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Article

Gene Expression Differences between High-Growth *Populus* Allotriploids and Their Diploid Parents

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Abstract: Polyploid breeding is important in *Populus* genetic improvement programs because polyploid trees generally display increased height growth compared to their diploid parents. However, the genetic mechanism underlying this phenomenon remains unknown. In the present study, apical bud transcriptomes of vigorous, fast growing *Populus* allotriploid progeny genotypes and their diploid parents were sequenced and analyzed. We found that these allotriploids exhibited extensive transcriptomic diversity. In total, 6020 differentially expressed genes (DEGs) were found when the allotriploid progeny and their parents were compared, among which 791 overlapped between the allotriploids and both parents. Many genes associated with cell differentiation and meristem development were preferentially expressed in apical buds of the fast growing *Populus* allotriploids compared to their diploid parents. In addition, many auxin-, gibberellin-, and jasmonic acid-related genes were also preferentially expressed in the allotriploids compared to their

parents. Our findings show that allotriploidy can have considerable effects on duplicate gene expression in *Populus*. In particular we identified and considered DEGs that provide important clues for improving our mechanistic understanding of positive heterosis of vigor- and growth-related traits in *Populus* allotriploids.

Keywords: *Populus* section Tacamahaca; allotriploid; apical bud; vigor and height growth traits; RNA-seq

1. Introduction

Polyploidization has played an important role in plant evolution and speciation [1]. Polyploids are typically grouped into allopolyploids and autopolyploids when multiple chromosome sets are derived from different species or the same species, respectively [2]. Natural populations of allopolyploids are more prevalent than those of autopolyploids. Thus, allopolyploids have succeeded in overcoming the limitations of polyploidy and may exhibit selective advantages, possibly attributable in part to heterozygosity and gene redundancy [3].

Polyploidization can be an important breeding strategy when seeking to develop new tree varieties for forestry purposes. Polyploidization is often accompanied by morphological and physiological changes that can afford advantages in growth rate and other traits of commercial interest. Propagation of polyploid trees is usually accomplished using vegetative organs, and many species are amenable to vegetative (asexual) propagation. This approach is advantageous also because it avoids fertility/reproductive problems inherent in polyploids. Thus, once new tree varieties are successfully identified and developed, long-term and sustainable cultivation is possible. Some triploid cultivars of *Populus* exhibit several desirable characteristics compared to their diploid progenitors, including faster growth, better pulpwood characteristics, improved timber quality, and higher levels of disease resistance [4–7]. Clearly, triploid breeding represents a powerful approach toward genetic improvement of *Populus* [8,9].

Populus species serve as model forest trees in plant molecular biology research. Growth and development is relatively rapid and growth in height in particular is readily studied. Both macro- and micro-observational data have shown that height growth is closely related to various characteristics of apical buds [10]. However, the underlying genetic causes of the increased growth rates of hybrids, including polyploids, compared to their parents (*i.e.*, heterosis) are not understood. Thus, identifying specific genes and metabolic pathways active in apical buds is of great interest. Artificially produced polyploids are excellent research materials in this context because the precise parental lines are known. Such research constructs have been widely used to determine how genes become duplicated during the early stages of polyploidization [11].

Several hormone-related genes play important roles in the maintenance of cells in apical buds (shoot tips) of plants, including amidase (*VrAMI1*), *PINFORMED*-3 (*VrPIN*3), and *isopentenyl transferase* (*VrIPT*) [12]. The GRAS-gene mediated signal from differentiating cells is essential for shoot meristem maintenance [13]. The homeobox gene *WUSCHEL* (*WUS*) is critical in terms of maintenance of the shoot apical meristem (SAM), as judged by both gene mutation [14] and by

genetically programmed elimination during flower development [15]. *WUS* expression is under negative feedback control by one of the *WUS* target genes (*CLAVATA3*), which maintains stem cell maintenance and differentiation in dynamic equilibrium [16,17]. Other major factors controlling SAM maintenance are the homeobox genes of the *KNOTTED* (*KNOX*) family.

Populus allotriploid artificial constructs can be used as model systems by which to better understand the molecular basis of height growth heterosis and the early stages of polyploidization in woody plants. In the present work, we sought to test the hypothesis that *Populus* (section Tacamahaca) allotriploid hybrids, with the same diploid parents but showing heterosis in terms of height growth, might exhibit considerable changes in gene expression compared to that of their parents. Furthermore, our hypothesis predicts that differentially expressed genes (DEGs) should participate in important biological processes associated with apical bud morphogenesis, leading to enhanced height growth in *Populus* allotriploid seedlings.

2. Materials and Methods

2.1. Plant Materials and RNA Preparation

Plant materials included diploid parents (2n = 2x = 38) and synthetic allotriploid (2n = 3x = 57) progeny plants of *Populus* section Tacamahaca. The allotriploid plants used were full-sib progeny, derived by embryo sac chromosome doubling via postmeiotic restitution (PMR) using a single cross-combination. Diploid eggs were induced by treating developing embryo sacs of *Populus* with colchicine solution. This treatment occurred when female catkins of *P. pseudo-simonii* × *P. nigra* "ZY3" had become 5.62 cm long, 84 h after they emerged from their bract scales, all stigmas were exposed and all pistils over the entire catkin were receptive. Observation of paraffin sections showed that embryo sac development of "ZY3", which initiated 12 h before pollination and finished 132 h after pollination, was a successive and asynchronous process. Generative cell division of pollen of the male parent *P.* × *beijingensis* took place 3–16 h after pollination. Catkins of "ZY3" were treated with colchicine 18–96 h after pollination. In the offspring seedlings, triploids were detected by chromosome counting (Figure 1) and flow cytometry measurement was performed using a flow cytometer (BD FACSCalibur, San Jose, CA, USA) [8,18].

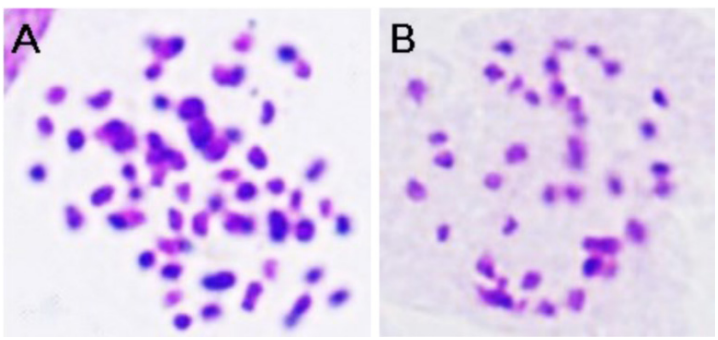

Figure 1. Number of chromosomes of diploid and triploid progenies—57 chromosomes in triploid (**A**) and 38 in diploid (**B**).

We selected 45 triploid seedlings derived by PMR and vegetatively propagated (see below) and grew them as clonal genotypes. In addition, we vegetatively propagated and grew nine ramets of each of the male and female parental clones. All ramets were grown in a uniform soil at three high-technology agricultural demonstration zones (Beijing, China). After six months of growth, we selected nine high-growth triploid genotypes (PMR-H) each differing significantly in plant height compared to their parents (Figure 2). The mean heights of the PMR-H, female parent, and male parent were 334.73, 189.00, and 218.67 cm, respectively (Figure 2). The selected nine PMR-H genotypes were all significantly taller than the non-selected 37 PMR genotypes.

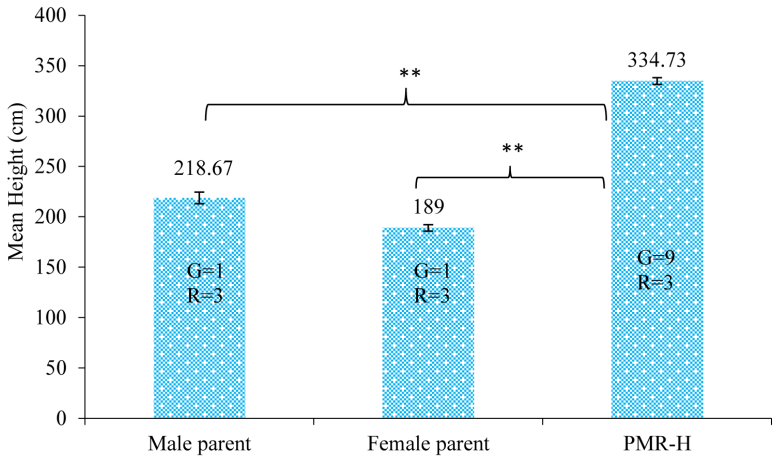

Figure 2. The mean heights of the high-growth allotriploid progeny (PMR-H), male parent, and female parent genotypes. Note: ** indicates a significant difference between means at the 0.01 level. G represents the number of genotypes. R represents the number of replicates for each genotype.

Vegetative propagation was accomplished using 15 cm shoots from one-year-old branches cut in April (spring growth). The shoot cuttings were planted in peat soil in plastic pots (25 cm in diameter × 25 cm in depth) and manually watered. All growth took place in a greenhouse of the National Engineering Laboratory for Tree Breeding under natural conditions of light and temperature (Beijing, China). Apical buds from 3-month-old rooted ramets were collected (between 9:00 and 10:00 AM) and frozen in liquid nitrogen prior to analysis.

The nine PMR-H genotypes were represented by one ramet per genotype or nine plants in total. Prior to RNA preparation their collected apical buds were divided into three pools of three plants each to provide tissue for three RNA samples. Tissues from individuals within each group of three PMR-H clones were pooled in equal amounts to ensure equal representation in each RNA pool. RNA was extracted separately from three replicate ramets of both parents. Total RNA was isolated using the TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer's protocol. RNA quality was confirmed by 1% (w/v) agarose gel electrophoresis using the RNA 6000 Nano Assay Kit and a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). The RNA samples were stored at −80 °C prior to analysis.

2.2. Library Construction and Illumina RNA-Sequencing

Nine cDNA libraries were constructed (three from pools of three trees each and six from individual trees) using the TruSeq™ RNA Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions. The protocol consists of the following steps: Poly-A-containing mRNA was purified from 10 μg total RNA using oligo(dT) magnetic beads and fragmented into 200–500 bp using divalent cations at 94 °C for 5 min. The cleaved RNA fragments were reverse-transcribed into first-strand cDNA using SuperScript II reverse transcriptase and random primers. After second-strand cDNA synthesis, fragments were end-repaired, A-tailed and ligated with indexed adapters. The products were purified and enriched by PCR to create the final cDNA libraries. Target bands were harvested by 2% agarose gel electrophoresis and quantified by Agilent 2100. The tagged cDNA libraries were pooled in equal ratios and used for 101-bp paired-end sequencing in a single lane of the Illumina HiSeq™ 2000 (Illumina, Inc.,San Diego, CA) with 51 + 7 cycles.

2.3. Mapping of Reads and Differential Gene Expression Analysis

The quality of raw sequence data was assessed using FASTQC software and mapped to the *Populus* genome (phytozome version 9.0) using Tohat (v2.0.8) [19,20] with the following criteria: read-mismatches 3; max-insertion-length 6; max-deletion-length 6; read-gap-length 7; read-edit-dist 12; b2-sensitive; solexa1.3-quals -p 22; min-coverage-intron 31; min-segment-intron 31. Based on the Tophat mapping results, we applied HT-seq method implemented on a Java platform (S. Cheng) to analyze gene expression.

The numbers of DEGs were estimated and validated using DEGseq software (Version 1.20.0) [21]. Unigene expression levels were calculated using the "fragments per kilobase of exon per million mapped reads" (FPKM) method. Transcripts exhibiting a false discovery rate (FDR) <0.05 and estimated absolute $\log_2$ fold-changes (FCs) >0.585 or <−0.585, with *p*-values <0.05 were considered to be significantly differentially expressed.

2.4. Gene Annotation

The Gene Ontology (GO) database (http://www.geneontology.org/) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.genome.ad.jp/kegg/) were used to annotate genes using corrected *p*-values <0.05 as the threshold. To functionally categorize *Populus* genes, GO enrichment analysis of each differentially-expressed gene was used. Gene ontology terms represent a dynamically-structured control vocabulary and they can be applied to describe functions of genes that can be classified into three major categories, namely biological process, molecular function, and cellular component, and their sub-categories. Putative transcription factors (TFs) among such genes were identified by querying "*Populus trichocarpa* Transcription Factors" in the Plant Transcription Factor Database (http://planttfdb.cbi.edu.cn/).

3. Results

3.1. Illumina Sequencing and Sequence Data Analysis

The reads were filtered using the following criteria: (1) delete reads having >20% base quality scores <20; (2) delete reads <50 bp; and (3) delete bases of the starting and ending regions having quality scores <15 (*i.e.*, trim ends) (Table S1). This provided an average of 29,779,557, 37,056,170 and 38,696,656 high-quality, filtered reads, 100 bp in length for the PMR-H, maternal, and paternal samples, respectively (Table 1). On average, the mapping rates for the PMR-H, maternal, and paternal samples were 83.1% (24,742,617 mapped reads), 82.0% (31,697,842 mapped), and 83.5% (30,985,626 mapped), respectively, and the unique mapping rates were on average 94.5%, 92.7%, and 92.8%, respectively (Table 1).

Table 1. Number of reads from high-growth allotriploid *Populus* progeny genotypes (PMR-H) and their diploid parents mapped to the *Populus trichocarpa* genome.

Sample	Replicates	Total Reads	Mapped Reads	Mapping Rate	Reads Uniquely Mapped	Unique Mapping Rate
PMR-H	1	30727624	25702386	83.6%	24393689	94.9%
PMR-H	2	30745554	25312468	82.3%	23762732	93.9%
PMR-H	3	27865492	23212997	83.3%	22002904	94.8%
Average		**29,779,557**	**24,742,617**	**83.1%**	**23,386,442**	**94.5%**
Maternal	1	34747702	28629766	82.4%	26582193	92.8%
Maternal	2	42295592	34608859	81.8%	32055183	92.6%
Maternal	3	39046676	31854903	81.6%	29553915	92.8%
Average		**38,696,656**	**31,697,842**	**82.0%**	**29,397,097**	**92.7%**
Paternal	1	43718240	37101310	84.9%	34418213	92.8%
Paternal	2	37805466	31331934	82.9%	29146283	93.0%
Paternal	3	29644804	24523634	82.7%	22693090	92.5%
Average		**37,056,170**	**30,985,626**	**83.5%**	**28,752,529**	**92.8%**

3.2. Differentially Expressed Genes between High-Growth Allotriploid Populus and Their Parents

When the PMR-H and male parent samples were compared, 4494 genes showed significant differential expression between them (Figure 3), including 2938 genes that were up-regulated and 1556 genes that were down-regulated. When PMR-H and the female parent were compared, 1526 genes showed significant differential expression (Figure 3), with PMR-H exhibiting 863 up-regulated and 663 down-regulated genes. We performed Venn diagram analysis to identify relationships between DEGs among samples and to identify genes associated with plant phenotypic changes and height growth. A total of 791 DEGs overlapped between the allotriploid progeny and the female parent, and between the allotriploid progeny and the male parent (Figure 3).

More DEGs were evident and exhibited larger expression differences when the PMR-H genotypes were compared with their male parent rather than their female parent, possibly associated with the way in which 2n gametes were formed. The triploid progeny used in the present study were obtained by treating developing embryo sacs of *Populus pseudo-simonii* × *Populus nigra* with colchicine to induce

2n eggs. Thus, triploid hybrid offspring have two sets of the maternal genome. The expression patterns of the 791 genes were evaluated by comparing the PMR-H expression values with the parental expression values, to detect a diverse spectrum of gene expression types and levels. This includes transgressive up- (I) and down-regulated (II) in the PMR-H, in addition to genes that were up-regulated *vs.* male parent (III) and down-regulated *vs.* the male parent (IV) (p-values < 0.05) (Figure 4A).

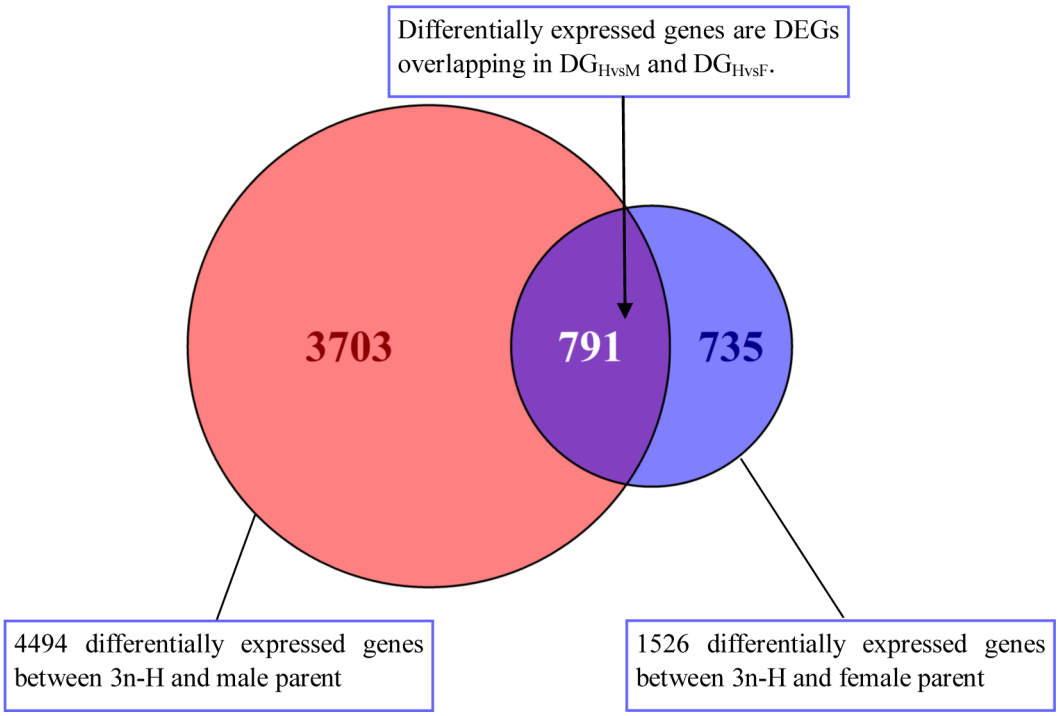

Figure 3. Venn diagrams of genes that are differentially expressed in diploid parents and high-growth allotriploid *Populus* progeny (PMR-H, indicated as 3n-H in figure). DG_{HvsM} and DG_{HvsF} refer to DEGs from the PMR-H *vs.* male parent, the PMR-H *vs.* female parent, respectively. We found 791 genes were common to both groups (DG_{HvsM} and DG_{HvsF}).

The largest numbers of genes (320, 40.5%) were up-regulated in the PMR-H compared to the parents (Table S2), while 275 (34.8%) genes were down-regulated (Table S3). In addition, 49 (6.2%) genes were up-regulated compared to male parent and down-regulated compared to female parent (Table S4), and 89 (11.3%) genes were up-regulated compared to female parent and down-regulated compared to male parent (p-values < 0.05) (Table S5).

We further pursued possible functions of four bins (I–IV) of differentially expressed genes (Figure 4B). Gene ontology analysis of 320 genes revealed enrichment of genes for the biological process sub-categories DNA and RNA methylation, cell proliferation, regulation of meristem growth, photosynthesis and nucleoside metabolic process (p-values < 0.05). The 275 down-regulated genes were mainly related to photosynthesis, response to stress, protein folding, nucleoside metabolic process and ethylene-activated signaling pathway (p-values < 0.05). In addition, 49 genes were significantly enriched for photosynthesis, translation, cell growth and metabolic process (p-values < 0.05). Another 89 genes were mainly associated with jasmonic acid (JA), fructose, lipid and carbohydrate metabolic processes (p-values < 0.05).

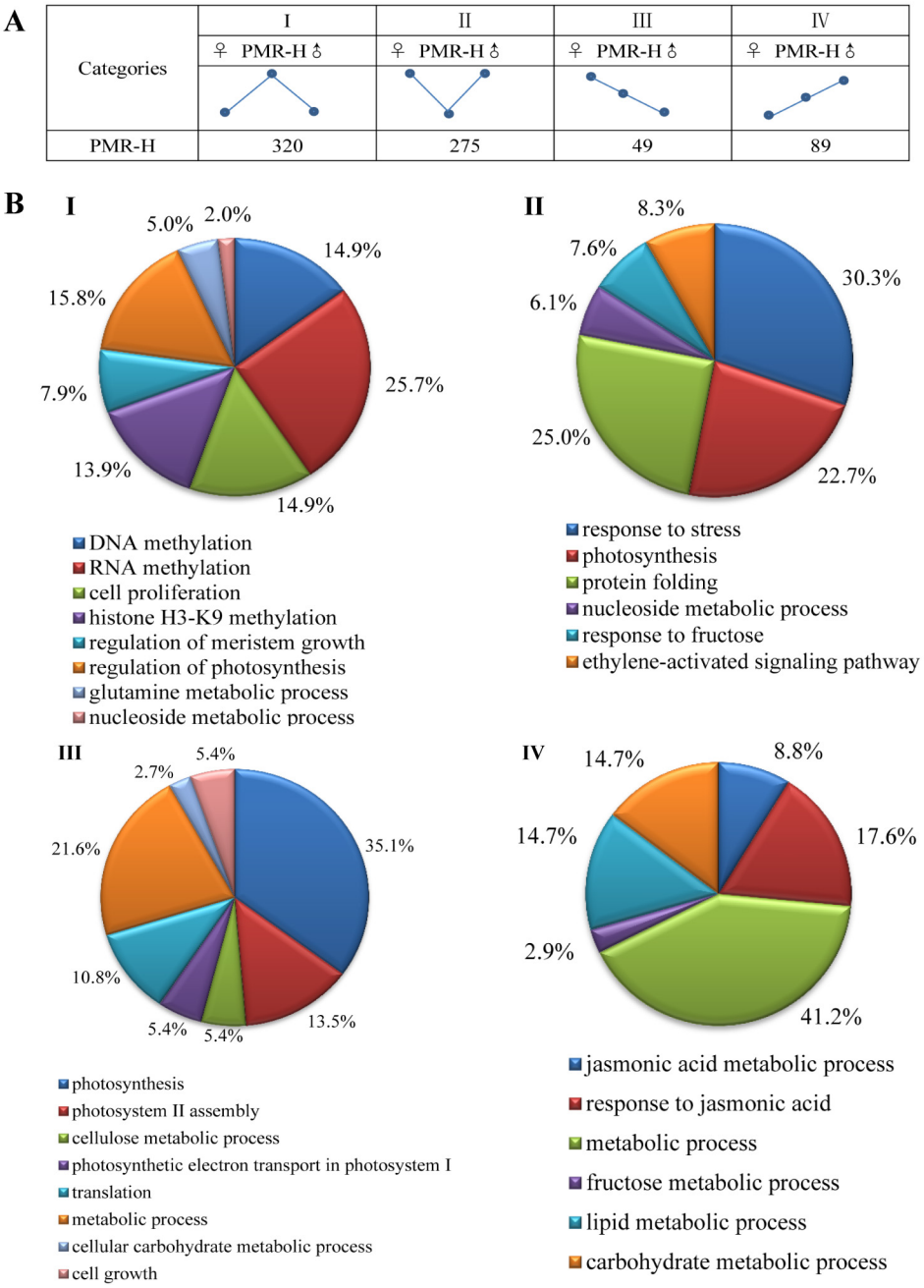

Figure 4. Differential gene expression categories (I–IV) in the high-growth allotriploid *Populus* progeny genotypes (PMR-H) relative to their diploid parents. (**A**) Four categories of the 791 DEGs in the overlapping region of the PMR-H and their parents; (**B**) Enriched GO terms of the genes in each category.

Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis for the DEGs revealed significant enrichment of specific pathways compared with the distribution of the whole transcriptome. Among the 320 genes that were up-regulated in the PMR-H compared to their parents, 116 genes had a KEGG pathway annotation, and the significantly enriched pathways (p-values < 0.05) were flavonoid biosynthesis, circadian rhythm–plant, pyrimidine and purine metabolism, glutathione metabolism and propanoate metabolism (Figure 5A). In addition, KEGG pathway analysis of 275 down-regulated genes indicated that these genes were involved in protein processing in endoplasmic reticulum,

porphyrin and chlorophyll metabolism, glyoxylate and dicarboxylate metabolism, glycolysis/ gluconeogenesis and fructose and mannose metabolism (Figure 5B).

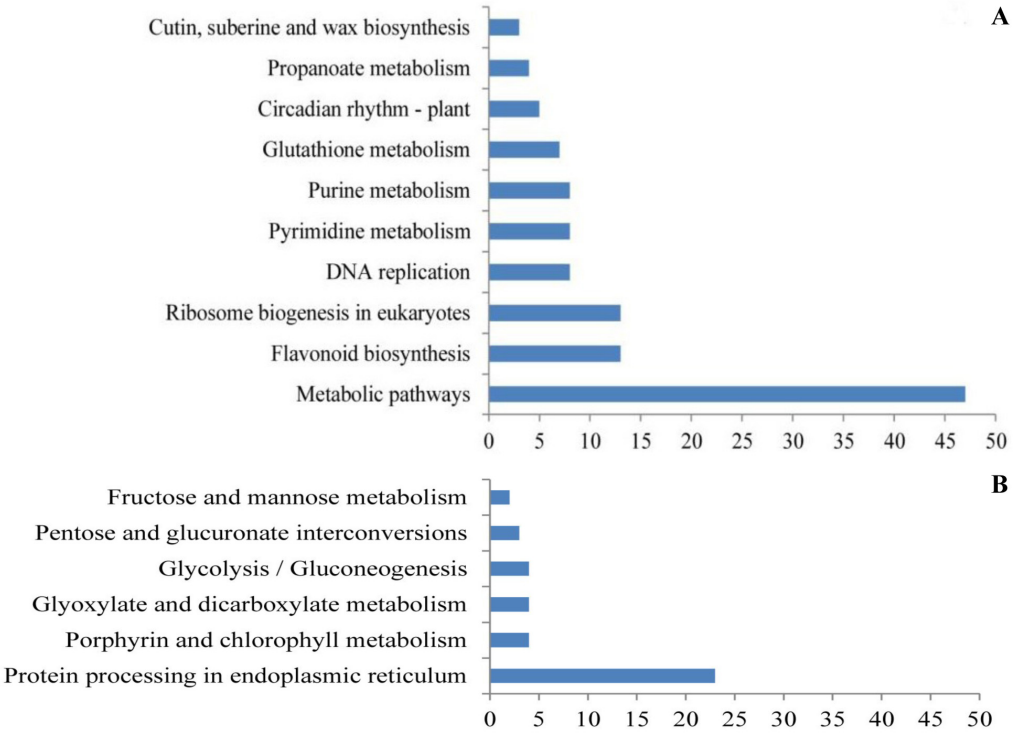

Figure 5. KEGG pathway assignments for (**A**) 320 up-regulated and 275 (**B**) down-regulated DEGs between high-growth allotriploid *Populus* progeny genotypes (PMR-H) and their diploid parents (p-values < 0.05).

3.3. Non-Additive Genes Expressed in High-Growth Allotriploid Populus Genotypes

To determine how hybridization and polyploidization changed mRNA profiles, we compared the expression levels of genes of the PMR-H with their mid-parent value (MPV; 1/3 of male parent + 2/3 of female parent mRNA expression levels). Genes exhibiting at least a two-fold difference in expression level between the PMR-H and the MPV (with FDRs < 0.05) were considered to be non-additive genes, and all others additive genes. In total, 1518 genes were non-additively expressed in PMR-H, accounting for about 4.71% of the total DEGs. Of these, 431 (28.40%) DEGs were up-regulated and 1087 (71.61%) were down-regulated.

The non-additive DEGs were functionally annotated using GO terms (p-values < 0.05) (Table 2). For example, in the biological process category, PMR-H up-regulated genes were mainly associated with cell proliferation, DNA methylation, histone H3-K9 methylation, the pyrimidine ribonucleotide biosynthetic process and the nucleobase-containing compound metabolic process, and PMR-H down-regulated genes were mainly associated with photosynthesis, unidimensional cell growth and the chlorophyll metabolic process (p-values < 0.05). In terms of the cellular components category, up-regulated genes involved in chlorophyll binding, transferase activity and carboxylic ester hydrolase activity were more highly transcribed in the apical buds of PMR-H, and down-regulated genes were mainly related to oxidoreductase activity and electron carrier activity (p-values < 0.05). In terms of the

molecular function category, PMR-H up-regulated genes were associated with chloroplast thylakoid, chloroplast thylakoid membrane and apoplast.

Table 2. Functional annotation of non-additive DEGs between high-growth allotriploid *Populus* progeny genotypes (PMR-H) and their parents based on GO terms.

Category	GO Term	Number of DEGs	
		Up-Regulated	Down-Regulated
BP	GO:0015979 Photosynthesis	5	63
	GO:0009826 unidimensional cell growth	2	14
	GO:0008283 cell proliferation	10	0
	GO:0006306 DNA methylation	54	0
	GO:0009220 pyrimidine ribonucleotide biosynthetic process	11	0
	GO:0051567 histone H3-K9 methylation	13	0
	GO:0006139 nucleobase-containing compound metabolic process	6	0
	GO:0015994 chlorophyll metabolic process	3	2
MF	GO:0016168 chlorophyll binding	10	3
	GO:0016491 oxidoreductase activity	8	112
	GO:0016747 transferase activity	23	0
	GO:0009055 electron carrier activity	6	44
	GO:0052689 carboxylic ester hydrolase activity	25	4
CC	GO:0009579 thylakoid	6	46
	GO:0009534 chloroplast thylakoid	32	5
	GO:0009535 chloroplast thylakoid membrane	54	3
	GO:0048046 apoplast	40	8

BP, biological process, MF, molecular function, CC, cellular component.

3.4. Cell Differentiation- and Meristem Development-Related Genes are Preferentially Expressed in High-Growth Allotriploid Populus Compared to Their Diploid Parents

We identified 33 DEGs (among the 791 DEGs) associated with cell differentiation and meristem development (Table 3). Five *mini-chromosome maintenance* (*MCM*) protein genes and *MSI2*, *MSH2* (*MUTS HOMOLOG 2*), *HTA3* (*HISTONE H2A 3*), *ATRPA70B* (*RPA70-KDA SUBUNIT B*), *AtBARD1* (*BREAST CANCER ASSOCIATED RING 1*), *SOM1* (*SOMNIFEROUS 1*), *ATL5* (*RIBOSOMAL PROTEIN L5*), and *ATML1* (*MERISTEM LAYER 1*) were preferentially expressed in the PMR-H, suggesting that the genes played roles in SAM maintenance. The genes *PSK3* (*PHYTOSULFOKINE 3 PRECURSOR*), *MAF5* (*MADS AFFECTING FLOWERING 5*), and *PSBO* (*PHOTOSYSTEM II SUBUNIT O*) were preferentially expressed in the parents. Of these genes, *MCM2–6* belongs to the MCM family of proteins. The MCM complex of plants remains in the nucleus throughout most of the cell cycle, becoming dispersed only in mitotic cells [22]. Also, *MCM3* and *MCM6* are known to be essential for both vegetative and reproductive growth, and development, in plants [23,24]. The gene *AtBARD1* regulates SAM maintenance in *Arabidopsis thaliana* by repressing *WUS* expression [25].

Table 3. Cell differentiation- and meristem development-related genes preferentially expressed in high-growth allotriploid *Populus* progeny genotypes (PMR-H) compared to their diploid parents.

Biological Function	Genes	Poplar Gene Model	TAIR Annotation	FoldChange (3n-H/male Parent)	FoldChange (3n-H/Female Parent)
Cell proliferation	MCM2	POPTR_0001s12700	AT1G44900	2.5	1.7
	MCM3	POPTR_0004s13660	AT5G46280	2.7	1.6
	MCM4	POPTR_0009s12440	AT2G16440	2.0	1.7
	MCM5	POPTR_0018s12080	AT2G07690	2.1	1.6
	MCM6	POPTR_0001s12380	AT5G44635	2.2	1.6
	BARD1	POPTR_0002s26070	AT1G04020	1.6	1.5
	MSH2	POPTR_0012s05670	AT3G18524	2.2	1.6
	HTA3	POPTR_0005s04250	AT1G54690	1.8	1.6
	MSI2	POPTR_0009s13750	AT2G16780	1.6	1.8
	RPA70B	POPTR_0015s06800	AT5G08020	2.3	1.8
	SOM1	POPTR_0019s15030	AT5G66750	2.4	2.0
	ATL5	Potri.014G197100	AT3G25520	1.7	1.5
	ATML1	POPTR_0011s00520	AT4G21750	3.9	2.4
	PSK3	POPTR_0014s00810	AT1G13590	0.6	1.8
	MAF5	POPTR_0003s16840	AT5G65080	0.2	0.3
	PSBO	POPTR_0007s12070	AT5G66570	0.6	0.7
	CRB	POPTR_0005s01370	AT1G09340	0.6	0.6
Meristem growth	LAX2	POPTR_0009s13470	AT2G21050	1.7	1.6
	CYP78A7	POPTR_0005s08640	AT5G09970	5.5	1.9
	JAG	Potri.008G121200	AT1G68480	3.6	2.1
	ANT	POPTR_0002s11550	AT4G37750	2.3	1.7
	GA20-Oxidase	POPTR_0007s04360	AT5G07200	0.4	0.6
	HEN2	Potri.018G146100	AT2G06990	2.4	2.9
	JP630	POPTR_0010s05000	AT1G23760	1.6	1.5
	SPL5	POPTR_0011s05480	AT3G15270	2.2	1.8
	PLP9	POPTR_0002s05350	AT3G63200	1.7	2.1
	RID3	POPTR_0014s02710	AT3G49180	2.3	1.7
	CHR17	POPTR_0010s02180	AT5G18620	2.2	1.7
	EXPA1	POPTR_0013s05730	AT1G69530	0.3	0.6
	ATEXPA10	POPTR_0010s17440	AT1G26770	0.5	0.6
	ATEXPB3	POPTR_0013s13780	AT4G28250	2.3	0.6
	MAN7	POPTR_0005s12250	AT5G66460	0.5	0.6
	LHT7	Potri.004G181100	AT4G35180	0.5	0.7

We found that the meristem growth-related genes *LAX2* (*LIKE AUXIN RESISTANT 2*), *PLP9* (*PATATIN-like protein 9*), *CYP78A7* (*CYTOCHROME P450, FAMILY 78*), *JAG* (*JAGGED*), *SPL5* (*SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 5*), *ANT* (*AINTEGUMENTA*), *RID3* (*ROOT INITIATION DEFECTIVE 3*), *HEN2* (*HUA ENHANCER 2*), *JP630*, and *CHR17* (*CHROMATIN REMODELING FACTOR17*) were preferentially expressed in the PMR-H compared to their parents. The gene LAX2 is a member of the AUX1 LAX family of auxin influx carriers, while LAX2 is a novel

gene required for axillary meristem (AM) formation and acts in concert with LAX1 in rice [26]. Mutation of *CYP78A* in *Arabidopsis* and rice suggested that CYP78A was a novel mobile factor regulating organ size and cell proliferation [27]. The JAG gene encodes a zinc-finger protein promoting cell proliferation and differentiation of leaf cells [28]. The ANT gene may coordinate proliferation with growth by maintaining the meristematic competence of cells during organogenesis [29]. Gibberellin 20-oxidase (GA 20-oxidase) regulates gibberellic acid (GA) levels during cotton fiber development, and such regulated genes may be targeted to manipulate cotton fiber initiation and elongation. Furthermore, GA 20-oxidase-regulated biosynthetic genes are involved in petiole elongation in Arabidopsis associated with exposure to end-of-day far-red (FR) light or long day from FR-rich incandescent lamps [30,31]. We found that GA 20-oxidase was down-regulated in the PMR-H compared to their parents, and gene function may have been associated with the level of gene expression.

3.5. Auxin-, Gibberellic Acid-, and Jasmonic Acid-Related Genes Are Preferentially Expressed in High-Growth Allotriploid Populus Progeny Genotypes Compared to Their Diploid Parents

In multicellular organisms, cellular communication is important to coordinate growth and to allow cell differentiation into new tissues and organs. Plant growth and development are regulated by small-molecule plant hormones including auxin, cytokinin, abscisic acid (ABA), GAs, and JA. These hormones govern and coordinate developmental processes. In the present study, many auxin-, GA-, and JA-response genes were preferentially expressed in the apical buds of the PMR-H trees (Table 4). We identified 18 differentially expressed auxin-related genes (among the 791 DEGs), of which 11 were preferentially expressed in PMR-H apical buds. At the cellular level, auxin regulates cell division, extension, and differentiation. At the whole-plant level, auxin plays an essential role in processes including apical dominance, lateral root formation, tropic responses, fruit growth and development, vascular differentiation, and embryogenesis. Chalcone synthase (CHS) is a key enzyme involved in the biosynthesis of flavonoids, regulation of auxin transport, and modulation of root gravitropism. The *SAUR78*, *SAUR51*, and *SAUR29* genes, members of the SAUR-like auxin-response protein family, are involved in elongation and growth [32]. These auxin-related genes were preferentially expressed in apical buds and may function to preserve auxin balance in the response to auxin stimuli and/or in regulating apical meristems [33,34].

Of the eight GA stimulus-related genes, both the F-box protein *SNE* (*SNEEZY*) and *ATHB22* (*ARABIDOPSIS THALIANA HOMEOBOX PROTEIN 22*) genes were preferentially transcribed in the PMR-H. Notably, we identified 10 JA-related genes, five of which were preferentially transcribed in the PMR-H, whereas the other five were preferentially transcribed in the male parent (Table 4). Jasmonic acid and the methyl ester methyl jasmonate (MeJA) are naturally occurring plant growth regulators, controlling morphological, physiological, and biochemical processes [35,36]. We identified seven JA-associated genes, of which *tMYB70* (*MYB DOMAIN PROTEIN 70*), *WRKY40* (*WRKY DNA-BINDING PROTEIN 40*), and *ANS* (*ANTHOCYANIDIN SYNTHASE*) were preferentially expressed in the PMR-H, whereas *SOT18* (*SULFOTRANSFERASE 18*), *SOT17* (*SULFOTRANSFERASE 17*), and *ACL5* (*ACAULIS 5*) were preferentially expressed in the male parent. The gene *NAC032* (*NAC DOMAIN CONTAINING PROTEIN 32*) was preferentially expressed in both male and female parents.

Table 4. Hormone-related genes preferentially expressed in high-growth allotriploid *Populus* progeny genotypes (PMR-H) compared to their diploid parents.

Hormone Types	Genes	Poplar Gene Model	TAIR Annotation	Log2 Ratio (PMR-H/male Parent) [a]	Log2 Ratio (PMR-H/Female Parent)
Auxin	CHS	POPTR_0003s17540	AT5G13930	0.86	1.21
	JP630	POPTR_0010s05000	AT1G23760	0.68	0.59
	TT7	POPTR_0013s07050	AT5G07990	0.69	0.60
	DOT3	POPTR_0007s06310	AT5G10250	1.43	0.96
	PDR12	POPTR_0001s14650	AT1G15520	1.38	1.01
	SAUR78	POPTR_0630s00200	AT1G72430	1.04	1.28
	SAUR51	POPTR_0004s17150	AT1G75580	−1.25	1.19
	SAUR29	POPTR_0004s17180	AT3G03820	−1.31	−1.16
	MYB78	POPTR_0010s15970	AT5G49620	1.42	1.24
	AAE18	POPTR_0001s04380	AT1G55320	0.70	1.15
	LAX2	POPTR_0009s13470	AT2G21050	0.72	0.70
	ARF18	POPTR_0002s17350	AT3G61830	0.64	0.61
	ARF2	POPTR_0003s16210	AT5G62000	1.03	0.63
	NAP9	POPTR_0012s01280	AT5G02270	−2.01	20
	GRXS13	POPTR_0017s04860	AT1G03850	−1.16	−0.97
	MI-1-P	POPTR_0007s05810	AT4G39800	−0.69	−0.80
	PBP1	POPTR_0004s02630	AT5G54490	−1.23	1.21
	GDSL-like	POPTR_0001s19220	AT3G16370	−0.94	−0.62
Gibberellin	XERICO	POPTR_0014s16830	AT2G04240	−0.97	−0.87
	EXPA1	POPTR_0010s17440	AT1G26770	−1.00	−0.73
	F-box protein SNE	POPTR_0014s16360	AT2G42660	0.64	1.12
	FKBP-like	POPTR_0015s08290	AT2G43560	−0.94	1.41
	GAST1	POPTR_0006s04300	AT3G10185	−1.85	−1.02
	ATHB22	POPTR_0002s10330	AT4G24660	0.62	0.71
	YAP169	POPTR_0007s04360	AT5G07200	−1.18	−0.61
Jasmonic Acid	tMYB70	POPTR_0009s09890	AT2G23290	1.82	1.33
	WRKY40	POPTR_0016s14490	AT1G80840	0.64	1.32
	MYB78	POPTR_0010s15970	AT5G49620	1.42	1.24
	ANS	POPTR_0003s11900	AT4G22880	1.61	1.30
	PDR12	POPTR_0001s14650	AT1G15520	1.38	1.01
	PBP1	POPTR_0004s02630	AT5G54490	−1.23	1.21
	SOT18	POPTR_0003s18750	AT1G74090	−0.73	0.91
	SOT17	POPTR_0003s18820	AT1G18590	−1.42	20.00
	ACL5	POPTR_0001s29530	AT5G19530	−1.42	2.39
	NAC032	POPTR_0007s04780	AT1G77450	−2.22	−1.32

[a] Log2 ratio represents relative expression of a gene in PMR-H compared to that in the parent plants; positive Log2 values means the genes were preferentially expressed in PMR-H, while negative Log2 means the genes were preferentially expressed in the parents.

3.6. Non-Additive Transcription Factor Gene Expression in High-Growth Populus Allotriploids

In total, 89 TFs from 21 families were differentially expressed in the PMR-H; a percentage of DEGs were different in the gene families, which included MYB (8.99%), HD-ZIP (3.37%), bZIP (3.37%), GRF (1.12%), M-type (1.12%), C2H2 (3.37%), NAC (11.24%), GRAS (2.45%), MYB_related (2.45%), bHLH (7.87%), WRKY (8.99%), TALE (1.12%), MIKC (7.87%), GATA (1.12%), AP2 (1.12%), HSF (2.25%), G2-like (1.12%), NF-YA (1.12%), ERF (28.09%), and Trihelix (1.12%). These non-additive TF genes play important biological roles in hormone signal transduction, regulation of transcription and cell differentiation, and regulation of carbohydrate metabolism (Table S6). Myeloblastosis is a key regulator of plant root-hair development in the tomato and *Arabidopsis* [37]. Homeodomain leucine-zipper (HD-ZIP) TFs act at sequence-specific DNA sites to control the expression levels of target genes, several of which are rapidly induced in response to altered environmental conditions and to integrate hormonal signals [38]. The NAC (no apical meristem (NAM), cup-shaped cotyledon (CUC)) TFs regulate xylem vessel differentiation by inducing genes required for secondary cell wall biosynthesis. Ethylene response factors (ERFs) are TFs forming a large family within the AP2/ERF superfamily and are implicated in a range of biological processes. Ethylene response factors are well characterized and regulate shoot development during initiation of organ primordia and growth [39]. They also maintain cell meristematic competence during shoot organogenesis. Moreover, growth-regulating factor (GRF) genes are plant-specific TFs involved in the response to ABA and leaf morphogenesis.

4. Discussion

Allopolyploids are derived by hybridization between different species, deriving from unreduced gametes or chromosome doubling. In allopolyploids, heterozygosity and intergenomic interactions generally result in plants that grow more vigorously and have better yield potential than their parents. Due to the general importance of understanding polyploidy inheritance and variation mechanisms, recent studies have focused on transcriptomic changes in polyploids using several model systems (e.g., *Arabidopsis*, cotton, *Brassica*, rice and wheat) since the importance of different mechanisms may vary among species [40–44]. Thus, investigations of additional taxa might be enlightening, particularly in woody plants. Here we used RNA-Seq to trace the transcriptomic changes between *Populus* allotriploid progeny and their diploid parents, finding numerous differentially expressed genes that can be used to investigate the molecular mechanism(s) underlying the variation and adaptation resulting from allopolyploidization.

In order to determine the key components and regulatory networks underlying high-growth rate phenotypes in *Populus* allotriploids, GO and KEGG pathway analysis were applied to identify key pathways linked to the differentially expressed genes. Interestingly, the transcripts related to metabolism were significantly enriched in high-growth allotriploids progeny relative to their diploid parents, which indicated that metabolism was the most up-regulated function in heterosis for growth-related traits in these *Populus* allotriploids. In a study of diploid *A. thaliana* hybrids, the correlation of heterosis and enhanced metabolic activities was also reported [45]. As a result, it is proposed that the activities of

metabolism are involved in the enhancement of some traits of *Populus* allotriploids such as faster height growth.

The enriched transcripts, especially those dramatically up- and down-regulated, are candidate genes for the observed heterosis of height growth in *Populus* allotriploids, including genes controlling cell differentiation and meristem development, hormone production and transcription factors. Most of the candidate genes were expressed at remarkably higher levels in the high-growth allotriploid progeny compared to their parents. Non-additive gene regulation in synthesized allopolyploids may increase the potential for fitness and selective adaptation [42,46]. In addition, non-additive gene expression in regulatory pathways is responsive to growth, development, and stresses in many polyploids [47]. In *Arabidopsis* allotetraploids, the non-additive gene regulation is involved in various biological pathways, and the changes in gene expression are developmentally regulated, which may provide the molecular basis for variation in selection and adaptation of new allopolyploid species [48]. In this study, non-additive genes were significantly enriched in biological processes related to hormone signal transduction, unidimensional cell growth, cell proliferation, and DNA methylation. DNA methylation is an important epigenetic factor for transcriptomic changes in allopolyploids, and changes in DNA methylation and their relationship with gene expression in allopolyploids have been investigated extensively [43,49]. These findings reveal that the morphological differences that developed between these high-growth *Populus* allotriploid progeny and their diploid parents may be caused by non-additive genes from multiple functional groups.

In this work, we show that a variety of TFs are differentially expressed in these *Populus* allotriploids, some of which have been greatly amplified [50,51]. Transcription factors regulate the expression of many genes and play critical roles in plant development and adaptation [52]. In synthesized *Arabidopsis* allotetraploids, the regulation of genome-wide non-additive gene expression may be partly controlled by TFs [48]. Our results provide a comprehensive view of TF gene families. For example, 89 TFs from 21 families were significantly differentially expressed in the apical buds of the high-growth *Populus* allotriploids progeny compared to their diploid parents; interestingly, all of these TF-related genes displayed non-additive expression patterns. We examined several multi-gene families in terms of their expression profiles and functions in plants. As studies of the NAC gene family increase in number, their importance in the developmental control of plant development is being elucidated. Several members of the NAC gene family were expressed in the apical buds of these high-growth *Populus* allotriploids, indicating that this gene family may regulate plant growth and development of the apical meristem. Several studies suggested that NAC genes are related to wood formation and the cell division process in various tissues. Our results suggest that TF genes play an important role in growth and developmental changes in *Populus* allotriploids.

5. Conclusions

In the present study, we used RNA-seq analysis to systematically investigate the transcriptome profiles of *Populus* allotriploid progeny genotypes and their diploid parents. We characterized differences in gene expression apparently resulting from polyploidization and identified genes that may be associated with the observed increased height growth of the allotriploids. The data obtained serve as a foundation for future research on polyploidy in *Populus*. We analyzed differentially

expressed genes between the high-growth *Populus* allotriploid progeny and their parents using the GO and KEGG databases to identify candidate transcripts that may contribute significantly to apical bud growth and development. Such differentially expressed genes are involved in a wide range of plant physiological processes that may be essential for development of the differences in morphology and physiology evident between these *Populus* allotriploids and their diploid parents. Our findings shed new light on variations in *Populus* allotriploidization and may contribute to identifying genes important for the genetic improvement of *Populus* and other forest trees in the near future.

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Author Contributions

Xiangyang Kang and Shiping Cheng conceived and designed the experiments; Shiping Cheng, Xiaohu Zhu, Ting Liao, Yun Li, Pengqiang Yao, Yujing Suo, Pingdong Zhang, and Jun Wang performed the experiments; Shiping Cheng analyzed the data. Xiangyang Kang, Shiping Cheng, and Xiaohu Zhu wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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