Molecular Plant • Volume 3 • Number 6 • Pages 1012–1025 • November 2010 RESEARCH ARTICLE

Comparative Transcriptional Profiling and Preliminary Study on Heterosis Mechanism of Super-Hybrid Rice

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ABSTRACT Heterosis is a biological phenomenon whereby the offspring from two parents show improved and superior performance than either inbred parental lines. Hybrid rice is one of the most successful apotheoses in crops utilizing heterosis. Transcriptional profiling of F_1 super-hybrid rice *Liangyou-2186* and its parents by serial analysis of gene expression (SAGE) revealed 1183 differentially expressed genes (DGs), among which DGs were found significantly enriched in pathways such as photosynthesis and carbon-fixation, and most of the key genes involved in the carbon-fixation pathway exhibited up-regulated expression in F_1 hybrid rice. Moreover, increased catabolic activity of corresponding enzymes and photosynthetic efficiency were also detected, which combined to indicate that carbon fixation is enhanced in F_1 hybrid, and might probably be associated with the yield vigor and heterosis in super-hybrid rice. By correlating DGs with yield-related quantitative trait loci (QTL), a potential relationship between differential gene expression and phenotypic changes was also found. In addition, a regulatory network involving circadian-rhythms and light signaling pathways was also found, as previously reported in *Arabidopsis*, which suggest that such a network might also be related with heterosis in hybrid rice. Altogether, the present study provides another view for understanding the molecular mechanism underlying heterosis in rice.

Key words: Heterosis; super-hybrid rice; transcriptional profiling; photosynthesis; carbon fixation; regulatory network.

INTRODUCTION

Heterosis, or hybrid vigor, refers to the phenomenon that the hybrid of two inbred lines shows superior performance than either parents and is a widely documented phenomenon in diploid organisms that undergo sexual reproduction. The tremendous impact of heterosis in rice breeding in China has resulted in a significant increase in productivity in the last three decades (Cheng et al., 2007). Now, the acreage of hybrid rice takes more than half of the total rice area in China and superior hybrid rice has been showing a increasing contribution to the grain yield (Normile, 2008). Given its importance

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doi: 10.1093/mp/ssq046, Advance Access publication 20 August 2010
Received 28 May 2010; accepted 22 July 2010

in breeding programs to world food security, considerable interest has been focused on how heterosis contributes to increased yield trait in hybrid crops.

Two hypotheses, the dominance (Bruce, 1910) and over-dominance hypotheses (East, 1936), have been proposed to explain the phenomenon of heterosis. However, they were coined before the molecular concepts of genetics were well understood and were not connected with molecular genetic principles (Birchler et al., 2003). Since the late 1980s, investigations of heterosis using molecular markers and quantitative trait loci (QTL) have yielded evidence for both the dominance (Xiao et al., 1995) and the over-dominance (Stuber et al., 1992) hypotheses. Since most phenotypic variations are regulated by many loci and changes in each responsible gene may influence traits by interacting with other genes, epistasis was also considered one of the genetic bases of heterosis (Yu et al., 1997). Heretofore, the consensus on the mechanism of heterosis was that no one hypothesis holds true for every phenomenon or every organism (Hochholdinger and Hoecker, 2007).

Recently, with the development of functional genomics, comparative gene expression profiling between hybrid triads has been studied in *Arabidopsis* (Wang et al., 2006), maize (Swanson-Wagner et al., 2006), rice (Song et al., 2007; Wang et al., 2010; Wei et al., 2009), and *Medicago sativa* (Li et al., 2009) by high-throughput gene expression profiling. These studies indicated that in hybrids, the expression of many genes does not exhibit the expected mid-parent value, and some potential association between differential gene expression and heterosis was suggested; for example, differential expression in genes involved in CO_2 assimilation (Bao et al., 2005; Wang et al., 2002) and energy metabolism (Wei et al., 2009) could be related to improved production in hybrid rice.

There were also other reports studying heterosis from different aspects. It was reported that allelic variation in gene expression may have an impact on hybrid vigor in maize (Guo et al., 2004; Springer and Stupar, 2007). In rice, the combined interplay between expression of transcription factors and polymorphic promoter *cis*-regulatory elements in hybrids was indicated as a plausible molecular mechanism underlying heterotic gene expression and heterosis (Zhang et al., 2008). Gene expression profiling in *Arabidopsis* had suggested that genes involved in the circadian rhythm, such as *LHY* and *CCA1*, both MYB-like transcription factors, are associated with heterosis (Ni et al., 2009). Epigenetic modification and small-RNA-directed gene regulation were also shown to be related to heterosis (Ha et al., 2009; He et al., 2010).

To date, despite the development of next-generation high-throughput sequencing technology, such as 454 and Solexa (von Bubnoff, 2008), serial analysis of gene expression (SAGE) and microarray are still effective tools in transcriptional profiling (Aya et al., 2009; Kim et al., 2009). Previously, we investigated the transcriptome profiles of super-hybrid rice *LYP9* and its parents by whole-genome oligonucleotide microarray (Wei et al., 2009). Here, we report our further research into transcriptional and physiological metabolism changes in another super-hybrid rice combination, *Liangyou-2186* (*SE21s* × *Minghui86*). Furthermore, we also found that differentially expressed genes (DGs) between hybrid and parents can be involved in certain regulatory networks, which suggested that complicated gene networks might be underlying heterosis. Results of the present study might help promote further understanding of mechanisms underlying heterosis.

RESULTS

SAGE Library Construction and Differential Expression Analysis

Leaves from the super-hybrid rice *LY2186* and its parental lines at grain-filling stage were used as raw materials for the construction of SAGE library. Three SAGE libraries of 207 266 tags were obtained, comprising 69 102, 69 110, and 69 064 tags from the male-sterile line *SE21s*, the restorer line *Minghui86* (*MH86*), and the F_1 hybrid *Liangyou-2186* (*LY2186*), respectively, which corresponded to 20 434, 19 122, and 21 751 unique tags in *SE21s*, *MH86*, and *LY2186*; they combined to form the total 41 776 unique tags (Figure 1A and Table 1). According to the SAGE principle, the 41 776 unique tags were used for alignment with the 10-base virtual tags extracted from the 3′-downstream sequences following the last *Nla*III site (CATG) of full-length cDNAs (FL-cDNA) in the Knowledge-based Oryza Molecular biological Encyclopedia (KOME) (Kikuchi et al., 2003). Altogether, 10 907 tags perfectly matched the FL-cDNA sequence and were defined as being annotated (Supplemental Table 1).

Based on the significance identification of a total of 41 776 unique SAGE tags, 1872 significant differentially expressed tags ($P < 0.05$) were found between *LY2186* and *MH86*, and 1788 between *LY2186* and *SE21s*; they were combined to yield 2617 differentially expressed tags (Figure 1B). Comparison of gene expression levels between the F_1 hybrid and its parents allowed classifying the differentially expressed tags into five expression patterns: above high-parent (AHP), high-parent level (HPL), mid-parent level (MPL), low-parent level (LPL), and below low-parent (BLP). What is interesting is that the dominant expression pattern (HPL and LPL) accounted for the majority (61%) of the total differentially expressed tags (Figure 1C).

FL-cDNA-matched differentially expressed tags were used to map genes from Rice Genome Annotation release 6.1 (Ouyang et al., 2007) and a total of 1294 (49.45%) tags were located. Among these tags, 1142 (88.25%) tags were assigned to single gene (1 versus 1), and 82 (6.34%) tags were those multiple tags that can be assigned to a single gene (*n* versus 1); altogether, 1183 differentially expressed genes (DGs) were acquired for further analysis. It should be noted that we also found that 70 tags (5.41%) showed ambiguous assignment to multiple genes (1 versus *n*), which were filtered out in the present study (Supplemental Table 2).

Gene Ontology (GO) slims from the Rice Genome Annotation release 6.1 were used for functional classification of

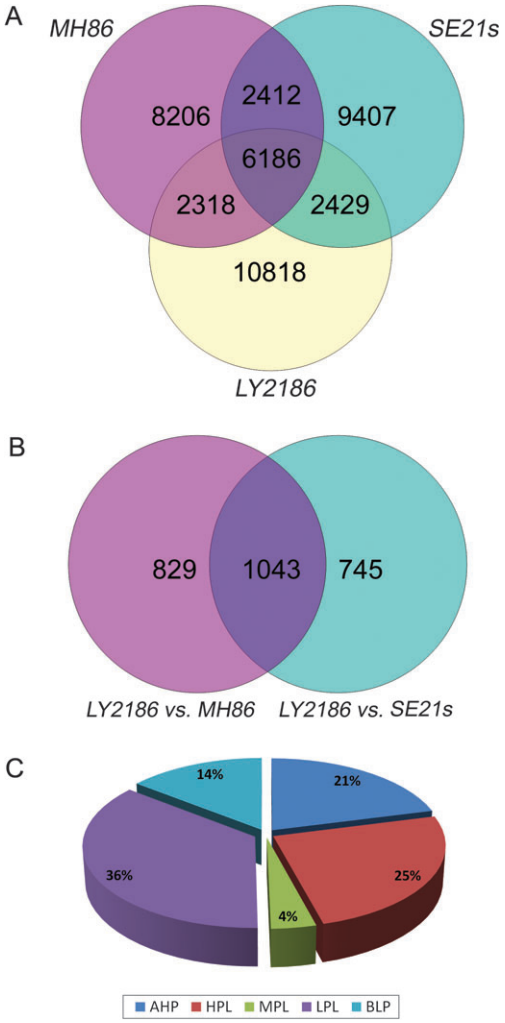

Figure 1. Overview of Serial Analysis of Gene Expression (SAGE) Libraries and Differentially Expressed Tags.

(A) Venn diagram of tags shared among three SAGE libraries.

(B) Venn diagram of tags with significant differential expression ($P < 0.05$) between *LY2186* and its parental lines.

(C) Expression patterns of tags with significant differential expression ($P < 0.05$) between F_1 hybrid and parents.

AHP, above high-parent; HPL, high-parent level; MPL, mid-parent level; LPL, low-parent level; BLP, below low-parent.

the 1183 DGs by the Web Gene Ontology Annotation Plot (WEGO) (Ye et al., 2006) and the results were plotted in Figure 2. Using all genes in the rice genome as background for significance testing, we found that DGs were significantly enriched in six cellular component categories, five molecular function categories, and eight biological process categories (denoted by stars). We further classified DGs at detailed levels based on Gene Ontology (GO) by the Micro Array Data Interface for Biological Annotation (MADIBA) web tool (Law et al., 2008) (Supplemental Tables 3–6). Interestingly, among the 26 significant DG-involved GO terms of cellular component (FDR corrected *P*-values < 0.05), over 50% were photosynthesis-related, such as photosystem I, chloroplast stroma, chloroplast, thylakoid membrane, and PSII-associated light-harvesting complex II, etc. (Table 2).

The Expression Patterns of Photosynthesis-Related Genes in Hybrid Rice *LY2186*

To investigate the metabolic pathways in which DGs were involved and enriched, metabolic pathway analysis was performed using the MADIBA web tool (Law et al., 2008), results of which showed that 207 out of 1183 DGs were involved in 12 functional categories (Supplemental Table 7). Among them, the majority were present in carbohydrate metabolism (72 DGs) and energy metabolism (64 DGs). Further analysis demonstrated that these 207 DGs were distributed in 91 out of a total of 141 metabolic pathways. Fisher's exact testing revealed only two metabolic pathways showing extreme significance ($P < 0.01$): the carbon fixation (P = 5.01E-10) and photosynthesis pathways (P = 6.53E-03) (Table 3 and Supplemental Figures 1 and 2). It should be noted that the starch and sucrose metabolism pathway was the second largest DG-involved pathway, following the carbon fixation pathway, although it does not reach the significant level (Supplemental Figure 3 and Supplemental Table 8).

In the two significant pathways, 27 DGs (encoding 18 unique enzymes) were detected in the carbon-fixation pathway (dark reactions) and 10 DGs (encoding three unique enzymes) in the photosynthesis pathway (light reactions). Interestingly, 20 of the 27 DGs in the carbon-fixation pathway were up-regulated (with expression pattern of AHP or HPL) in F_1 hybrid *LY2186*. As compared with the mid-value of parental lines, in the F_1 hybrid, the transcriptional levels of two DGs (Os01g11054 and Os02g14770) encoding phosphoenolpyruvate carboxylase (PEPC) were up-regulated by 20- and 4.5-fold, respectively. The gene encoding pyruvate phosphate dikinase (PPDK) and NADP-malate dehydrogenase (NADP-MDH) showed 1.5-fold and 3.5-fold higher expression in F_1 hybrid than the mean of the parental lines, respectively. Besides, DGs involved in the Calvin cycle were also found up-regulated expression in hybrid, including genes encoding fructose-bisphosphate aldolase (FBA), sedoheptulose-1,7-bisphosphatase (SBP), fructose-1,6-bisphosphatase (FBP), triosephosphate isomerase (TPI), phosphoribulokinase (PRK), and ribulose bisphosphate carboxylase (Rubisco), etc. (Table 4 and Supplemental Table 8).

Confirmation of Differential Gene Expression

To validate the DG expression involved in carbon fixation and examine whether this result can be extended to other hybrid rice, the expression levels of 14 DGs in the carbon-fixation pathway (Supplemental Table 9) were examined by real-time quantitative PCR (qPCR) in three hybrid rice combinations: *Liangyou-2186* (*MH86* × *SE21s*), *Liangyou-pei9* (*PA64S* × *93–11*), and *Shanyou-63* (*ZS97A* × *MH63*). Twelve out of the 14 genes except *NADP-malic enzyme* (*NADP-ME*) and *Rubisco* were confirmed in *LY2186* hybrid triads, and all 14 genes were up-regulated in *SY63* hybrid triads (Figure 3A and 3C); 11 DGs,

Table 1. Number of Unique Tags in SAGE Libraries of *LY2186* Super-Hybrid Rice Combination.

	SE21s		*LY2186*		*MH86*		*Combination*	
Copy number[a]	Number[c]	%[d]	Number	%	Number	%	Number	%
>100	52	0.25	49	0.23	57	0.30	207	0.50
Matched[b]	46	0.66	42	0.57	51	0.76	164	1.50
11–100	771	3.77	784	3.60	835	4.37	2539	6.08
Matched	562	8.01	563	7.64	578	8.64	1662	15.24
2–10	6242	30.55	6587	30.28	5942	31.07	13 371	32.01
Matched	3301	47.04	3551	48.18	3054	45.65	5551	50.89
Once	13 369	65.43	14 331	65.89	12 288	64.26	25 659	61.42
Matched	3108	44.29	3214	43.61	3007	44.95	3530	32.36
Total	20 434	100.00	21 751	100.00	19 122	100.00	41 776	100.00
Matched	7017	100.00	7370	100.00	6690	100.00	10 907	100.00

a Category based on the copy number of tags.
b Matched refers to the number of unique tags that match full-length cDNAs (Kikuchi et al., 2003).
c Number refers to the number of unique tags in each category.
d % refers to the proportion of unique tags in each category in total unique tags.

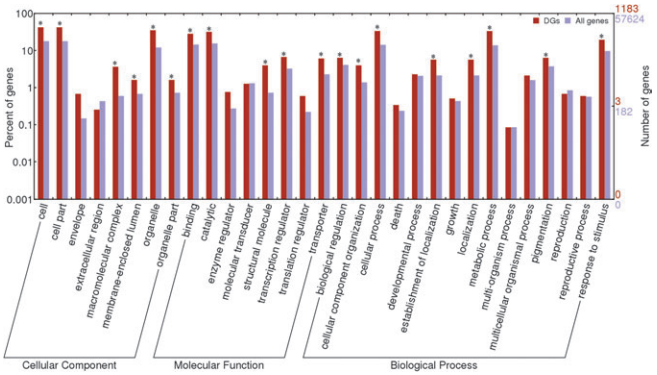

Figure 2. Comparison of Gene Ontology (GO) Classification between Differentially Expressed Genes (DGs) and All Genes from the Rice Genome.

The gene ontology slims are from the Rice Genome Annotation (Ouyang et al., 2007). GO terms at level 2 are plotted. Star (*) indicates remarkable relationship, if the *P*-value is below the significant level of 0.05.

with three exceptions (*NAD-MDH*, *TPI*, and *GAPDH*), were confirmed up-regulated expressions in *LYP9* hybrid triads (Figure 3B). Altogether, more than 80% of DGs in the carbon-fixation pathway were verified, which demonstrated the satisfactory quality of the SAGE library and the data analysis.

Activities Measurement of Enzymes Involved in Carbon Fixation in Hybrid Rice

Although some DGs involved in the carbon-fixation pathway exhibited higher transcription levels in the F_1 hybrid than in its parents, the corresponding enzyme activities might not always display a similar trend. To examine whether corresponding photosynthesis enzymes also exhibited higher catalytic activities, activities of PEPC, NADP-MDH, NADP-ME, PPDK, Rubisco, PGK, TPI, FBA, FBP, and RPI in the carbon-fixation pathway were examined in *LY2186* hybrid triads. The enzymes including PEPC, NADP-MDH, NADP-ME, PPDK, PGK, and TPI showed significantly increased activity in the F_1 hybrid (by 39.79–80.03%, $P < 0.01$; for PEPC, $P < 0.05$) (Table 5). These results demonstrated that enzymatic activities were consistent with the corresponding gene expression pattern in the SAGE library.

To visualize enzyme activity in the carbon-fixation pathway, principal component analysis (PCA) (Jolliffe, 2002) was performed with a BIPLOT analysis (Figure 4), by plotting the objects (rice lines) and variables (enzymes) in the same plot to enable direct interpretation of the observed effects. The result showed that the similarity between parents was higher than that between the F_1 hybrid and either parent during the grain-filling stage. The direction in the plot from the parents towards the F_1 hybrid line directly reflects an increase in activity of four enzymes—PEPC, NADP-MDH, NADP-ME, and PPDK—which confirms transcript-level observations and might strengthen our deduction that these enzymes play an important differentiating role in the F_1 hybrid.

Examination of Photosynthetic Rate in Hybrid Rice

Furthermore, to check whether the up-regulated gene expression and increased enzymatic activity can lead to physiological changes in hybrid rice or not, we examined photosynthetic characteristics of the flag leaf at the grain-filling stage in three hybrid rice combinations: *LY2186* (*MH86* × *SE21s*), *LYP9* (*PA64s* × *93–11*), and *SY63* (*ZS97A* × *MH63*). As compared with the average photosynthetic level of the parental lines, all three F_1 hybrids showed higher net photosynthetic rates (P_n) in flag leaves, by 10.4, 11.3, and 11.7%, respectively (Figure 5A, 5D, and 5G). More detailed measurements of Apparent Quantum Yields (AQY)—the slope of linear low light part (<200 μmol m^{-2} s^{-1}) of the light response curves—showed a significant improvement in apparent quantum yield (by 30.9, 32.1, and

Table 2. Significant GO Terms of DGs in the GO Annotation Analysis of Cellular Component (FDR-Corrected *P*-Values < 0.05).

GO term	Definition	FDR corrected *P*-value[a]
GO:0009522	Photosystem I	0
GO:0009923	Fatty acid elongase complex	0
GO:0030076	Light-harvesting complex	8.35E-07
GO:0009570	Chloroplast stroma	3.36E-06
GO:0030093	Chloroplast photosystem I	1.17E-04
GO:0009507	Chloroplast	1.89E-04
GO:0009782	Photosystem I antenna complex	5.00E-04
GO:0042651	Thylakoid membrane	1.03E-03
GO:0005840	Ribosome	1.07E-03
GO:0009579	Thylakoid	1.26E-03
GO:0009783	Photosystem II antenna complex	3.70E-03
GO:0009538	Photosystem I reaction center	3.78E-03
GO:0009517	PSII associated light-harvesting complex II	5.42E-03
GO:0009543	Chloroplast thylakoid lumen	7.73E-03
GO:0005843	Cytosolic small ribosomal subunit (sensu Eukaryota)	1.17E-02
GO:0009328	Phenylalanine-tRNA ligase complex	1.83E-02
GO:0005829	Cytosol	1.90E-02
GO:0005739	Mitochondrion	3.87E-02
GO:0005677	Chromatin silencing complex	3.87E-02
GO:0005749	Mitochondrial respiratory chain complex II	3.87E-02
GO:0005830	Cytosolic ribosome (sensu Eukaryota)	3.87E-02
GO:0005838	Proteasome regulatory particle (sensu Eukaryota)	3.87E-02
GO:0009533	Chloroplast stromal thylakoid	4.25E-02
GO:0015934	Large ribosomal subunit	4.36E-02
GO:0015935	Small ribosomal subunit	4.84E-02
GO:0009941	Chloroplast envelope	4.91E-02

a *P*-values calculated using a hypergeometric test, which determines whether the number of times that a GO term appears in the cluster is significant, relative to its occurrence in the genome (Benjamini and Hochberg, 1995).

20.7%) in the three F_1 hybrids than in the parental lines (Figure 5B, 5E, and 5H). We can also see that all three F_1 hybrid rice lines showed a lower CO_2 compensation point than that of their parents (*LY2186* and *LYP9*, Figure 5C and 5F) or the mid-parent value (*SY63*, Figure 5I).

Mapping DGs to Yield-Related QTL of Small Intervals

QTL are intervals across a chromosome identifying a particular region of the genome as containing one or more genes associated with the trait being measured. To survey the association between gene expression variation and phenotypic changes in hybrid triads, we mapped DGs to rice QTL collected by Gramene (www.gramene.org). We were able to map 1158 of 1183 (97.9%) DGs to 3017 QTL, which could be classified into nine categories, including Yield, Vigor, Quality, etc. Hybrid rice is superior to its parents mainly in yield, so we further investigated the QTL of the yield category and found 1101 DGs (93.1%) could be mapped to 785 yield-related QTL, many of which are well characterized, such as *seed weight*, *seed number*, and *filled grain number*, etc. (Supplemental Table 10). More interestingly, we found that DGs could be mapped to QTL of small intervals (spanning no more than 100 genes) and 110 DGs (9.3%) were found located in 173 yield-related QTL of small intervals (Figure 6).

We further examined the relationship of DGs involved in photosynthesis, carbon fixation, and starch and sucrose metabolism with yield-related QTL and found that quite a few DGs could be mapped to yield-related QTL, including all 10 DGs involved in photosynthesis, 25 out of the 27 DGs involved in carbon fixation, and 13 out of the 15 DGs involved in starch and sucrose metabolism. In yield-related QTL of small intervals, we found six DGs, including three involved in photosynthesis (Os01g46980, *vacuolar ATP synthase subunit E*; Os02g51470, *ATP synthase delta chain*; and Os07g05400, *ferredoxin-NADP reductase*), two in carbon fixation (Os01g67860, *fructose-bisphosphate aldolase cytoplasmic isozyme*; and Os10g42100, *pyruvate kinase isozyme G*), and one in starch and sucrose metabolism (Os04g53310, *soluble starch synthase 3*); all these DGs seemed have good relationships with the QTL they were located in.

Table 3. Top Ten Differentially Expressed Genes (DGs) Enriched in Metabolic Pathways.

Metabolic pathway[a]	No. of enzymes[b]	No. of genes[c]	*P*-value[d]
Carbon fixation*	18	31	5.01E-10
Photosynthesis*	3	10	6.53E-03
Reductive carboxylate cycle (CO_2 fixation)	5	8	7.75E-02
Valine, leucine, and isoleucine biosynthesis	6	7	1.02E-01
Oxidative phosphorylation	5	8	1.32E-01
Citrate cycle (TCA cycle)	6	8	2.49E-01
Inositol metabolism	2	5	3.15E-01
Biosynthesis of ansamycins	1	1	3.41E-01
Selenoamino acid metabolism	5	5	3.97E-01
Gamma-Hexachlorocyclohexane degradation	5	6	4.37E-01

a Pathway analysis based on MADIBA (Law et al., 2008).
b Number of enzymes encoded by DGs in the pathway.
c Number of DGs clustered in the pathway.
d *P*-value by Fisher's exact test; the top 10 pathways with *P*-value are listed; * pathway with *P*-value < 0.05 is considered as significant.

Construction of Regulatory Network in DGs

Genes often interact with other genes to accomplish the whole functions in a cell and these complex gene interactions could contribute to many biological characteristics (Barabasi and Oltvai, 2004). To examine whether some interconnection exists in DGs, we investigated the relationship among DGs using Pathway Studio software (Nikitin et al., 2003) and found a gene regulatory network (Figure 7). This network consisted of circadian rhythm-related genes such as *LATE ELONGATED HYPOCOTYL* (*LHY*) and *GIGANTEA* (*GI*); phytochrome-mediated light signaling-related genes such as *phytochrome interacting factor 3* (*PIF3*), which is involved in the phoytochrome-mediated light signaling pathway, receiving the light signal from photo-activated phytochrome molecules at the first step (Castillon et al., 2007); and stress-tolerance-related genes such as *salt tolerance* (*STO*) and *radical-induced cell death 1* (*RCD1*) (Supplemental Table 11). We also found downstream targets of these regulators, such as photosynthesis-related genes *ribulose-bisphosphate carboxylase small chain 1A* (*RBCS1A*) and *chlorophyll a/b binding protein 1* (*CAB1*), regulated by PIF3; and starch synthesis-related gene, *granule-bound starch Synthase I* (*GBSS1*), regulated by LHY. Of note, PIF3 and LHY played a central role in the regulations, facilitating integration in the network.

DISCUSSION

Despite its critical importance to agriculture, a mechanistic understanding of heterosis has not been achieved. Differential gene expression between the hybrid and its parental cultivars has been hypothesized to contribute to heterosis (Swanson-Wagner et al., 2006; Wang et al., 2006; Song et al., 2007; Li et al., 2009; Wei et al., 2009). In the present study, we attempted to survey the relationship between the transcriptional profiles of gene expression and heterosis in super-hybrid rice *LY2186* combination. Comparison of transcriptional profiles of *LY2186* showed that of 41 776 detected tags, only 1183 DGs were detected (2.8%), which implied that only a small number of genes were responsible for the changes in phenotypic performance in the F_1 hybrid.

Increased Photosynthesis Efficiency in Hybrid Rice

Metabolic pathway analysis demonstrated that DGs were involved in 91 pathways, but were significantly enriched in the carbon-fixation and photosynthesis pathways (*P* = 5.01E-10 and 6.53E-03, respectively). Photosynthesis, including two phases—light reactions (photosynthesis pathway) and dark reactions (carbon fixation pathway)—is a key process converting CO_2 into organic compounds using solar energy (Rascher and Nedbal, 2006). We found 10 DGs involved in the photosynthesis pathway, including six genes encoding F-type ATPase, three encoding ferredoxin-NADP reductase, and one cytochrome b6-f complex-encoding gene, which have important roles in photosynthesis and are responsible for photosynthetic electron transfer in thylakoids (Merchant and Sawaya, 2005). Furthermore, GO annotation analysis of cellular component indicated the DGs significantly enriched in photosynthesis-related organelles. Significant changes in expression patterns of these key genes implied alterations in photosynthetic efficiency.

In the carbon-fixation pathway, our results showed that 20 out of 27 DGs involved in this pathway exhibited higher expression in the F_1 hybrid than in its parent (Table 4), which was further validated by qPCR (Figure 3A). These results suggested higher carbon-fixation efficiency in the F_1 hybrid than in the parents, which was further supported by significantly increased activity of enzymes in the carbon-fixation pathway (39.79–80.03%) (Table 5). The PCA of enzymatic activities of key enzymes involved in the carbon-fixation pathway provided more evidence that the F_1 hybrid had more enhanced carbon fixation efficiency characteristics than its parents (Figure 4). Photosynthetic efficiency testing of flag leaves at the grain-filling stage revealed increases in the net photosynthetic rate (P_n) of 24.1 and 10.6% in the F_1 hybrid (*LY2186*) compared to the maternal (*SE21s*) and paternal lines (*MH86*), respectively (Figure 5A). In addition, two other hybrid rice combinations, *LYP9* and *SY63*, also exhibit similar trends based on qPCR (Figure 3B and 3C) and photosynthetic rate testing (Figure 5D and 5G). Of photosynthetic characteristics, the F_1 hybrids had higher CO_2 assimilation, apparent quantum yield, and lower CO_2 compensation points than the parents in all of three hybrid combinations (Figure 5). Increased photosynthetic efficiency has been mentioned in F_1 hybrids of rice (Bao et al., 2005; Zhang et al., 2007a) and wheat (Yang et al., 2007), and our results explained that

Table 4. Transcription Levels of DGs Involved in Carbon Fixation Pathways.

Gene definition	Locus ID[a]	Tag	Copy number			Ratio[b]	Pattern[c]
			SE21s	*LY2186*	*MH86*		
PEPC	Os01g11054	GCCTTGCCGG	4	9	0	4.5	AHP
	Os02g14770	ATGAGATGGT	1	10	0	20.0	AHP
NADP-MDH	Os08g44810	CTTCCAGGAG	2	14	6	3.5	AHP
NADP-ME	Os01g09320	TGTACCACCA	42	44	35	1.1	AHP
PPDK	Os05g33570	GTAATGTACC	19	48	44	1.5	AHP
PEPCK	Os03g15050	CGTGTCTGTT	9	11	1	2.2	HPL
PK	Os10g42100	GTTCCAATTG	5	7	2	2.0	HPL
NAD-MDH	Os03g56280	TAAAATCACT	19	24	39	0.8	MPL
	Os08g33720	AGGGCGATAA	6	3	8	0.4	LPL
	Os10g33800	CCTCAACTAA	2	7	0	7.0	AHP
Rubisco	Os12g17600	TTCGGGTGCA	6	0	13	0.0	BLP
	Os12g19381	TTCGGCTGCA	231	177	142	0.9	MPL
	Os12g19470[d]	CTCTACAACC	1	7	0	14.0	AHP
	Os12g19470[d]	TAATATGATG	328	263	407	0.7	BLP
PGK	Os05g41640	TACCATTCTA	25	74	34	2.5	AHP
GAPDH	Os03g03720	ACTGTCGAAG	111	211	104	2.0	AHP
	Os04g38600[d]	TGTAATACCG	68	69	100	0.8	MPL
	Os04g38600[d]	TTGCTTGGGA	349	214	348	0.6	BLP
TPI	Os01g05490	TGAGTTTCAG	43	54	45	1.2	AHP
	Os09g36450	CTGCTGTTCG	9	15	6	2.0	AHP
FBA	Os01g67860	CTATCTTTTT	3	5	13	0.6	LPL
	Os06g14740	ACTTCAGGAC	5	9	3	2.3	AHP
	Os11g07020[d]	AATCTTTTCT	270	581	469	1.6	AHP
	Os11g07020[d]	CTTGTGATTC	339	383	581	0.8	MPL
FBP	Os03g16050	CCTACGGAGA	20	20	13	1.2	HPL
SBP	Os04g16680[d]	TCAAGGACAC	21	36	26	1.5	AHP
	Os04g16680[d]	TTGTGCTTCC	5	9	2	2.6	HPL
TKL	Os06g04270	AAGGTCTTGT	23	18	9	1.1	MPL
RPE	Os03g07300	TGATACTACC	98	104	100	1.1	AHP
RPI	Os07g08030	GCGACTTCGG	26	25	11	1.4	HPL
PRK	Os02g47020	AGGGTGCTCC	81	98	1	2.4	AHP

a IDs of DGs based on the Rice Genome Annotation (Ouyang et al., 2007).
b Ratio = *LY2186*/[(*SE21s*+*MH86*)/2].
c Expression pattern: AHP, above high-parent; HPL, high-parent level; MPL, mid-parent level; LPL, low-parent level; BLP, below low-parent.
d One locus has more than one tag matched.

increased photosynthetic capability in the F_1 hybrid caused by enhanced efficiency in the carbon fixation and the photosynthesis pathway due to the up-regulated genes in the carbon-fixation pathway at both transcriptional level and translational level, which probably play some roles in the formation of hybrid vigor.

It should be noted that genes annotated as PEPC, NADP-MDH, NADP-ME, PPDK, etc., known involved in C_4 carbon fixation pathways, were found significantly up-regulated in F_1 hybrid than its parental lines. However, rice is known as a C_3 plant; even with this result, we are still not sure whether C_4 circulation occurred in hybrid rice. Whatsoever, in the tested hybrid rice, the annotated C_4 genes' overall higher expression is a very interesting phenomenon, which may be another way, though not definite at all for now, to help in understanding the mystery of heterosis in rice.

The Roles of Carbohydrate Metabolism and Other Pathways in Heterosis

Besides photosynthesis, other metabolic pathways, such as sucrose and starch pathways, oxidative phosphorylation, citrate cycle (TCA cycle), and stress-resistant pathway, etc., may also contribute to heterosis (Yao et al., 2005). It should be noted that 72 DGs were enriched in carbohydrate metabolism

(Supplemental Table 7). In the sucrose and starch pathway, although the *P*-value was not significant (9.12E-01), it was one of the pathways containing the most DGs (15 DGs) (Supplemental Table 7). Interestingly, we found that the transcriptional expression level of *Granule-bound starch synthase I* (*GBSSI*, *Os07g22930*) was up-regulated, which exhibits circadian oscillation and is controlled by the transcription factors CCA1 and LHY in *Arabidopsis* (Tenorio et al., 2003). In addition, DGs were also found in the inositol phosphate metabolism pathway, which is considered to play an important role in plant growth, development, and cellular signal transduction (Zhang et al., 2007b). The observation of performance trait also showed that hybrid rice surpassed its parents both in biomass and in harvest index (Table 6). Moreover, *LY2186* and *LYP9* are both super-hybrid rice cultivars, and exhibited increased harvest indices compared with that of *SY63*, which is a traditional hybrid rice cultivar, suggesting yield vigor of super-hybrid rice might be related to not only high photosynthesis efficiency, but also other aspects such as distribution efficiency of photosynthetic products.

DGs Are Associated with Yield-Related QTL

QTL provide links between genotype and phenotype for complex traits, and QTL analysis had been widely used in heterosis study (Garcia et al., 2008; Lippman and Zamir, 2007; Meyer et al., 2010). DGs between F_1 hybrid and parents are derived from the heterozygosis of the combined hybrid genomes and may be associated with phenotypic changes in the F_1 hybrid. In the present study, we tried to investigate links between DGs, QTL, and heterosis.

Mapping DGs to known QTL revealed about 1158 DGs (97.9%) located in QTL and 1101 (93.1%) in yield-related QTL. Similar results were also obtained in transcriptomic analysis of *LYP9* hybrid triads by microarray technology (Wei et al., 2009). More interestingly, in the two significant DG-enriched pathways, carbon fixation and photosynthesis pathways, three DGs encoding vacuolar ATP synthase subunit E, ATP synthase delta chain, and ferredoxin-NADP reductase were involved in the photosynthesis pathway, and two DGs encoding pyruvate kinase (PK) and FBA in the carbon-fixation pathway were located at yield-related QTL.

The potential association among DGs, QTL, and heterosis was also suggested within many QTL regions: examples are *soluble starch synthase 3* (Os04g53310) to AQCY010 for *filled grain number*, *photosystem I reaction center subunit psaK* (Os07g05480) to AQF079 for *grain yield*, and *fructose-bisphosphate aldolase cytoplasmic isozyme* (Os01g67860) to AQFF020 for *harvest index*. *LATE ENLONGATED HYPOCOTYL* (*LHY*, Os08g06110) (Murakami et al., 2007) can be mapped to yield-related QTL, and a recent report indicated its counterpart was closely associated with heterosis in *Arabidopsis* (Ni et al., 2009). Interestingly, Os08g06110 can be located to yield-related QTL of small intervals, involved in biomass yield, seed weight, and spikelet number, etc. QTL of small intervals are rather fine-mapped and of increased biological significance.

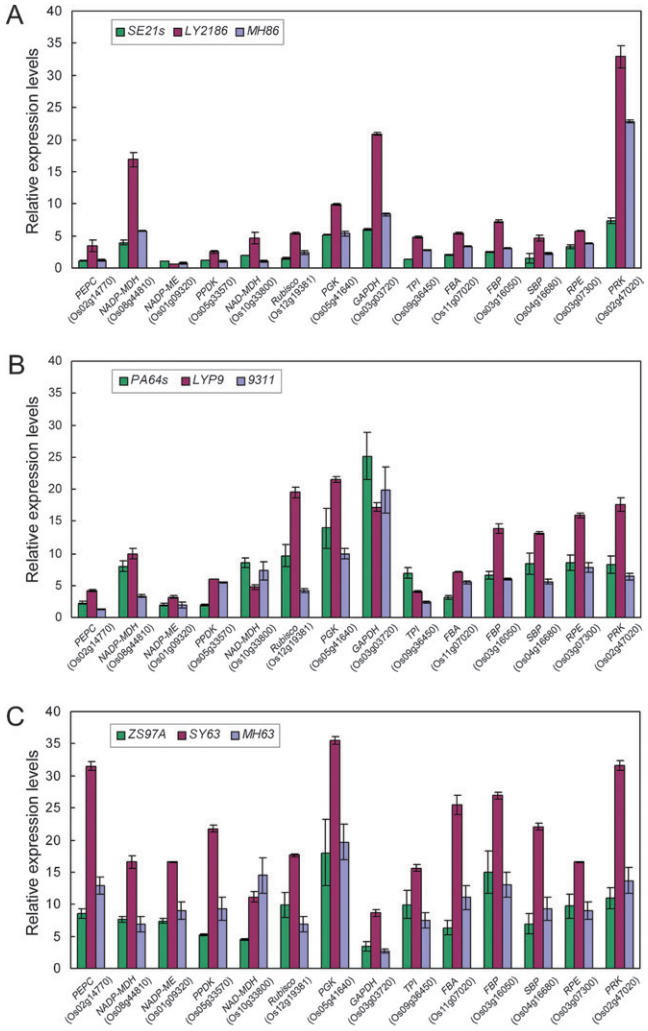

Figure 3. Real-Time Quantitative PCR of DGs in the Carbon-Fixation Pathway.

(A) Hybrid rice combination *LY2186*, **(B)** *LYP9*, and **(C)** *SY63*. Data are means ± SE of three replicates.

Recently, using a fine-mapping approach, the altered expression of *tb1* was characterized as the cause of quantitative phenotypic changes in maize (Clark et al., 2006). *Ghd7* was isolated by map-base cloning and considered a crucial factor for increasing productivity of an elite hybrid rice cultivar, *Shanyou 63* (Xue et al., 2008).

Implications to Mechanism of Heterosis

In this research, we detected multiple expression patterns of DGs, including dominance (HPL and LPL) and over-dominance (AHP and BLP) (Figure 1C). This suggested that heterosis is a complex issue. It is difficult to decipher its molecular basis using only one hypothesis; related hypotheses such as the dominance (Bruce, 1910), over-dominance (East, 1936), or epistatic hypotheses (Yu et al., 1997) may all contribute to heterosis in rice.

Table 5. Activity of Enzymes Involved in Carbon Fixation Pathways.

Enzymes	Activity (nmol mg^{-1} min^{-1})[1]			Activity (nmol min^{-1} cm^{-2})[2]		
	SE21s	*LY2186*	*MH86*	*SE21s*	*LY2186*	*MH86*
PEPC	74.19 ± 3.89^{ab}	95.87 ± 8.06^{a}	60.60 ± 4.85^{b}	0.75 ± 0.04^{ab}	1.04 ± 0.07^{a}	0.52 ± 0.04^{b}
NADP-MDH	34.55 ± 0.95^{b}	49.52 ± 0.66^{a}	20.46 ± 0.72^{c}	0.35 ± 0.009^{b}	0.45 ± 0.006^{a}	0.18 ± 0.006^{c}
NADP-ME	10.95 ± 0.35^{b}	16.43 ± 0.42^{a}	10.12 ± 0.89^{b}	0.11 ± 0.03^{b}	0.15 ± 0.04^{a}	0.09 ± 0.01^{b}
PPDK	16.95 ± 0.33^{b}	30.46 ± 1.21^{a}	18.98 ± 1.08^{b}	0.17 ± 0.03^{b}	0.28 ± 0.01^{a}	0.16 ± 0.01^{b}
Rubisco	119.05 ± 1.05^{a}	101.20 ± 2.55^{b}	97.42 ± 2.49^{b}	1.20 ± 0.01^{a}	0.93 ± 0.02^{b}	0.84 ± 0.02^{b}
PGK	60.88 ± 7.78^{b}	112.10 ± 9.98^{a}	99.49 ± 4.47^{a}	0.61 ± 0.08^{b}	1.03 ± 0.11^{a}	0.86 ± 0.04^{a}
TPI	111.33 ± 2.89^{b}	236.00 ± 11.29^{a}	197.57 ± 8.40^{a}	1.12 ± 0.03^{b}	2.16 ± 0.10^{a}	1.71 ± 0.08^{a}
FBA	257.15 ± 6.03^{b}	271.14 ± 5.53^{ab}	288.54 ± 3.04^{a}	2.59 ± 0.06^{b}	2.49 ± 0.05^{ab}	2.50 ± 0.02^{a}
Stromal FBP	16.78 ± 1.40^{b}	16.73 ± 2.03^{b}	37.03 ± 4.35^{a}	0.17 ± 0.01^{b}	0.15 ± 0.02^{b}	0.32 ± 0.01^{a}
Cytosolic FBP	9.92 ± 0.66^{b}	10.59 ± 1.39^{b}	17.85 ± 0.57^{a}	0.10 ± 0.01^{b}	0.10 ± 0.01^{b}	0.15 ± 0.01^{a}
RPI	2.09 ± 0.13^{a}	2.57 ± 0.40^{a}	1.98 ± 0.30^{a}	0.02 ± 0.001^{a}	0.02 ± 0.004^{a}	0.02 ± 0.001^{a}

1 Enzyme activity calculated by 1 unit per mg protein.
2 Enzyme activity calculated by 1 unit per cm^2 leaf.
The data are means ± SE of three replicates. One-way analysis of variance (ANOVA) was performed by using GraphPad Prism4 software. Different letters indicate significant differences ($P < 0.05$) in Bonferroni's post tests following an ANOVA.

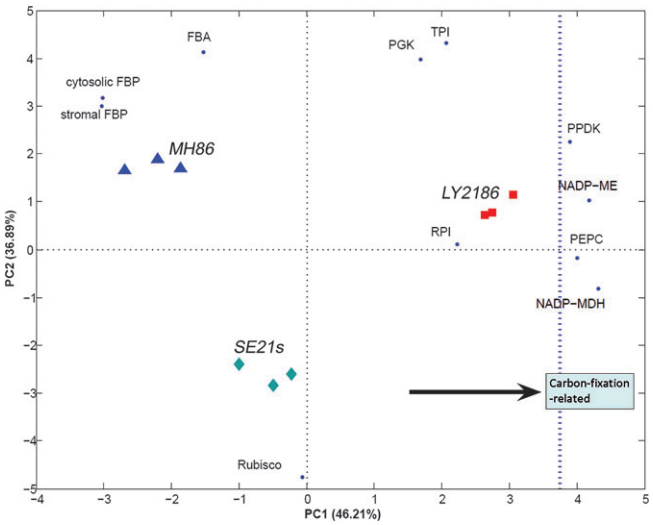

Figure 4. Principal Component Analysis (PCA) of Enzyme Activity Patterns in *LY2186* Combination.

BIPLOT visualization of PCA of enzyme activity patterns of the carbon-fixation pathway. The rice lines are indicated in red (*LY2186*), green (*SE21s*), or blue (*MH86*), and the contribution of the enzymes to positioning in the plot is provided. The two parent lines (*MH86* and *SE21s*) are more similar to each other than either to the hybrid line (*LY2186*). The positioning of the hybrid line towards the right side of the plot (indicated by an arrow) underpins the increase in the carbon-fixation-related enzyme activity as compared with its parents.

Recently, Ni et al. (2009) reported a model related to circadian rhythms to explain heterosis, in which F_1 hybrid and allopolyploid of *Arabidopsis* gained advantages from the control of circadian-mediated physiological and metabolic pathways. In this model, two key factors, *CIRCADIAN CLOCK ASSOCIATED 1* (*CCA1*) and *LATE ENLONGATED HYPOCOTYL*(*LHY*) (Alabadi et al., 2001), were epigenetically modified and repressed in the F_1 hybrid and allopolyploid during the day and further induced the expression of downstream genes involved in photosynthesis and carbohydrate metabolic pathways. The regulatory network of metabolic pathways involved in circadian rhythms was also reported to increase fitness in animals and plants (Michael et al., 2003; Wijnen and Young, 2006).

By Pathway Studio analysis, we investigated the regulatory network of DGs and finally focused on LHY, a transcription factor of the *MYB* family, also called *OsCCA1* in the case of rice (Murakami et al., 2007). Moreover, another two factors in circadian rhythms, PSEUDO RESPONSE REGULATOR (APRR) (Kaczorowski and Quail, 2003) and GIGANTEA (GI) (Gould et al., 2006), were also included in the network. Meanwhile, *LHY* is regulated by a transcription factor encoded by a DG (Os01g18290), PHYTOCHROME INTERACTING FACTOR 3 (PIF3), which is involved in the phytochrome-mediated light signaling pathway, receiving the light signal from photoactivated phytochrome molecules at the first step. PIF3 plays an important role in response to light (Castillon et al., 2007). Some DGs involved in photosynthesis, carbon-fixation, starch, and sucrose metabolism pathways could be regulated by the above factors (Dodd et al., 2005), and might result in yield traits vigor and heterosis. As mentioned above, all detected DGs involved in the circadian-rhythm network, including *LHY* or *OsCCA1* (Murakami et al., 2007), could be mapped to yield-related QTL. The similarity of the regulatory network between rice and *Arabidopsis* may imply that the circadian rhythms regulatory network in hybrid might be one of the molecular mechanisms underlying heterosis in hybrid plants. However, it can not be excluded that the accumulation of small advantages of dominance and over-dominance at a large number of loci in the heterozygotic genome can also

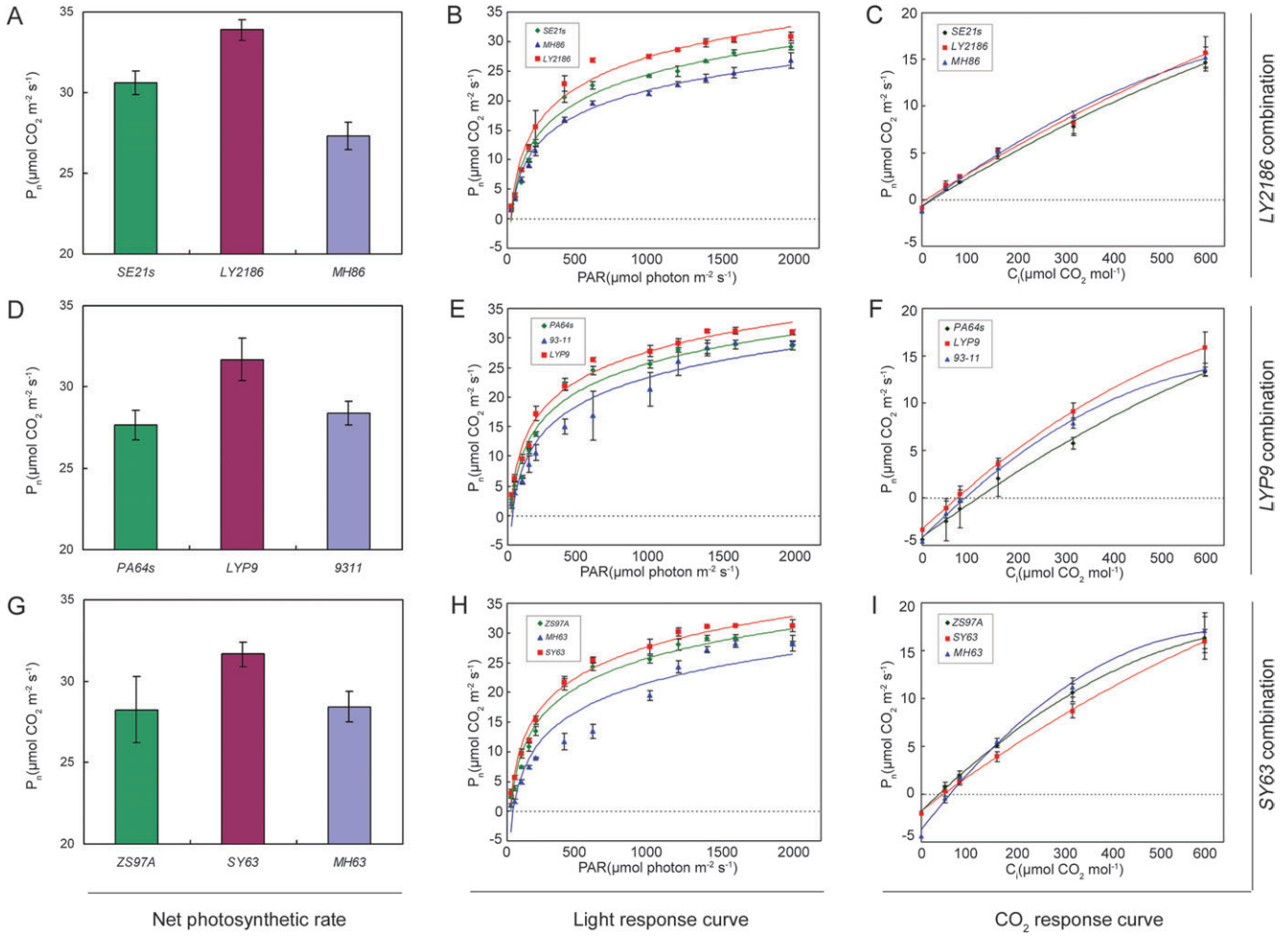

Figure 5. Comparisons of Photosynthesis Characters in Three Hybrid Rice Combinations.

(A–C) Photosynthesis characters of hybrid rice combination *Liangyou-2186*, **(D–F)** *Liangyou-pei9*, and **(G–I)** *Shanyou-63*. (A, D, G) Net photosynthetic rate (P_n); (B, E, H) light response curve; (C, F, I) CO_2 response curve. PAR, photosynthetic activity rate; C_i, CO_2 concentration. Data are means ± SE of three replicates.

contribute to heterosis. Altogether, though the hybrid vigor or heterosis is undoubtedly one of the most complex issues, our findings might provide another view to the understanding the mystery of heterosis.

METHODS

Plant Materials

Three hybrid rice combinations, *Liangyou-2186* (super-hybrid rice *LY2186* and its parental lines, sterile line *SE21s* and restorer line *MH86*), *Liangyou-pei9* (super-hybrid rice *LYP9* and its parental lines, sterile line *PA64s* and restorer line *93–11*), and *Shanyou-63* (traditional hybrid rice *SY63* and its parental lines, sterile line *ZS97A* and restorer line *MH63*) were planted in the same field. Samples were collected and photosynthesis characters were measured at the same time and under the same environmental conditions.

SAGE Library Construction

Total RNA was extracted from the flag leaves at the grain-filling stage of hybrid rice *Liangyou-2186* and its parental lines, *SE21s* and *Minghui86* (*MH86*), and mRNA was isolated for SAGE library construction using the I-SAGE kit (Invitrogen) according to the manufacturer's protocol. The cloned concatemers were sequenced with an ABI3730 auto-sequencer (Perkin–Elmer), and SAGE tags were extracted by the SAGE2000 software. Differentially expressed tags were defined by use of IDEG6 (Romualdi et al., 2003), with the significance threshold set at $P < 0.05$, on the basis of Audic–Claverie statistics (Audic and Claverie, 1997).

Annotation of SAGE Tags

According to the SAGE principle, 10-bp tags were extracted from the 3′-downstream sequence after the last *Nla*III site (CATG) of the FL-cDNAs in KOME (Kikuchi et al., 2003) to

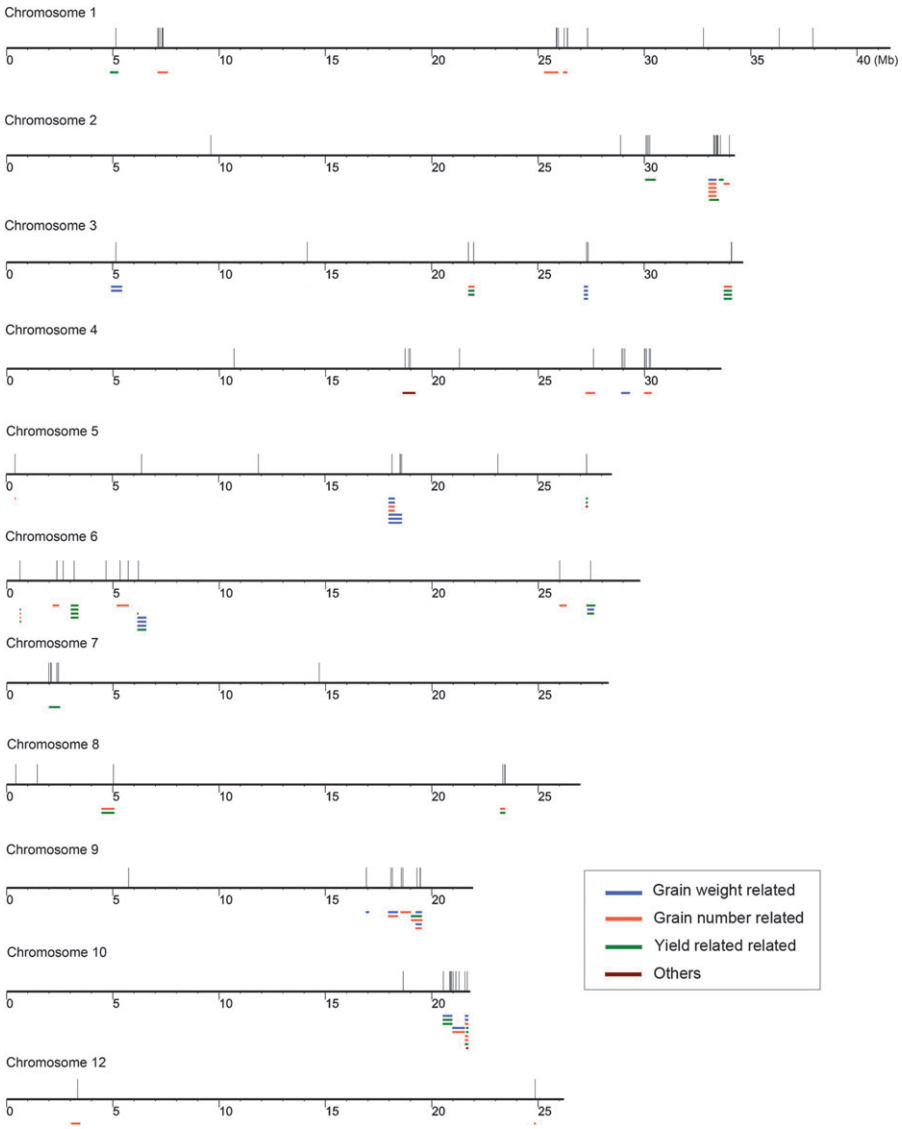

Figure 6. Distribution of DGs Located in Yield-Category QTL of Small Intervals.

Yield-category QTL of small intervals (number of genes ⩽ 100) that harbor DGs were aligned with the gene coordinates in Rice Genome Annotation release 6.1. The long horizontal lines represent the rice chromosomes, the short horizontal lines in different colors QTL intervals, and the short vertical lines DGs.

generate virtual tags as a reference. Tags in each library of the hybrid rice *Liangyou-2186* combination were aligned with the reference for annotation. On the basis of the Rice Genome Annotation release 6.1, tags that matched FL-cDNA clones were mapped to the exact genome locus and annotated gene models (Ouyang et al., 2007). Gene ontology (GO) annotation analysis of DGs was performed by the WEGO (Ye et al., 2006) and MADIBA (Law et al., 2008) web tools.

Metabolic Pathway Analysis of DGs

Analysis of the metabolic pathways of DGs involved was performed using MADIBA (Law et al., 2008). The locus ID of DGs by the Rice Genome Annotation was used for clustering and mapping gene products (enzymes) onto metabolic pathways by use of the Kyoto Encyclopedia of Genes and Genomes (KEGG) representation (Kanehisa et al., 2004); a *P*-value was calculated for each pathway by Fisher's exact test.

Real-Time Quantitative PCR (qPCR)

The flag leaves at the grain-filling stage of all three hybrid rice combinations were used for total RNA extraction with TRIzol (Invitrogen). Reverse transcription was performed using the SuperScript II First-Strand Synthesis System for RT–PCR (Invitrogen) according to the manufacturer's protocol. Real-time PCR was performed on an MJ Chromo 4 detection system in 96-well reaction plates with parameters recommended by the manufacturer (95°C for 5 min; 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s; 72°C for 5 min). Each PCR reaction was

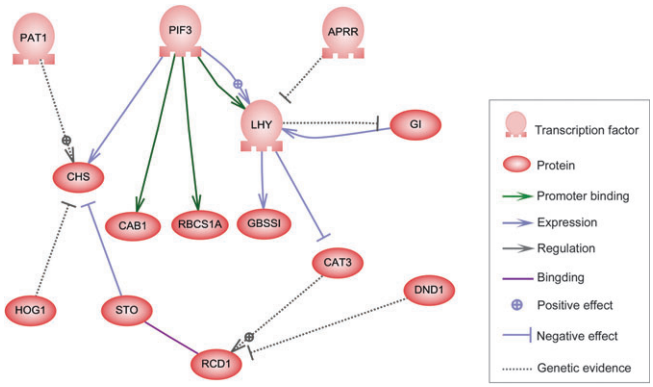

Figure 7. Gene Network of DGs in Hybrid Rice by Pathway Studio Analysis.

DGs were used for direct interaction analysis by Pathway Studio 6.2. Interaction searching includes promoter binding, expression, regulation, and binding. APRR, PSEUDO-RESPONSE REGULATOR; CAB1, CHLOROPHYLL A/B BINDING PROTEIN 1; CAT3, CATALASE 3; CHS, CHALCONE SYNTHASE; DND1, DEFENSE NO DEATH 1; GBSSI, GRANULE-BOUND STARCH SYNTHASE I; GI, GIGANTEA; HOG1, HOMOLOGY-DEPENDENT GENE SILENCING 1; LHY, LATE ELONGATED HYPOCOTYL; PAT1, PHYTOCHROME A SIGNAL TRANSDUCTION 1; PIF3, PHYTOCHROME INTERACTING FACTOR 3; RBCS1A, RUBISCO SMALL SUBUNIT 1A; RCD1, RADICAL-INDUCED CELL DEATH1; STO, SALT TOLERANCE.

Table 6. Comparison of Harvest Index for the Three Hybrid Rice Combinations.

Rice breed	Biomass. plant^{-1} (g)	Production increased (%)[a]	Grain yield. plant^{-1} (g)	Harvest index	Harvest index increased (%)[b]
SE21s	40.27 ± 0.77		5.57 ± 0.53	–	
LY2186	72.60 ± 1.06	19.80%	23.10 ± 0.57	0.32	18.52%
MH86	60.60 ± 1.84		16.53 ± 0.55	0.27	
PA64s	44.08 ± 1.90		6.50 ± 0.78	–	
LYP9	48.47 ± 1.30	12.40%	17.13 ± 1.61	0.35	20.69%
93–11	43.12 ± 0.66		12.32 ± 1.05	0.29	
ZS97A	38.97 ± 1.79		5.17 ± 0.87	–	
SY63	58.07 ± 1.06	32.97%	17.10 ± 1.03	0.29	7.41%
MH63	43.67 ± 1.27		11.97 ± 0.61	0.27	

a Production increased (%) = (Dry weight of hybrid rice/Dry weight of restorer line – 1) × 100%.
b Harvest index (HI) increased (%) = (HI of hybrid rice/HI of restorer line – 1) × 100%.
The data are means ± SE.

performed in triplicate and *ACTIN1* as internal control was included (for primers, see Supplemental Table 9). Specificity of the amplification was verified according to the melting curve by Opticon monitor™ analysis software. Statistical analyses involved the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Measurement of Enzyme Activity

Total protein from collected leaf samples was extracted for enzyme activity measurements. Three gram leaf samples were ground to powder in liquid nitrogen and extracted with buffer (50 mM Tris-HCl, pH 8.0, 10% (v/v) glycerol, 2% (w/v) PVP, 1 mM PMSF, 0.1% (v/v) Triton X-100, 10 mM $MgCl_2$, 1 mM EDTA, and 15 mM mercaptoethanol), then centrifuged at 15 000 *g* for 10 min at 4°C. The supernatant was used for protein quantification and enzyme activity analysis. Activities of PEPC (Meyer et al., 1988), NADP-MDH (Holaday et al., 1992), NADP-ME (Leegood, 1990), PPDK (Salahas et al., 1990), Rubisco (Ueno and Sentoku, 2006), PGK (Kuntz and Krietsch, 1982), TPI (Gracy, 1975), FBA (Haake et al., 1998), RPI (Domagk and Alexander, 1975), stromal FBP, and cytosolic FBP (Leegood, 1990) were measured according to indicated references. Statistical analyses were performed by GraphPad Prism4 software, using the one-way ANOVA method. Significant differences ($P < 0.05$) were analyzed by Bonferroni's post tests following an ANOVA. PCA was used for reduction of the dimensionality of the enzyme activity data. Visualization was based on the BIPLOT method after auto-scaling of the data (Jolliffe, 2002). All calculations involved use of Matlab (The MathWorks, Natick, USA) and the PLS toolbox (Eigenvector Research, Wenatchee, USA).

Measurement of Photosynthetic Characters

The flag leaves at the grain-filling stages for all three field-grown hybrid rice combinations were measured. Photosynthetic characters from leaf samples of hybrid rice combinations in the grain-filling stage were measured under sunny conditions by a portable photosynthesis analyzer (CIRAS-1 system). The photosynthetic rate, light response curves, and CO_2 response curve were calculated and analyzed according to previous methods (Nogues and Baker, 2000). The apparent quantum yield (AQY) for flag leaves was measured under low-light conditions: 0, 25, 50, 100, and 150 μmol photon m^{-2} s^{-1}.

Mapping DGs to QTL

Rice QTL data with physical positions on the rice genome were acquired from Gramene (www.gramene.org), gene loci, and their coordinates were obtained from Rice Genome Annotation release 6.1. Based on the physical positions of both gene loci and QTL, DGs were mapped to QTL. QTL of small intervals spanning no more than 100 genes were extracted and mapped with DGs in the rice chromosomes.

Regulatory Network Analysis

The gene network of DGs was analyzed by Pathway Studio software (version 6.2) (Nikitin et al., 2003), with the Unigene ID of DGs from NCBI (www.ncbi.nih.gov/) as input for the direct interaction analysis. The regulatory network was constructed by searching the ResNet Plant Database, employing four interaction types, namely promoter binding, expression, regulation, and binding. False interactions and signal genes without interactions with others were removed according to the original references recorded by the software.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This work was supported by the grants from the Major Project of China on New varieties of GMO Cultivation (2008ZX08001-001, 2008ZX08012-002), the Research Project on Rice Functional Genes related to the Yield Performance of the High Technology Research and Development Program (863) of China (2006AA10A101), the National Program on Key Basic Research Projects (973) of China (2004CB720406, 2007CB109000), the Innovation Foundation of Chinese Academy of Science (KSCX2-SW-306 and KSCX1-SW-03), the Program for Strategic Scientific Alliances between China and the Netherlands (KNAW-PSA04-PSA-BD-04 for P.B.F.O. and M.W., KNAW-CEP project 04CDP022 for Y.X., and the CAS-KNAW Joint PhD Training Programme (05-PhD-02) for Y.Z. Funding to pay the Open Access publication charges for this article was provided by the Major Project of China on New varieties of GMO Cultivation (2008ZX08001-001).

ACKNOWLEDGMENTS

We thank Prof. Longping Yuan and Huaan Xie for kindly providing the seeds of hybrid rice, Prof. G.E. Edwards for helpful advice, Prof. C.R. Buell and Dr J. Hamilton from the Rice Genome Annotation Project Team for providing helpful information about FL-cDNA related to the gene models from the Rice Genome Annotation, and Prof. Xiujie Wang for support in regulatory network analysis. No conflict of interest declared.

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