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An expression profiling analysis of hybrid millet and its parents at grain filling stage

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ABSTRACT. Heterosis has been widely used in crop breeding and production. However, a shortage of genes known to function in heterosis significantly limits our understanding of the molecular basis underlying heterosis. Here, we report 740 differentially expressed genes (DEGs) in the leaves of the hybrid millet Zhang No.5 and its parents at the grain filling stage determined using Solexa Illumina digital gene expression. Of the 740 DEGs, 546 were from the hybrid and its parents and most were up-regulated in the hybrid. Particularly, a large number of DEGs related to starch and carbohydrate metabolism and 2 DEGs encoding chlorophyll a/b binding proteins were up-regulated in hybrid millet. Moreover, all DEGs were enriched in the biological process and molecular function, and no DEGs were found to be enriched in the cellular component of GO terms. Pathway enrichment using KEGG showed that several DEGs were enriched in the circadian rhythm pathway. Further analysis revealed that the altered circadian rhythm, which mediates photosynthesis and carbohydrate accumulation, may play an important role in heterosis of the hybrid millet.

Key words: Differentially expressed gene; Grain filling stage; Heterosis; Hybrid millet

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INTRODUCTION

Heterosis is a biological phenomenon in which the offspring from 2 parents show improved and superior performance compared to either inbred parental line. The application of heterosis in agriculture has led to revolutionary increases in the productivity for a number of agricultural species. However, the mechanism of heterosis remains largely unknown. The genes in hybrids are obtained from the parents, and new genes are not introduced. Thus, heterosis may originate from differentially expressed genes (DEGs) (Doebley and Lukens, 1998). Therefore, studies of gene expression levels may reveal the molecular mechanism of heterosis. Great progress has been made in rice and maize. Romagnoli et al. (1990) showed that mRNAs are differentially synthesized and expressed in the F_1 primary root tips compared to in parental lines. Xiong et al. (1998) and Wu et al. (2001) found that heterosis was related to inhibition of genes expression in hybrids and not related to expression of the dominant gene. Ni et al. (2001) showed that over-expressed and hybrid specific expressed genes may play important roles in wheat heterosis. Based on these results, DEGs may be closely related to heterosis.

High-throughput sequencing can greatly improve the efficiency of identifying DEGs, which will provide information to increase the understanding of the molecular basis underlying heterosis. Wei et al. (2009) investigated transcriptome profiles of superhybrid rice LYP9 and its parental cultivars 93-11and PA64s in 7 tissues and identified 3926 DEGs as a potential group of heterosis-related genes. Song et al. (2010) found 1183 DEGs to be significantly enriched in pathways such as photosynthesis and carbon-fixation, and most key genes involved in the carbon-fixation pathway exhibited up-regulated expression in F_1 hybrid rice. Qin et al. (2013), provided a repertoire of genes useful for identifying genes involved in maize ear trait heterosis. The large number of genes identified in these studies should be examined to understand the underlying molecular basis of heterosis.

Although foxtail millet (*Setaria italica* P. Beauv.) is an important cereal crop, hybrid millet was bred successfully until 2000. The yield of hybrid millet was increased greatly (Zhao et al., 2000), but little were known about the heterosis of foxtail millet. Determining the foxtail millet genome sequence may lay a foundation for molecular and biology studies of heterosis (Zhang et al., 2012). In this study, DEGs were identified by high-throughput sequencing to identify a potential group of heterosis-related genes for further study of the mechanism of heterosis in millet.

MATERIAL AND METHODS

Plant materials

The hybrid millet Zhang No.5 and parental inbreds were used in this study. The female was a foxtail millet of a photoperiod-sensitive male sterile line. The yield of the hybrid millet was significantly higher than that of the male.

Plants of hybrid millet and its parents were grown in a field. Flag leaves were collected at the beginning of the grain filling stage. All samples were rapidly stored at -80°C until RNA extraction.

RNA samples were isolated from each sample using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions, quantified using a NanoDrop1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA), and labeled.

Illumina cDNA library preparation and sequencing

After RNA extraction, mRNAs were purified using oligo-dT-conjugated magnetic beads, and then the mRNA was fragmented into small pieces using divalent cations at high temperature. From these cleaved RNA fragments, 2 cDNA strands were synthesized. A linear polymerase chain reaction (PCR) amplification for 15 cycles was performed for the cDNA to enrich the samples for the desired fragments. The resulting fragments were purified by 2% certified low-range ultra-pure agarose electrophoresis. After denaturation, single-chain molecules were fixed onto TBS380 (PicoGreen, Life Technologies, Carlsbad, CA, USA). As sequencing template, each molecule developed into a single molecule cluster through *in situ* amplification. Sequencing by synthesis was performed with a read length of 50 base pairs using the Hiseq2000 (Majorbio, Shanghai, China) according to manufacturer protocols.

Aligning short reads to foxtail millet reference genome

The original data were evaluated to obtain the clean data using the software SeqPrep (https://github.com/jstjohn/SeqPrep) and sickle (https://github.com/najoshi/sickle). All high-quality sequences were mapped to the reference genome from the phytozome web site (http://www.phytozome.net/foxtailmillet.php).

Screening of DEGs and gene annotation

A rigorous algorithm was performed to identify DEGs between cultivars. In this study, $P \leq 0.01$, false discovery rate ≤ 0.05, and the absolute value of the log2Ratio ≥ 1 (2-fold change) were used as the thresholds to judge the significance of gene expression differences. The results were analyzed using the software Tophat (http://tophat.cbcb.umd.edu/) and Cuffdiff (http://cufflinks.cbcb.umd.edu/). After mapping the reference sequences, the homologs of DEGs with known genes were assigned by homology searching against the National Center for Biotechnology Information Non-Redundancy protein database using BLASTx with an E-value $\leq$1E-10. In addition, Gene Ontology (GO) and pathway enrichment analysis mapped all DEGs in the GO database (http://www.geneontology.org/) into significantly enriched GO terms (Bonferroni corrected P value ≤ 0.05) and DEGs in the Kyoto Encyclopedia of Genes and Genomes database (http://www.kegg.jp/kegg/pathway.html) into metabolic or signal transduction pathways (P value ≤ 0.05) through comparisons with the whole genome background.

Validation of DEGs using real-time-quantitative PCR

The flag leaves collected at the same time as those used for expression profile analysis but from different plants were used for real-time PCR to validate the DEGs identified by expression profile analysis. A sample of 15 DEGs was selected. Primers were designed using Primer 5 (**Table S1**) and synthesized by ShengGong Cooperation (Shanghai, China). Total RNA was extracted using TRIzol Reagent (Invitrogen) from approximately 100 mg frozen leaves and treated with DNase I (TaKaRa, Shiga, Japan) to remove potential DNA contamination. Relative quantitative analysis was conducted using the Applied Biosystems 7900HT with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Three technical replicates were included on each plate. Relative expression levels were computed using the $2^{-\Delta\Delta Ct}$ method. Two biological replicates were used for real-time PCR analysis.

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RESULTS

Summary information of DEGs

Our data were derived from the leaves of 3 cultivars at grain-filling stage. After removing low-quality sequences and trimming adapter sequences, totals of 11,031,026; 9,984,122; and 10,299,335 high-quality sequences were generated from female, hybrid, and male plants. Next, we blasted these sequences against the genome sequence of foxtail millet (http://www.phytozome.net/foxtailmillet.php) using the software TopHat (http://tophat.cbcb.umd.edu/). BLAST comparison revealed that 78-81% of the sequences could be mapped to the foxtail millet genome (Table 1).

Table 1. Summary information of DEGs.

Sample name	Number of high-quality sequence	Number of high-quality base (bp)	Number of blasting sequence	Percentage of mapping to the genome (%)
Female	11,031,126	548,195,981	8,698,190	78.85
F_1	9,984,122	496,502,744	8,046,531	80.59
male	10,299,335	511,783,387	8,342,377	81.00

Real-time PCR experiments verified the high-throughput sequencing data

To evaluate the validity of gene expression analysis using high-throughput sequencing, we performed real-time PCR analysis for 15 genes. The expression based on real-time PCR showed consistent results with those obtained from the sequencing data, demonstrating the satisfactory quality of the sequencing and the data analysis (Table 2).

Table 2. Validation of DEGs by real-time PCR.

Gene	Annotation	Sequencing		Real-time PCR	
		log2FC (F_1/Female)	log2FC (F_1/Male)	F_1/Female	F_1/Male
Si026463	Chalcone and stilbene synthase family protein	-0.29	3.86	0.47	4.35
Si032777	Chlorophyll A-B binding family protein	-1.53	3.55	0.40	2.23
Si031983	UDP-D-glucose/UDP-D-galactose 4-epimerase 1	-2.15	-3.90	0.47	0.41
Si006946	Peroxidase superfamily protein	1.77	3.93	1.23	8.34
Si031242	Basic pathogenesis-related protein 1	3.49	5.31	1.90	7.68
Si023769	Thioredoxin H-type 5	4.09	4.41	2.49	31.23
Si000306	Alpha-glucan phosphorylase 2	0.25	1.82	1.51	4.08
Si030429	WRKY DNA-binding protein 18	5.78	8.39	1.01	1.33
Si029769	UDP-glucosyltransferase $74F_2$	2.29	2.81	1.95	3.65
Si035705	Calreticulin precursor	2.49	2.50	1.73	4.61
Si013398	MYB family transcription factor	-0.14	-1.60	0.77	0.45
Si014685	MYB family transcription factor	-1.18	-1.20	0.17	0.45
Si033968	Phytochrome B	-0.41	-1.73	0.49	0.40
Si033984	Phytochrome A	-7.06	-1.87	0.32	0.33
Si000698	Phytochrome interacting factor 3	-2.10	-2.74	0.51	0.65

Positive numbers indicate genes in F_1 that were up-regulated, and the negative numbers indicate genes in F_1 that were down-regulated based on the sequencing data; numbers above 1 indicate genes in F_1 that were up-regulated and numbers less than 1 indicate genes in F_1 that were down-regulated from real-time PCR.

Differential expression analysis of hybrid millet and its parents

According to the BLAST results from all 3 samples, we identified 740 genes significantly differentially expressed in each pairwise comparison (**Table S2**). Exceeding our

expectations, the larger number of DEGs (487 DEGs) was found between females and males, while the lowest number of DEGs (177) was found between hybrids and females. A total of 446 DEGs were identified between the hybrid and males. A total of 359 genes, accounting for 48.5% of all DEGs, appeared to be differentially expressed in more than 1 cultivar, including 11 genes (1.5% of total DEGs) in 3 cultivars (Figure 1A). Both up- and down-regulation was observed in different cultivars. However, up-regulation dominated in the hybrid, with 132 up-regulated genes (74.6%) of 177 between the F_1 and female and 277 (62.1%) up-regulated genes of 446 between the F_1 and male. The number of up-regulated genes was 278 of 487 DEGs between females and males (Figure 1B).

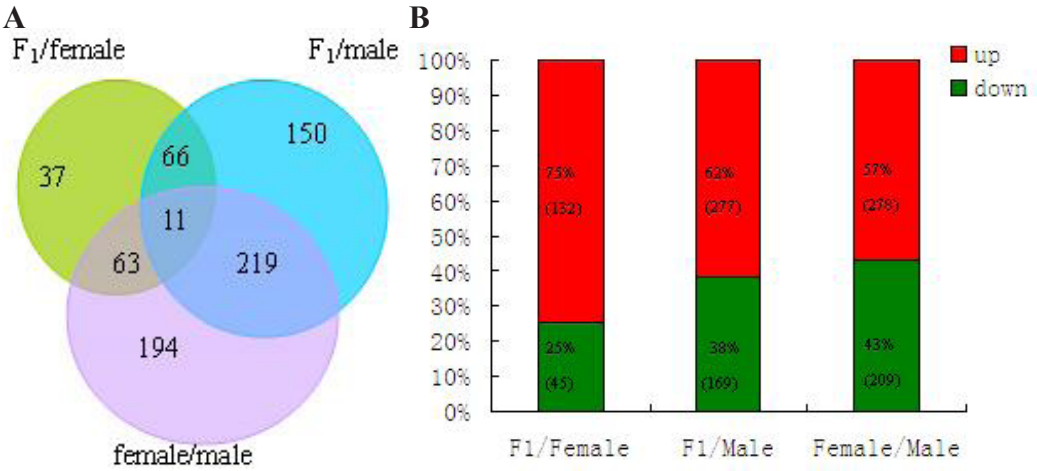

Figure 1. Genes differentially expressed between various cutivars. **A.** Genes differentially expressed. **B.** Percentages of up- and down-regulated genes.

GO terms and pathway enrichment of DEGs

We examined the functional categories of the DEGs using the goatools software (https://github.com/tanghaibao/goatools). We categorized the 740 DEGs according into the general categories of biological process, molecular function, and cellular component (Table 3). The DEGs between the hybrid and the female were found in only 5 GO terms. DEGs between hybrid and male were found in 34 GO terms. The number of GO terms between female and male was largest as the largest number of DEGs were observed between these groups. Although the number of GO terms between cultivars differed, DEGs were all enriched in the biological process and molecular function categories. No DEGs were enriched in the cellular component GO terms, which did not agree with the results that no DEGs between the hybrid maize and its parents were identified in molecular function (Qin et al., 2013). These results suggest that the basis of heterosis differs in different crops. In addition, pathway enrichment analysis was used to analyze potential involvement of genes in cellular metabolic pathways. The DEGs between F_1 and female were enriched in 6 pathways. Twenty-five DEGs between F_1 and male were found in the 12 pathways, with some involved in more than 1 pathway. The

pathways containing the top 3 largest numbers of DEG were phenylpropanoid biosynthesis (5 DEGs), antigen processing and presentation (4 DEGs), and amino sugar and nucleotide sugar metabolism (4 DEGs). Furthermore, the 5 DEGs (Si026463, Si033984, Si033968, Si014685, Si013398) were found in the circadian rhythm pathway. Twenty-one DEGs between female and male were found in 12 pathways, 6 of which were shared with the enriched pathway from F_1 and male (Table 4).

DEGs involved in circadian rhythms

The increase in biomass or vitality in F_1 is an important aspect of heterosis. A study by Ni et al. (2009) suggested that the increased biomass or vitality in F_1 originated from altered circadian rhythms. In this study, several DEGs involved in the circadian rhythms were identified. Two genes encoding the circadian clock genes CCA1 and LHY (Si014685, Si013398) were down-regulated in hybrids. CCA1 and LHY, which are partially redundant MYB-domain transcription factors with incompletely overlapping functions, were considered to be the central oscillators of the circadian clock (Alabadi et al., 2001; Mizoguchi et al., 2002). The red/far-red light sensing phytochromes PHYA and PHYB transduce light signals to reset the circadian clock. In this study, the expression of PHYA and PHYB (Si033984, Si033968) were low in the hybrids. Furthermore, the gene (Si000698) encoding the phytochrome-interacting basic helix-loop-helix transcription factor 3 was down-regulated. The expression alteration of DEGs involved in circadian rhythm suggest that these genes play important roles in heterosis.

DEGs involved in carbohydrate metabolism

Numerous studies have revealed that the metabolism of starch is affected by circadian rhythms (Lu et al., 2005; Graf et al., 2010; Shen et al., 2014). Altered circadian rhythms regulate starch accumulation in hybrids and allopolyploids in *Arabidopsis* (Ni et al., 2009). In this study, a large number of DEGs related to carbohydrate metabolism were identified. The DEGs consisted of the genes related to starch synthesis and the genes responsible for starch degradation, including 3 genes encoding glycosyl hydrolases family 17 (Si002182, Si004560, Si022625), 2 genes encoding amylase (Si021452, Si035044), 2 genes encoding carbohydrate phosphorylase (Si000306, Si034019), 1 gene encoding aldehyde dehydrogenase (Si000743), 3 genes encoding UDP-glucoronosyl/UDP-glucosyl transferase (Si006342, Si029769, Si040038), 2 genes responsible for transporter family protein (Si030126, Si030450), the trehalose synthase gene (Si000238), and the UDP-glucose 4-epimerase gene (Si031983). These genes were all up-regulated in the hybrid except for 2 genes (Si000238, Si031983). These DEGs suggest that carbohydrate metabolism is vigorous in the hybrid. The up-regulation of glucosyl transferase indicates that starch metabolism in the hybrid is particularly vigorous.

DEGs involved in photosynthesis

Hybrids nearly always show higher photosynthetic capacity, which has been observed in *Arabidopsis* hybrids (Fujimoto et al., 2012), super-hybrid rice (Wang et al., 2000; Song et al., 2010), maize hybrids (Tollenaar et al., 2004), and hybrid wheat (Yang et al., 2007). Similarly, hybrid millet showed higher photosynthetic capacity because of the higher chlorophyll

Table 3. Significant GO terms of DEGs in the GO annotation analysis (false-discovery rate-corrected P values, 0.05).

GO Term	Description	P_fdr
F_1/Female		
GO:0009607	Response to biotic stimulus	0.015
GO:0006952	Defense response	0.033
GO:0005488	Binding	0.046
GO:0001071	Nucleic acid binding transcription factor activity	0.046
GO:0003700	Sequence-specific DNA binding transcription factor activity	0.046
Female/Male		
GO:0043531	ADP binding	0
GO:0008219	Cell death	0
GO:0016265	Death	0
GO:0012501	Programmed cell death	0
GO:0006915	Apoptotic process	0
GO:0043168	Anion binding	0
GO:0032553	Ribonucleotide binding	0
GO:0000166	Nucleotide binding	0
GO:1901265	Nucleoside phosphate binding	0
GO:0036094	Small molecule binding	0
GO:1901363	Heterocyclic compound binding	0
GO:0097159	Organic cyclic compound binding	0
GO:0032555	Purine ribonucleotide binding	0
GO:0032550	Purine ribonucleoside binding	0
GO:0001883	Purine nucleoside binding	0
GO:0035639	Purine ribonucleoside triphosphate binding	0
GO:0017076	Purine nucleotide binding	0
GO:0032549	Ribonucleoside binding	0
GO:0001882	Nucleoside binding	0
GO:0032559	Adenyl ribonucleotide binding	0
GO:0005524	ATP binding	0
GO:0030554	Adenyl nucleotide binding	0
GO:0043167	Ion binding	0
GO:0009987	Cellular process	0
GO:0003674	Molecular function	0
GO:0008150	Biological process	0
GO:0005488	Binding	0
GO:0005509	Calcium ion binding	0.002
GO:0009055	Electron carrier activity	0.005
GO:0020037	Heme binding	0.011
GO:0016491	Oxidoreductase activity	0.011
GO:0046906	Tetrapyrrole binding	0.012
GO:0046872	Metal ion binding	0.012
GO:0065008	Regulation of biological quality	0.017
GO:0008152	Metabolic process	0.017
GO:0019725	Cellular homeostasis	0.017
GO:0042592	Homeostatic process	0.018
GO:0003824	Catalytic activity	0.024
GO:0005515	Protein binding	0.024
GO:0044710	Single-organism metabolic process	0.03
F_1/Male		
GO:0008219	Cell death	0
GO:0016265	Death	0
GO:0043531	ADP binding	0
GO:0012501	Programmed cell death	0
GO:0006915	Apoptotic process	0
GO:0032559	Adenyl ribonucleotide binding	0
GO:0005524	ATP binding	0
GO:0030554	Adenyl nucleotide binding	0
GO:0032553	Ribonucleotide binding	0
GO:0008150	Biological process	0
GO:0032555	Purine ribonucleotide binding	0
GO:0032550	Purine ribonucleoside binding	0
GO:0001883	Purine nucleoside binding	0
GO:0035639	Purine ribonucleoside triphosphate binding	0

Continued on next page

Table 3. Continued.

GO Term	Description	P_fdr
GO:0017076	Purine nucleotide binding	0
GO:0032549	Ribonucleoside binding	0
GO:0001882	Nucleoside binding	0
GO:1901363	Heterocyclic compound binding	0
GO:0097159	Organic cyclic compound binding	0
GO:0003674	Molecular function	0
GO:0043168	Anion binding	0
GO:0043167	Ion binding	0
GO:0036094	Small molecule binding	0
GO:0000166	Nucleotide binding	0
GO:1901265	Nucleoside phosphate binding	0
GO:0009055	Electron carrier activity	0
GO:0016705	Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen	0.002
GO:0005488	Binding	0.002
GO:0009987	Cellular process	0.002
GO:0003824	Catalytic activity	0.003
GO:0044424	Intracellular part	0.007
GO:0020037	Heme binding	0.007
GO:0046906	Tetrapyrrole binding	0.007
GO:0004645	Phosphorylase activity	0.031

Table 4. Enriched pathways for DEGs.

Pathway term	Sample No.	Genes	P value
F_1/female			
DDT degradation	1	Si004534	0.019218
Chlorocyclohexane and chlorobenzene degradation	1	Si004534	0.023018
Novobiocin biosynthesis	1	Si017185	0.030577
Cysteine and methionine metabolism	2	Si017185, Si001369	0.036295
Phenylpropanoid biosynthesis	2	Si013626, Si009896	0.042438
Tropane, piperidine and pyridine alkaloid biosynthesis	1	Si017185	0.049226
F_1/male			
Antigen processing and presentation	4	Si021434, Si000619, Si035705, Si001225	4.28E-05
Flavonoid biosynthesis	3	Si035224, Si001126, Si026463	0.000641
Phenylpropanoid biosynthesis	5	Si001126, Si000743, Si016467, Si013626, Si030593	0.001335
Legionellosis	3	Si021434, Si034887, Si000619	0.005765
Circadian rhythm-plant	5	Si026463, Si033984, Si033968, Si014685, Si013398	0.00666
Fatty acid biosynthesis	2	Si006496, Si010296	0.011449
Amino sugar and nucleotide sugar metabolism	4	Si035310, Si021771, Si031983, Si008781	0.012824
Measles	2	Si021434, Si000619	0.022244
MAPK signaling pathway	2	Si021434, Si000619	0.024029
Phenylalanine metabolism	3	Si001126, Si016467, Si030593	0.027878
Toxoplasmosis	2	Si021434, Si000619	0.038044
Nitrogen metabolism	2	Si021521, Si016467	0.038044
Female/male			
Nitrogen metabolism	4	Si021521, Si016467, Si013224, Si016715	0.001408
Flavonoid biosynthesis	3	Si035224, Si026463, Si021789	0.002496
Antigen processing and presentation	3	Si021434, Si000619, Si009497	0.003932
Naphthalene degradation	2	Si036012, Si026503	0.006268
Retinol metabolism	2	Si036012, Si026503	0.009872
Metabolism of xenobiotics by cytochrome P450	2	Si036012, Si026503	0.009872
Phenylpropanoid biosynthesis	5	Si016467,Si000743, Si021789, Si009896, Si030593	0.010047
Chloroalkane and chloroalkene degradation	2	Si036012, Si026503	0.014189
Drug metabolism - cytochrome P450	2	Si036012, Si026503	0.014189
Porphyrin and chlorophyll metabolism	3	Si010922, Si026773, Si001132	0.017846
Legionellosis	3	Si021434, Si034887, Si000619	0.020622
Fatty acid biosynthesis	2	Si006496, Si010296	0.027804

a/b value compared with that of conventional millet (Liu et al., 2012). In this study, 2 genes (Si031233, Si032777) encoding chlorophyll a/b binding proteins were up-regulated, which is significant for photosynthesis. Qin et al. (2013) identified 4 DEGs encoding chlorophyll a/b binding proteins in the developing top ear shoots between a maize heterotic F_1 hybrid (Mo179 x B73) and its parental inbreds using maize microarrays. These results suggest that the differential expression of chlorophyll a/b may play an important role in the higher photosynthetic capacity of the hybrid. In addition, the expression of 2 genes (Si011039, Si026463) encoding chalcone synthase were increased in the hybrid, which is important for protecting photosynthetic organs against ultraviolet light (Chappell and Hahlbrock, 1984). This protection may be another important factor for the increased photosynthetic capacity.

DISCUSSION

Differential gene expression underlies the molecular basis of differential trait heterosis. However, nearly all traits exhibiting heterosis are quantitatively inherited and controlled by numerous genes. High-throughput sequencing enables identification of a large number of genes, which will increase the understanding of the heterosis mechanism. Genome-wide changes in gene expression have been documented in intraspecific hybrids of *Arabidopsis thaliana* (Andorf et al., 2010; Fujimoto et al., 2012; Shen et al., 2012), rice (Wei et al., 2009; He et al., 2010, Song et al., 2010), maize (Swanson-Wagner et al., 2006; Guo et al., 2006; Stupar and Spinger, 2006), and wheat (Wang et al., 2006), and in allopolyploids of *Arabidopsis* (Wang et al., 2006; Shi et al., 2012) and wheat (Qi et al., 2012). Nevertheless, heterosis-related DEGs and/or their regulatory mechanisms vary significantly between species (Qin et al., 2013). In this study, we identified 740 DEGs between hybrid millet and its parent lines. Most of these genes were up-regulated in the hybrid. Furthermore, nearly all of the genes have been functionally annotated. Therefore, this study provides a repertoire of genes useful for studying the underlying molecular basis of heterosis.

Recent studies have indicated that over 30% of the *A. thaliana* transcriptome is driven by the circadian clock (Harmer et al., 2000; Covington et al., 2008), thus potentially regulating numerous metabolic pathways (Covington et al., 2008), such as starch metabolism, chlorophyll synthesis, and jasmonate and salicylates accumulation (Danielle et al., 2013), among others. CCA1 and LHY, as key elements of the circadian clock, were down-regulated and induced heterosis in hybrids and allopolyploids (Ni et al., 2009). Our results agreed with those of the previous study. In this study, CCA1 and LHY were down-regulated in hybrid millet. In addition, PHYA and phytochrome-interacting basic helix loop helix transcription factor 3 were down-regulated. PHYA is thought to be the only active photoreceptor that can mediate far-red light input to the circadian rhythm. Down-regulation of PHYA in the hybrid millet may induce down-regulation of CCA1and LHY, which then induce a series of downstream events. The greatest changes induced by the circadian rhythm in this study were the increased metabolism of starch and the chlorophyll because the DEGs (Si031233, Si032777) encoding chlorophyll a/b binding proteins and the DEGs related to carbohydrate metabolism were up-regulated. The increased chlorophyll level enhanced the photosynthetic capacity and altered carbohydrate accumulation, resulting in the heterosis of biomass and yield of the hybrid millet (Figure 2). Although the hybrid vigor or heterosis is undoubtedly complex, our findings increase the understanding of heterosis.

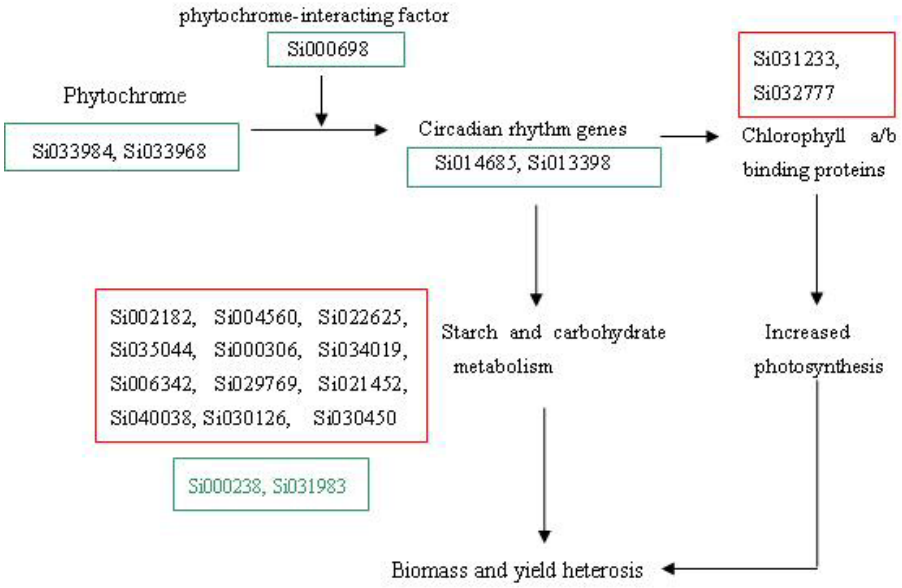

Figure 2. Schematic representation of DEGs involved in the circadian rhythm pathway and the biological process regulated by the circadian clock. The diagram was adapted from 2 papers (Ni et al., 2009; Chen, 2013). The DEGs are indicated in colored rectangular boxes, with red for DEGup and green for DEGdown.

Based on our results, circadian clock genes were clearly related to heterosis. Because light is a key factor in setting the circadian clock, the difference in photoreceptors may induce differential expression of circadian clock genes. At least 3 families of photoreceptors have been identified as transducing light signals to reset the clock, including the blue light-sensing cryptochromes, the red/far-red light sensing phytochromes (PHYA, PHYB, PHYD, PHYE) (Yanovsky et al., 2000), and a family of three F-box proteins, including ZEITLUPE (Baudry et al., 2010). In this study, the down-regulation of circadian clock genes in hybrid millet were induced by red/far-red light sensing the phytochromes (PHYA, PHYB). The characteristics of hybrids may be inherited from the female of a photoperiod-sensitive male sterile line. The difference in expression of genes encoding PHYA and PHYB (Si033984, Si033968) between the hybrid and female was not significant. However, no DEG encoding any phytochromes was found in a study by Song et al. (2010), although circadian clock genes were also down-regulated in hybrid rice. This indicates that the causes of differential expression of circadian clock genes are complex. Additionally, the basis of heterosis differs between species.

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Supplementary material

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