	uuguacacagaauaggugaaau	16.25	16.82	4.66	15.36
ptc-miR6457b	uuaguuuggcagccucuucuc	289.87	1835.74	670.36	1441.69
ptc-miR6459a-5p	agcucaagcacaaauucgauc	40.64	15.52	0.00	39.24

Prediction of miRNA targets and enrichment analysis

In order to further understand the biological function of miRNAs specific expressed in triploid group, prediction of miRNA targets was performed. A total of 2367 potential target genes were predicted among which 2336 genes were targets for known miRNAs and 31 genes were for novel miRNAs (Table 5; Table S1). Noticeably, targets of specific expressed miRNAs in triploid group were associated with various transcription factors involved in extensive regulation pathways and physiological processes (Table 5). For example, miR169, expression level of which in triploid group was beyond four times more than in the other libraries.

Table 5. Part targets of representative specfic expressed miRNAs in triploid offspring group

miRNA	GeneID	InterPro ID	InterPro description
ptc-miR169q	POPTR_0006s14740	IPR001289	CCAAT-binding transcription factor, subunit B
	POPTR_0006s21640	IPR001289	CCAAT-binding transcription factor, subunit B
	POPTR_0016s06860	IPR001289	CCAAT-binding transcription factor, subunit B
	POPTR_0017s11180	IPR000719	Protein kinase, catalytic domain
	POPTR_0018s07220	IPR001289	CCAAT-binding transcription factor, subunit B
	POPTR_0010s14600	IPR011598	Helix-loop-helix DNA-binding
ptc-miR171a-5p	POPTR_0001s00480	IPR005202	Transcription factor GRAS
ptc-miR171e	POPTR_0001s03090	IPR001806	Ras GTPase
	POPTR_0002s14500	IPR005202	Transcription factor GRAS
	POPTR_0003s11020	IPR005202	Transcription factor GRAS
	POPTR_0005s12730	IPR005202	Transcription factor GRAS
	POPTR_0006s27110	IPR008942	ENTH/VHS
	POPTR_0007s12480	IPR005202	Transcription factor GRAS
	POPTR_0014s05910	IPR005202	Transcription factor GRAS
	POPTR_0014s05940	IPR005202	Transcription factor GRAS
ptc-miR3627b	POPTR_0013s00750	IPR000330	SNF2-related
	POPTR_0006s07240	IPR001757	ATPase, P-type, K/Mg/Cd/Cu/Zn/Na/Ca/Na/H-transporter
ptc-miR396g-3p	POPTR_0003s06640	IPR009637	Transmembrane receptor, eukaryota
	POPTR_0006s03850	IPR009060	UBA-like
	POPTR_0006s07930	IPR011989	Armadillo-like helical
	POPTR_0010s19080	IPR001117	Multicopper oxidase, type 1

Gene ontology (GO) enrichment analysis was performed to obtain further functional understanding on differentially expressed miRNAs and their targets in triploid group compared with their parents and full sib group (TOM vs DFP; TOM vs DMP; TOM vs DOP). According to the blastx results, differential targets of all the three comparison sets were enriched in ADP binding (GO: 0043531; FDR<0.05) of molecular function (Figure 5). The full results in each comparison set were listed in Table S2-4. Kyoto Encyclopedia of Gene and Genomes (KEGG) was a major public database of biological systems integrating genomic, chemical and systemic functional information (Kanehisa *et al*., 2008). Results showed that differential target genes of all the three comparison sets were enriched in Plant-pathogen interaction pathway (FDR < 0.05) (Figure 6). The top 20 enriched pathways in each comparison set were listed in Table S5-7. According to GO and KEGG enrichment analysis, target genes of specific expressed in triploid offspring group potentially influenced the disease resistance of triploid offspring's through adenosine metabolic pathway.

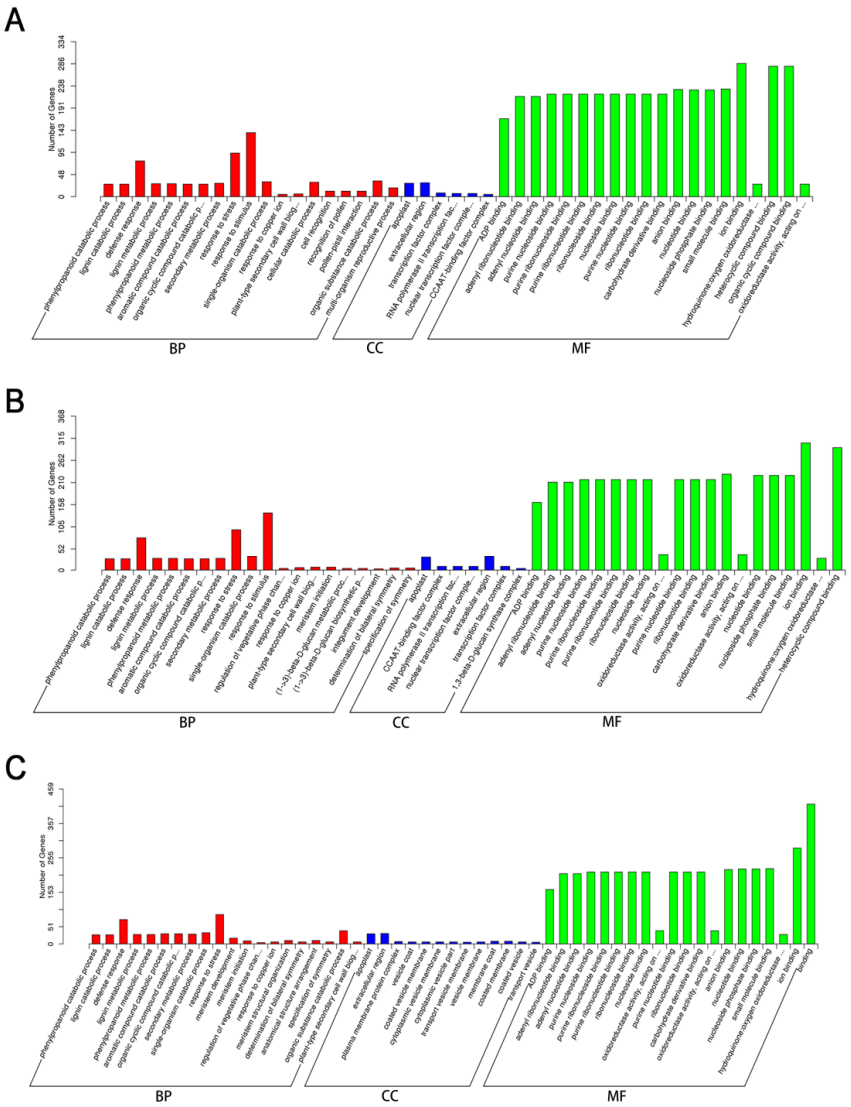

Figure 5. Gene ontology (GO) enrichment of target genes predicted from specific expressed miRNAs in triploid offspring group. BP: biological process; CC: cellular component; MF: molecular function. **(A)** Triploid offspring group compared to female parent. **(B)** Triploid offspring group compared to male parent. **(C)** Triploid offspring group compared to diploid full sib group

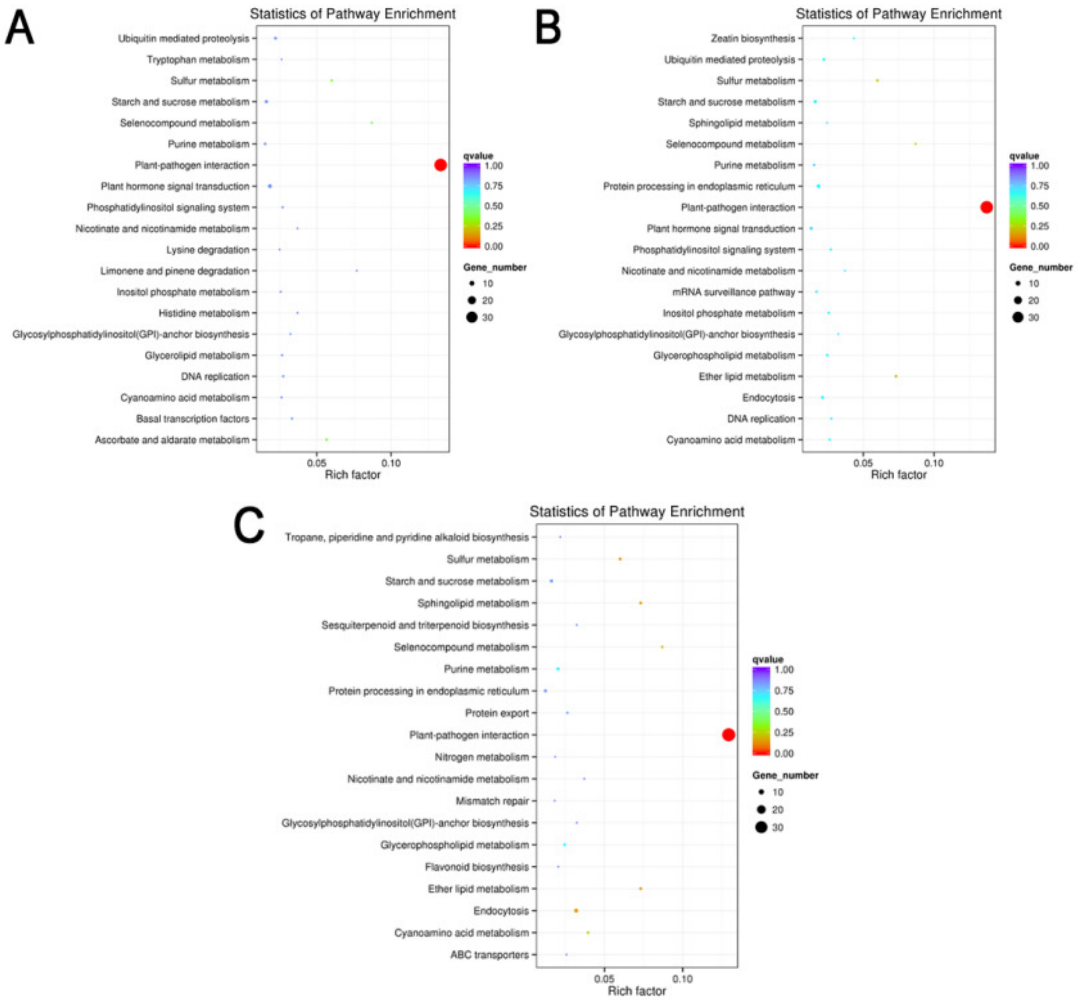

Figure 6. Kyoto Encyclopedia of Gene and Genomes (KEGG) enrichment of target genes predicted from specific expressed miRNAs in triploid offspring group. **(A)** Triploid offspring group compared to female parent. **(B)** Triploid offspring group compared to male parent. **(C)** Triploid offspring group compared to diploid full sib group

Discussion

miRNAs involving in an ancient but active epigenetic regulation network participated in extensive variety of genes associated with almost all biological process of eukaryotes (Vazquez *et al*., 2010). Many studies had discussion on the essential impact of miRNAs during WGD events. But the potential role of miRNAs in newly formed polyploids was restrictedly investigated in *Arabidopsis* and some crops (Wang *et al*., 2006; Li *et al*., 2014) probably because of the limitation of research materials. In this study, a synthetic triploid group of poplar, a model species of woody plants, obtained in our previous work provided an appropriate opportunity for us to have further understanding on variation of miRNAs in newly formed polyploid. According to our results, the genome-wide changes of miRNA level were activated rapidly and affected various levels of responses after WGD: (1) most of the miRNAs exhibited nonadditive expression and the down-regulation of miRNAs in triploid offsprings was probably an important regulatory mechanism of polyploid advantages (2) miRNA might involve in RdDM regulation pathway on noncoding region of genome in the newly formed triploids. (3)

Specific expressed miRNAs in response to WGD were associated with large-scale transcription factors in adenosine metabolic pathway that involved in regulation network of disease resistance.

Like changes of protein-coding gene expression after WGD (Jackson and Chen, 2010; Yoo *et al*., 2014), miRNAs in polyploid of some plant species were found to have non additive expression (deviating from the mid-parent value, MPV) immediately after WGD. In study of *Arabidopsis*, about 51% miRNAs in leaves and 40% in flowers expressed differently between an allotetraploid and MPV (Ha *et al*., 2009). In a nascent hexaploid wheat, 23.7% miRNAs were found to be non-additively expressed when compared with their MPVs (Li *et al*., 2014). In this study, nonadditive expression of miRNAs was dominant in a total number of 240 miRNAs both in triploid (195, 81.25%) and diploid (190, 79.17%) offsprings. While nonadditive miRNAs in triploid offsprings were tended to down-regulated (102, 52.31%) in comparison to diploid offsprings (92, 48.42%). Down-regulation of miRNAs meant the original balance between miRNAs and their targets was broken. And negative regulation of miRNAs on their target genes was reduced. Thus, the result indicated that adjustment and regulation over genome-wide miRNAs was rapidly activated after WGD in poplar, most of which were down-regulated potentially leading to polyploid advantages (Suo *et al*., 2017).

Length and the first base of miRNA were crucial characters involving the recognition of AGO and subsequent function in different epigenetic regulation pathways (Axtell, 2013; Montgomery *et al*., 2008). In our study, most of the novel miRNAs were 24nt in length (43.4%) and had an increasing bias of adenosine in the 5' terminal base compared to known miRNAs. 24nt miRNAs with a 5' terminal adenosine was accorded with preferential recruitment of AGO4 (Mi *et al*., 2008). Previous research had demonstrated that this kind of miRNAs directed DNA methylation at some of their target loci or *in trans* (Chellappan *et al*., 2010; Wu *et al*., 2010). Results in our study suggested that novel miRNAs of poplar had distinct function compared with known miRNAs strongly associating with DNA methylation pathway which means that novel miRNAs in this study was probably undergoing sub functionalization and neofunctionalization. It was worth mentioning that novel_124, expression level of which was extremely lower in triploid group than in the male parent and diploid full sib group, had characters (with a 5' terminal adenosine and was 24nt in length) that could be recognized by AGO4 and no target gene was predicted. These results suggested that novel miRNAs generated rapidly after WGD in poplar were potentially involved in regulation pathway of DNA methylation on noncoding region. It was the first time that associated miRNA with regulation of DNA methylation after WGD in *Populus* which was worth to be further studied.

Variation of miRNA expression in plant polyploids caused nonadditive expression of target genes involving in growth vigor and adaptation (Chen, 2007; Ha *et al*., 2009; Ng *et al*., 2012; Chen, 2013). In nascent hexaploid wheat, nonadditive repression of miR9009 resulted in upregulation of its target *R* gene analogs (RGAs) which was finally leaded to the enhanced powdery mildew resistance of newly synthesized wheat (Li *et al*., 2014). Non additive regulation of grass-specific miRNAs might contribute to significant performance in disease resistance (Li *et al*., 2014). In synthetic *Populus* triploid populations, decrease of miRNA negative regulation on target genes were mainly related to the functional regulation of the genome in response to the abiotic stress (Suo *et al*., 2017). Enrichment analysis in our study showed a strong correlation between target genes of specific expressed miRNAs in triploid offsprings and plant-pathogen interaction. Besides, the specific expressed miR169 was reported to be associated with various abiotic stress (Zhao *et al*., 2009; Xu *et al*., 2013), or involved in developmental and some other biological processes of plants (Wenkel *et al*., 2006; Edwards *et al*., 1998). Therefore, these results drew a similar conclusion that specific expressed miRNAs in triploid offsprings probably influenced disease resistance and adaptation of polyploid. The relationship between miRNAs and disease resistance predicted in our study might be validated in further researches.

Conclusions

In conclusion, the material system including two parents and their triploid and diploid offspring groups used in our study provided an ideal model for research of rapid responses after WGD in woody plant due to its explicit genetic relationships and the genomic data sequenced in poplar. Results in this study revealed an important role of miRNAs that participated in the rapid responses after WGD in poplar. As a conservative and active epigenetic regulation method, miRNA was presumed in this study to affect multiple levels of responses for example DNA methylation of non-coding region in genome and post-transcriptional regulation of disease resistance-related pathway. Meanwhile, expression of miRNAs was being regulated immediately after WGD. polyploidization mainly leaded to down-regulation of miRNAs which might a potential reason for polyploid advantages. These conclusions provided some significant directions and pointcuts for further researches on WGD rapid response in woody plant.

Authors' Contributions

Kaiyuan ZHU conceived and designed the experiments. Wenting XU, Xue WANG, Huichun LIU, Jiaqiang ZHANG, Jianghua ZHOU, Rongxin GOU performed the experiments. Wenting XU and Xue WANG executed data analysis. Wenting XU wrote the paper.

All authors read and approved the final manuscript.

Ethical approval (for researches involving animals or humans)

Not applicable.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

References

Abrouk M, Zhang R, Murat F, Li A, Pont C, Mao L, Salse J (2012). Grass microRNA gene paleohistory unveils new insights into gene dosage balance in subgenome partitioning after whole-genome duplication. The Plant Cell 24:1776-1792. *https://doi.org/10.1105/tpc.112.095752*

Axtell MJ (2013). Classification and comparison of small RNAs from plants. Annual Review of Plant Biology 64:137-159. *https://doi.org/10.1146/annurev-arplant-050312-120043*

Barakat A, Wall PK, DiLoreto S, dePamphilis CW, Carlson JE (2007). Conservation and divergence of microRNAs in *Populus*. BMC Genomics 8:481. *https://doi.org/10.1186/1471-2164-8-481*

Birchler J, Yang H (2022). The multiple fates of gene duplications: Deletion, hypofunctionalization, subfunctionalization, neofunctionalization, dosage balance constraints, and neutral variation. The Plant Cell 34:2466-2474. *https://doi.org/10.1093/plcell/koac076*

Carrington JC, Ambros V (2003). Role of microRNAs in plant and animal development. Science 301:336-338. *https://doi.org/10.1126/science.1085242*

Chalhoub B, Denoeud F, Liu S, Parkin IAP, Tang H, Wang X, ... Wincker P (2014). Early allopolyploid evolution in the post-Neolithic *Brassica napus* oilseed genome. Science 345:950-953. *https://doi.org/10.1126/science.1253435*

Chávez Montes R, Rosas-Cárdenas D, De Paoli E, Accerbi M, Rymarquis LA, Mahalingam G, ... De Folter S (2014). Sample sequencing of vascular plants demonstrates widespread conservation and divergence of microRNAs. Nature Communications 5:3722. *https://doi.org/10.1038/ncomms4722*

Chellappan P, Xia J, Zhou X, Gao S, Zhang X, Coutino G, ... Jin H (2010). siRNAs from miRNA sites mediate DNA methylation of target genes. Nucleic Acids Research 38:6883-6894. *https://doi.org/10.1093/nar/gkq590*

Chen ZJ (2007). Genetic and epigenetic mechanisms for gene expression and phenotypic variation in plant polyploids. Annual Review of Plant Biology 58:377-406. *https://doi.org/10.1146/annurev.arplant.58.032806.103835*

Chen ZJ (2013). Genomic and epigenetic insights into the molecular bases of heterosis. Nature Reviews Genetics 14:471-482. *https://doi.org/10.1038/nrg3503*

Ding M, Chen ZJ (2018). Epigenetic perspectives on the evolution and domestication of polyploid plant and crops. Current Opinion in Plant Biology 42:37-48. *https://doi.org/10.1016/j.pbi.2018.02.003*

Edwards D, Murray JAH, Smith AG (1998). Multiple genes encoding the conserved CCAAT-Box transcription factor complex are expressed in *Arabidopsis*. Plant Physiology 117:1015-1022. *https://doi.org/10.2307/4278359*

Friedlander MR, Mackowiak SD, Li N, Chen W, Rajewsky N (2011). miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Research 40:37-52. *https://doi.org/10.1093/nar/gkr688*

Gyorfy MF, Miller ER, Conover JL, Grover CE, Wendel JF, Sloan DB, Sharbrough J (2021). Nuclear-cytoplasmic balance: whole genome duplications induce elevated organellar genome copy number. The Plant Journal 108:219-230. *https://doi.org/10.1111/tpj.15436*

Ha M, Lu J, Tian L, Ramachandran V, Kasschau KD, Chapman EJ, ... Chen ZJ (2009). Small RNAs serve as a genetic buffer against genomic shock in *Arabidopsis* interspecific hybrids and allopolyploids. Proceedings of the National Academy of Sciences 106:17835-17840. *https://doi.org/10.1073/pnas.0907003106*

Hughes TE, Langdale JA, Kelly S (2014). The impact of widespread regulatory neofunctionalization on homeolog gene evolution following whole-genome duplication in maize. Genome Research 24:1348-1355. *https://doi.org/10.1101/gr.172684.114*

Initiative AG (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 408:796-815. *https://doi.org/10.1038/35048692*

Jackson S, Chen ZJ (2010). Genomic and expression plasticity of polyploidy. Current Opinion in Plant Biology 13:153-159. *https://doi.org/10.1016/j.pbi.2009.11.004*

Kanehisa M, Araki M, Goto S, Hattori M, Hirakawa M, Itoh M, ... Yamanishi Y (2008). KEGG for linking genomes to life and the environment. Nucleic Acids Research 36:D480-484. *https://doi.org/10.1093/nar/gkm882*

Kim JH, Woo HR, Kim J, Lim PO, Lee IC, Choi SH, ... Nam HG (2009). Trifurcate feed-forward regulation of age-dependent cell death involving miR164 in *Arabidopsis*. Science 323:1053-1057. *https://doi.org/10.1126/science.1166386*

Li A, Liu D, Wu J, Zhao X, Hao M, Geng S, ... Mao L (2014). mRNA and small RNA transcriptomes reveal insights into dynamic homoeolog regulation of allopolyploid heterosis in nascent hexaploid Wheat. The Plant Cell 26:1878-1900. *https://doi.org/10.1105/tpc.114.124388*

Liu Z, Xin M, Qin J, Peng H, Ni Z, Yao Y, Sun Q (2015). Temporal transcriptome profiling reveals expression partitioning of homeologous genes contributing to heat and drought acclimation in wheat (*Triticum aestivum* L.). BMC Plant Biology 15:1-20. *https://doi.org/10.1186/s12870-015-0511-8*

Lynch M, Conery JS (2000). The Evolutionary fate and consequences of duplicate genes. Science 290:1151-1155. *https://doi.org/10.1126/science.290.5494.1151*

Mallory AC, Vaucheret H (2006). Functions of microRNAs and related small RNAs in plants. Nature Genetics 38:S37-S37. *https://doi.org/10.1038/ng1791*

Mao X, Cai T, Olyarchuk JG, Wei L (2005). Automated genome annotation and pathway identification using the KEGG orthology (KO) as a controlled vocabulary. Bioinformatics 21:3787-3793. *https://doi.org/10.1093/bioinformatics/bti430*

Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, ... Qi Y (2008). Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5' terminal nucleotide. Cell 133:116-127. *https://doi.org/10.1016/j.cell.2008.02.034*

Montgomery TA, Howell MD, Cuperus JT, Li D, Hansen JE, Alexander AL, ... Carrington JC (2008). Specificity of ARGONAUTE7-miR390 interaction and dual functionality in TAS3 trans-acting siRNA formation. Cell 133:128-141. *https://doi.org/10.1016/j.cell.2008.02.033*

Ng DW, Lu J, Chen ZJ (2012). Big roles for small RNAs in polyploidy, hybrid vigor, and hybrid incompatibility. Current Opinion in Plant Biology 15:154-161. *https://doi.org/10.1016/j.pbi.2012.01.007*

Ozkan H, Levy AA, Feldman M (2001). Allopolyploidy-induced rapid genome evolution in the Wheat (*Aegilops-Triticum*) group. The Plant Cell 13:1735. https: //doi.org/10.2307/3871315

Peer YV, Mizrachi E, Marchal K (2017). The evolutionary significance of polyploidy. Nature Reviews Genetics 18:411-424. *https://doi.org/10.1038/nrg.2017.26*

Qiao X, Li Q, Yin H, Qi K, Li L, Wang R, ... Paterson AH (2019). Gene duplication and evolution in recurring polyploidization–diploidization cycles in plants. Genome Biology 20:38. *https://doi.org/10.1186/s13059-019-1650-2*

Ren YY, Chen L, Zhang YY, Kang XY, Zhang ZY, Wang YW (2012). Identification of novel and conserved *Populus tomentosa* microRNA as components of a response to water stress. Functional and Integrative Genomics 12:327-339. *https://doi.org/10.1007/s10142-012-0271-6*

Renny-Byfield S, Wendel JF (2014). Doubling down on genomes: polyploidy and crop plants. American Journal of Botany 101:1711-1725. *https://doi.org/10.3732/ajb.1400119*

Shi T, Rahmani RS, Gugger PF, Wang M, Li H, Zhang Y, ... Chen J (2020). Distinct expression and methylation patterns for genes with different fates following a single whole-genome duplication in flowering plants. Molecular Biology and Evolution 37:2394-2413. *https://doi.org/10.1093/molbev/msaa105*

Soltis PS, Marchant DB, Van de Peer Y, Soltis DE (2015). Polyploidy and genome evolution in plants. Current Opinion in Genetics & Development 35:119-125. *https://doi.org/10.1016/j.gde.2015.11.003*

Stull GW, Qu XJ, Parins-Fukuchi C, Yang YY, Yang JB, Yang ZY, ... Yi TS (2021). Gene duplications and phylogenomic conflict underlie major pulses of phenotypic evolution in gymnosperms. Nature Plants 7:1015-1025. *https://doi.org/10.1038/s41477-021-00964-4*

Suo Y, Min Y, Dong C, Wang Y, Cheng S, Kang X (2017). MicroRNA expression changes following synthesis of three full-sib *Populus* triploid populations with different heterozygosities. Plant Molecular Biology 95:215-225. *https://doi.org/10.1007/s11103-017-0627-3*

Vanneste K, Van de Peer Y, Maere S (2013). Inference of genome duplications from age distributions revisited. Molecular Biology and Evolution 30:177-190. *https://doi.org/10.1093/molbev/mss214*

Vazquez F, Legrand S, Windels D (2010). The biosynthetic pathways and biological scopes of plant small RNAs. Trends in Plant Science 15:337-345. *https://doi.org/10.1371/journal.pone.0015224*

Wang J, Tian L, Lee HS, Wei NE, Jiang H, Watson B, ... Chen ZJ (2006). Genome wide nonadditive gene regulation in *Arabidopsis* allotetraploids. Genetics 172:507-517. *https://doi.org/10.1534/genetics.105.047894*

Wang JL, Tian L, Madlung A, Lee HS, Chen M, Lee JJ, ... Chen ZJ (2004). Stochastic and epigenetic changes of gene expression in *Arabidopsis* polyploids. Genetics Society of America 167:1961-1973. *https://doi.org/10.1534/genetics.104.027896*

Wen M, Shen Y, Shi S, Tang T (2012). miREvo: An Integrative microRNA Evolutionary Analysis Platform for Next-generation Sequencing Experiments. BMC Bioinformatics 13:140. *https://doi.org/10.1186/1471-2105-13-140*

Wendel JF, Jackson SA, Meyers BC, Rod AW (2016). Evolution of plant genome architecture. Genome Biology 17:37. *https://doi.org/10.1186/s13059-016-0908-1*

Wendel JF, Jackson SA, Meyers BC, Wing RA (2016). Evolution of plant genome architecture. Genome Biology 17:37. *https://doi.org/10.1186/s13059-016-0908-1*

Wenkel S, Turck F, Singer K, Gissot L, Gourrierec JL, Samach A, Coupland G (2006). CONSTANS and the CCAAT box binding complex share a functionally important domain and interact to regulate flowering of *Arabidopsis*. The Plant Cell 18:2971-2984. *https://doi.org/10.1105/tpc.106.043299*

Wu HJ, Ma YK, Chen T, Wang M, Wang XJ (2012). PsRobot: a web-based plant small RNA meta-analysis toolbox. Nucleic Acids Research 40:W22-W28. *https://doi.org/10.1093/nar/gks554*

Wu L, Zhou H, Zhang Q, Zhang J, Ni F, Liu C, ... Qi Y (2010). DNA methylation mediated by a microrna pathway. Molecular Cell 38:465-475. *https://doi.org/10.1016/j.molcel.2010.03.008*

Xu MY, Zhang L, Li WW, Hu XL, Wang MB, Fan YL, ... Wang L (2013). Stress-induced early flowering is mediated by miR169 in *Arabidopsis thaliana*. Journal of Experimental Botany 65:89-101. *https://doi.org/10.1093/jxb/ert353*

Xu W, Guo L, Zhang Y, Zhao J, Wei Z, Zhang J (2019). An improved method for high-temperature induced embryo sac chromosome doubling in *Populus simonii* Carr. × *P. nigra* var. *italica* (Moench.) Kochne, an interspecifc hybrid of *Tacamahaca* and *Aigeiros* poplars. Scientific Reports 9:551. *https://doi.org/10.1038/s41598-018-37297-w*

Yoo MJ, Liu X, Pires C, Soltis PS, Soltis DE (2014). Nonadditive gene expression in polyploids. Annual Review of Genetics 48:485-517. *https://doi.org/10.1146/annurev-genet-120213-092159*

Zhang L, Zhao J, Bi H, Yang X, Zhang Z, Su Y, ... Ma T (2021) Bioinformatic analysis of chromatin organization and biased expression of duplicated genes between two poplars with a common whole-genome duplication. Horticulture Research 8:62. *https://doi.org/10.1038/s41438-021-00494-2*

Zhao B, Ge L, Liang R, Li W, Ruan K, Lin H, Jin Y (2009). Members of miR-169 family are induced by high salinity and transiently inhibit the NF-YA transcription factor. BMC Molecular Biology 10:29. *https://doi.org/10.1186/1471-2199-10-29*

Zhao M, Meyers BC, Cai C, Xu W, Ma J (2015). Evolutionary patterns and coevolutionary consequences of miRNA genes and microRNA targets triggered by multiple mechanisms of genomic duplications in soybean. Plant Cell 27:546-562. *https://doi.org/10.1105/tpc.15.00048*

Zhou L, Chen J, Li Z, Li X, Hu X, Huang Y, ... Zhang X (2010). Integrated profiling of microRNAs and mRNAs: microRNAs located on Xq27.3 associate with clear cell renal cell carcinoma. PLoS One 5:e15224. *https://doi.org/10.1371/journal.pone.0015224*