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Research Article

Expression Analysis of miRNAs and Highly-expressed Small RNAs in Two Rice Subspecies and their Reciprocal Hybrids

Fangfang Chen[1,2], Guangming He[2,3,4], Hang He[4], Wei Chen[4], Xiaopeng Zhu[5], Manzhong Liang[6], Liangbi Chen[6] and Xing Wang Deng[3,4*]

[1]Graduate Program in Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China
[2]National Institute of Biological Sciences, Zhongguancun Life Science Park, Beijing 102206, China
[3]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520, USA
[4]Peking-Yale Joint Center of Plant Molecular Genetics and Agrobiotechnology, College of Life Sciences, Peking University, Beijing 100871, China
[5]Institute of Biophysics, the Chinese Academy of Sciences, Beijing 100101, China
[6]Department of Botany, College of Life Sciences, Hunan Normal University, Changsha 410081, China
[*]Corresponding author
Tel: +1 203 432 8908; Fax: +1 203 432 5726; E-mail: xingwang.deng@yale.edu
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Abstract

Heterosis, or hybrid vigor, is the phenomenon whereby progeny of two inbred lines exhibit superior agronomic performance compared with either parent. We analyzed the expression of miRNAs and highly expressed small RNAs (defined according to Solexa sequencing results) in two rice (*Oryza sativa*) subspecies (*japonica* cv. Nipponbare and *indica* cv. 93-11) and their reciprocal hybrids using microarrays. We found that of all the 1141 small RNAs tested, 140 (12%, 140 of 1141) and 157 (13%, 157 of 1141) were identified being significantly differentially expressed in two reciprocal hybrids, respectively. All possible modes of action, including additive, high- and low- parent, above high- and below low-parent modes were exhibited. Both F1 hybrids showed non-additive expression patterns, with downregulation predominating. Interestingly, 15 miRNAs displayed stark opposite expression trends relative to mid-parent in reciprocal hybrids. Computational prediction of targets of differentially expressed miRNAs showed that they participated in multifaceted developmental pathways, and were not distinguishable from the targets of non-differentially expressed miRNAs. Together, our findings reveal that small RNAs play roles in heterosis and add a new layer in the understanding and exploitation of molecular mechanisms of heterosis.

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Introduction

Heterosis refers to the phenomenon that heterozygous F1 offspring exhibits phenotypic traits in terms of biomass, speed of development, yield etc. superior compared to the two parents. Heterosis has been described by Charles Darwin in maize in 1876. The phenomenon has been successfully applied in the breeding of crops and animals, and has made significant contributions toward meeting people's growing needs for food and energy. Approximately 95% of the US maize acreage and 55% of rice acreage in China are planted with hybrids. It has been estimated that the use of heterosis in maize increases yields by 15% annually (Duvick 1999). Furthermore, 10%–20% yield increase has been observed in hybrid rice cultivars

(Li et al. 2008).Though heterosis has been exploited for a long time, its molecular mechanisms are still enigmatic.

Numerous efforts have been undertaken to unravel the molecular and genetic mechanisms of heterosis, but no consensus could be reached to date (Birchler et al. 2003; Birchler et al. 2006; Hochholdinger and Hoecker 2007; Lippman and Zamir 2007; Chen 2010). Two genetic concepts to explain heterosis are the dominance (Davenport 1908) and over-dominance (Shull 1908; East 1908) hypothesis. The first one postulates that deleterious alleles that are present in one inbred parental line will be complemented by functional alleles from the opposite parent. The over-dominance hypothesis refers to the idea that heterosis is the result of allelic interactions at one or multiple loci in hybrids, and the allelic interactions will result in superior traits compared with the homozygous parental inbred lines. These two models did have experimental support in the early days (Stuber et al. 1992; Guo et al. 2003; Gibson et al. 2004) before molecular concepts were formulated. However, with the development of molecular research tools, they are insufficient to describe mechanisms of heterosis at the molecular level. More and more studies show that heterosis depends on multiple mechanisms that are involved in both genetic and epigenetic contexts (Hoecker et al. 2006; Meyer et al. 2007; Springer and Stupar 2007; Chen 2010; He et al. 2010).

Small RNAs of 20–30 nt acting in gene-silencing systems falls into two major categories, miRNAs and siRNAs (small interfering RNAs) (Bartel 2004). They are essential regulatory molecules playing important roles in developmental regulation, responses to biotic and abiotic stresses, and epigenetic control of transposable elements in most eukaryotes (Baulcombe 2004; Vaucheret 2006). miRNAs and siRNAs are distinguished by their biogenesis instead of their function (Bartel 2004). miRNAs are derived from long, single-stranded RNAs (ssRNAs), which can fold and form a stem-loop structure, and then are processed by Drosha/Dicer family proteins (Bartel 2004; Du and Zamore 2005; Kim 2005). However, siRNAs are produced from long double-stranded RNAs (dsRNAs) that originate from products of bidirectional transcription or RNA-dependent RNA polymerases (RDRs) (Bartel 2004; Vaucheret 2006).

Here, we report the application of a microRNA microarray system to examine expression of annotated rice miRNAs and highly-expressed small RNAs in two rice subspecies and their reciprocal hybrids to exploit mechanisms of heterosis. The definition of highly-expressed small RNAs used here comes from a Solexa sequencing dataset (He et al. 2010) using the same materials as in this study.

A total of 1141 small RNAs including all of the 142 unique annotated miRNA sequences representing more than 300 miRNA loci from Rice miRBase 11.0 were examined on the microarray, showing that the most common mode of action in both F1 reciprocal hybrids is non-additive. Twelve to thirteen percent of small RNAs exhibited significantly differential expression among genotypes. More small RNAs were downregulated with low parent and below low parent modes predominating. All possible action modes were observed. The expression patterns of eight randomly selected representative miRNAs were validated by using quantitative real-time polymerase chain reaction (PCR). Taken together, our findings provide a fresh insight into the complex relationships between small RNAs and heterosis.

Results

Heterosis performance of two rice subspecies in their reciprocal hybrids

Rice, one of the most important staple foods, is widely planted around the world. Hybrid rice accounts for 50% of the total rice acreage in many rice producing countries such as China, India, and Indonesia (Chen 2010) and exhibits growth vigor beginning with the seedling stage. The materials used in the present study are the above-ground seedling tissue at the four-leaf stage of two rice subspecies (*O. sativa* ssp. *japonica* cv. Nipponbare and *O. sativa* ssp *indica* cv. 93-11) and their reciprocal hybrids, taking advantage of the available complete genome sequences of the two parental lines (Goff et al. 2002; Yu et al. 2002). At the four-leaf stage, F1 hybrids already show taller growth, stronger growth potential and larger biomass (**Figure 1A**, data not shown) than their parents, and this growth vigor continues to the reproductive growth stage (tillering stage, **Figure 1B**) with accompanying yield increase.

Identification and characterization of differentially expressed small RNAs

We used microRNA microarray technology from LC Sciences to test the expression of 1 141 selected small RNAs from Nipponbare, 93-11 and their reciprocal hybrids. The 1 141 selected small RNAs include two parts, the first part is 142 unique microRNA sequences representing more than 300 annotated miRNAs loci based on Sanger version 11.0 (http://www.mirbase.org/), and the second part is 999 sequences ranging at 16–26 nt chosen from small RNAs Solexa sequencing dataset (He et al. 2010). The adapters from raw Solexa sequencing reads were computationally removed and the remaining sequences were aligned to the well-annotated *japonica* rice genome sequence. After removing rRNA, tRNA, and sn/snoRNA, the remaining small RNAs reads from the four samples were pooled and we selected the 999 sequences with the most reads to fill the microarray's capacity. Four biological replicates were analyzed to provide a higher degree of statistical power.

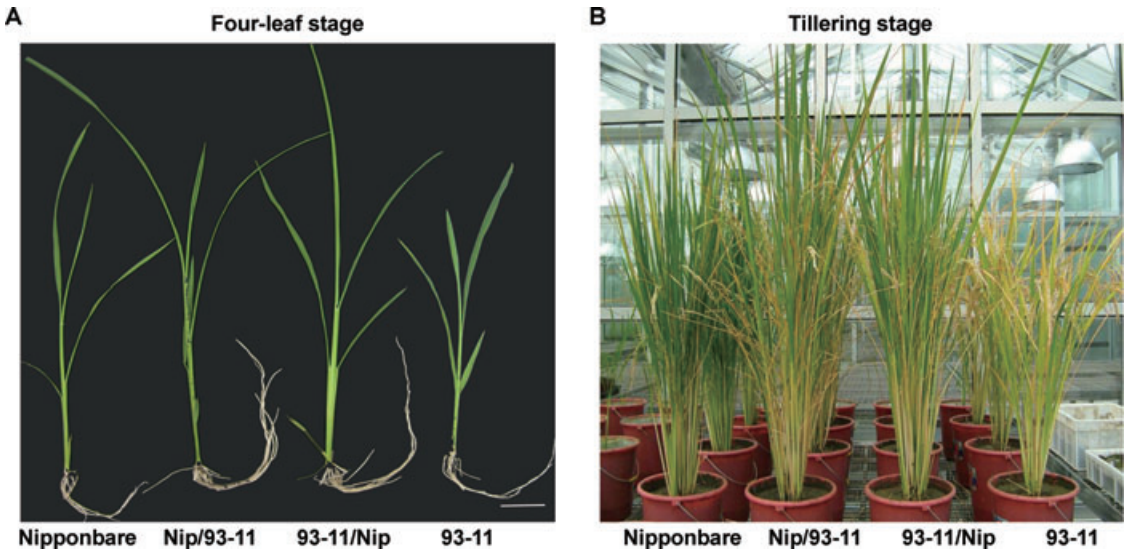

Figure 1. Heterosis in reciprocal hybrids between rice subspecies of Nipponbare and 93-11.

Growth vigor of reciprocal hybrids at **(A)** four-leaf stage and **(B)** tillering stage.
Bar = 2 cm. (Modified from Supplementary Figure 1A of He et al. (2010)

Total RNAs extracted from four-leaf stage seedlings were isolated to purify small RNAs < 300 nt. After being labeled with fluorescent dyes, the isolated small RNAs were hybridized to the microRNA microarray, which contains more than 3000 spots (see Materials and Methods). Four biological replicates were analyzed to provide a higher degree of statistical power.

The processed microarray data were used to determine small RNAs differential expression in pairwise comparisons. In these comparisons, approximately 11%–14% of small RNAs showed a differential expression in each comparison that was statistically significant, with about the same number of small RNAs that were upregulated or downregulated, respectively (**Figure 2A, B**). A total of 140 ($P < 0.01$, False Discovery Rate (FDR) = 0.082) and 157 ($P < 0.01$, FDR = 0.073) small RNAs exhibited statistically significant differential expression in two reciprocal hybrids. Among these small RNAs, 83% (116 out of 140) and 82% (128 out of 157) showed non-additive expression pattern, while the remaining 17% (24 out of 140) and 18% (29 out of 157) of small RNAs were not distinguishable from an additive expression pattern in the reciprocal hybrids (**Figure 2C**). We observed that more small RNAs were downregulated than upregulated in both F1 hybrids compared with the mid-parent value (**Figure 2D**). Low-parent and below low-parent were the predominant modes in both hybrids (**Figure 2E and F**). Considering that small RNAs usually work as negative regulators to repress translation or cleave transcripts, our results support a previous study that used the same rice strains (He et al. 2010). In that study, the authors showed that siRNA clusters were more downregulated than upregulated in hybrids, which led the authors of that study to conclude that this points to a suppressive effect of siRNAs in hybrids. All possible action modes, including additive, high- and low-parent, above high- and below low-parent modes were detected in our study, which suggests their association with heterosis. This is consistent with studies in maize and rice, which showed that multiple modes of action are involved in heterosis (Swanson-Wagner et al. 2006; Zhang et al. 2008; He et al. 2010).

As miRNAs play documented roles in regulating gene expression, we also specifically focused our analyses on miRNAs (**Table 1**). We found that 47 of the 142 miRNAs ($P < 0.01$, FDR = 0.030) showed differential expression in at least one of the hybrids (**Table 2**). Further investigation revealed that these differentially expressed miRNAs fell into several families (miR156, miR159, miR162, miR164, miR166, miR167, miR168, miR396, miR397, miR399 etc.). Moreover, distinct miRNAs from one family tended to exhibit similar modes of action in both hybrids. These can be explained as members of a miRNA family with functional redundancy, which often target the same sets of genes (Jones-Rhoades et al. 2006; Sieber et al. 2007). Similarly to all other identified small RNAs, miRNAs also showed more downregulation than upregulation. However, we also found 15 miRNAs that displayed opposite expression trends relative to the mid-parent value in two hybrids (**Table 2**, highlighted with asterisk). Since the overall modes of action in the two reciprocal hybrids was similar and because they showed the same overt phenotypes, we may conclude that small RNAs exert functions in a co-operating network in which no single miRNA plays the decisive role for heterosis.

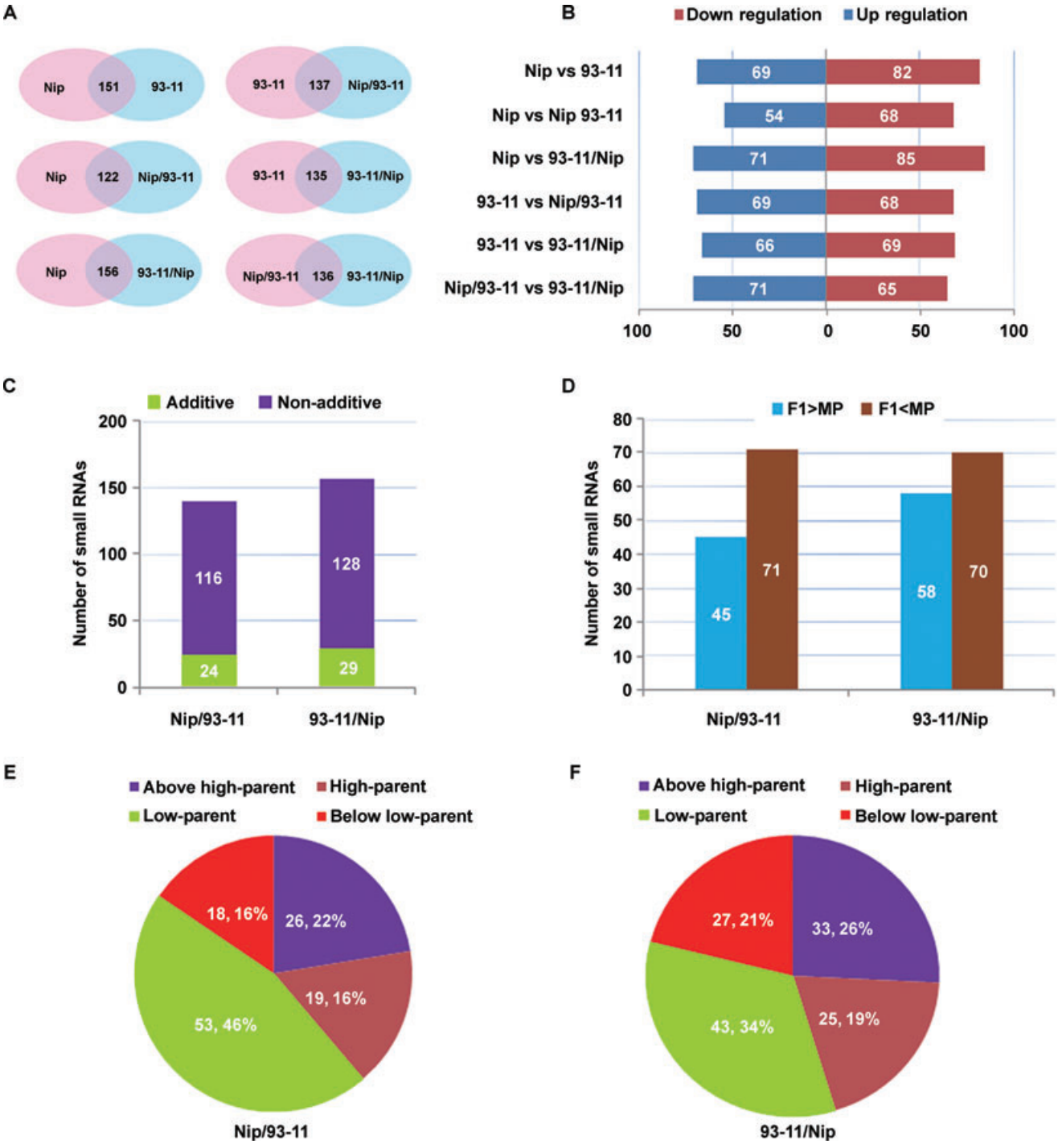

Figure 2. Diversity of small RNAs in composition and expression between parents and hybrids.

(A) Venn diagram and **(B)** statistical analysis of differentially expressed small RNAs among genotypes.
(C) Additive and non-additive variation of small RNAs expression in the reciprocal hybrids. Additive, hybrids show an expression level equal to mid-parent value. Non-additive, hybrids show an expression level deviating from mid-parent value.
(D) Differential expression of small RNAs between F1 hybrids and their parents. MP, mid-parent value.
(E) and **(F)** Non-additive expression patterns of small RNAs in Nip/93-11 and 93-11/Nip.

Table 1. Statistical analysis of differentially expressed miRNAs

F1 hybrid	Total	Additive	Non-additive	High-parent	Low-parent	Above high-parent	Below low-parent
Nip/93-11	40	8	32	6	19	4	3
93-11/Nip	43	9	34	13	7	13	1

$P < 0.01$.

Targets of differentially expressed miRNAs are involved in multiple developmental pathways

microRNAs play extensive roles in regulating multiple aspects of organismal development. They are produced from independently transcribed genetic loci and serve as negative regulators of gene expression by targeting mRNA degradation or translational repression (Bartel 2004; Baulcombe 2004; Jones-Rhoades et al. 2006; Fait et al. 2008). Plant miRNAs mainly function by transcript cleavage with sequences highly complementary to their targets, while animal miRNAs only partially complement with their targets to repress translation. This allows fast and confident bioinformatic identification of plant miRNA targets, which have been experimentally verified in many studies (Rhoades et al. 2002; Jones-Rhoades and Bartel 2004; Adai et al. 2005). A typical computational prediction of rice miRNA targets from Archak et al. showed diverse functions of rice targets including transcription, catalysis, binding, and transporter activity (Archak and Nagaraju 2007).

A recent study by Wu et al. (2009) generated a comprehensive target prediction of 77 rice miRNA families including all annotated miRNAs and newly identified miRNAs. By applying a bioinformatic method with a cutoff mispairing score of $\leq$4.0 (Allen et al. 2005), they predicted 311 targets. Meanwhile, Li et al. (2010) and Zhou et al. (2010) applied a "degradome sequencing" approach and confirmed nearly 200 rice targets of conserved and non-conserved miRNA families from Nipponbare and 93-11, respectively. Since all miRNAs tested in our study were included in this prediction, we made a comprehensive investigation of these predicted especially the experimentally confirmed rice miRNA targets (see supplemental Table S1). As mentioned above, the differentially expressed miRNAs mainly clustered in the following families: miR156, miR159, miR162, miR164, miR166, miR167, miR168, miR396, miR397, and miR399 family. Investigation revealed that targets of differentially expressed miRNAs include functions like transcription regulator activity (GAMYB TF, TCP family TF, transcription regulator, MADS-box TF, scarecrow-like TF, retrotransposon protein etc.), binding activity (squamosa promoter-binding-like protein, calcium-binding protein, copper ion binding protein etc.), catalytic activity (L-ascorbate oxidase, laccase, calcium-transporting ATPase, endoribonuclease Dicer etc.), and other activities such as structural formatting, growth-regulating factor and transporters. However, the targets of non-differentially expressed miRNAs show in general the same activities, which led us to conclude that miRNAs might act in cooperation with other regulatory mechanisms to control gene expression that contributes to heterosis.

Validation of miRNA expression via qRT-PCR

Eight miRNAs were randomly selected from the 47 miRNAs with differential expression in hybrids for expression validation via quantitative reverse transcription polymerase chain reaction (qRT-PCR). These eight miRNAs included all modes of action. We conducted these experiments following a stem-loop qRT-PCR method. Stem-loop primers, specific forward primer for every miRNA and universal reverse primer are designed as described (**Table 3**) (Varkonyi-Gasic et al. 2007; Schmittgen et al. 2008). RNA samples from three biological replicates of Nipponbare, 9311, Nip/93-11 and 93-11/Nip were treated in the experiments with three technical replications in each plate. Our results showed that six out of eight miRNAs (miR444b.1, miR168b, miR168a, miR156k, miR156l, miR319a) exhibited a relatively good match with the microarray data, showing the same modes of action as deduced from the microarray data (**Figure 3**). qRT-PCR results of miR1432 and miR528 exhibited a discrepancy with microarray data, while the relative trends to mid-parent value still match (**Figure 3**).

Discussion

In the present study, we investigated molecular mechanisms underlying heterosis from a unique perspective by detecting the annotated miRNAs and highly expressed small RNAs in two rice subspecies and their reciprocal hybrids. Approximately 12–13% of all the tested small RNAs showed differential expression in the two hybrids. We found that most small RNAs exhibited a non-additive pattern in both reciprocal hybrids with higher proportions of low parent and below low parent modes, and observed that more small RNAs were downregulated than upregulated in F1 hybrids. This is consistent with a study by He et al. (2010), which used the same rice lines as in our study. The authors of that study showed that more siRNA clusters were downregulated than upregulated in hybrids, which supports the hypothesis that small RNAs function as negative regulators. However, in that study, most siRNA clusters showed additive pattern in both hybrids, which is opposite to our

Table 2. miRNAs with differential expression in Nip/93-11 or 93-11/Nip

miRNA[a]	Nip/93-11		93-11/Nip	
	Significant pattern	Action mode	Significant pattern	Action mode
miR1432	N ≈ N9 ≈ 9	No variation	N ≈ 9N > 9	HP
*miR156a	N ≈ N9 < 9	LP	N < 9N ≈ 9	HP
*miR156K	N ≈ N9 < 9	LP	N < 9N ≈ 9	HP
*miR156l	N ≈ N9 < 9	LP	N < 9 < 9N	AHP
miR159a	N > N9 ≈ 9	Additive	9 < N < 9N	AHP
miR159c	N ≈ N9 ≈ 9	No variance	9 < N < 9N	AHP
miR159d	N ≈ N9 ≈ 9	No variance	9 ≈ N < 9N	AHP
miR159e	N ≈ N9 ≈ 9	No variation	9 < N < 9N	AHP
miR159f	N > N9 ≈ 9	Additive	9 < N < 9N	AHP
miR162a	N < N9 ≈ 9	Additive	N < 9N ≈ 9	Additive
miR162b	N < N9 < 9	Additive	N < 9N ≈ 9	HP
miR164a	9 ≈ N < N9	AHP	N ≈ 9N ≈ 9	No variation
miR164d	9 ≈ N < N9	AHP	N ≈ 9N ≈ 9	No variation
miR164e	9 ≈ N < N9	AHP	N ≈ 9N ≈ 9	No variation
*miR166a	N < 9 < N9	AHP	N ≈ 9N < 9	LP
*miR166e	N < N9 < 9	LP	N < 9N < 9	HP
miR166g	N < N9 ≈ 9	Additive	N < 9N < 9	HP
*miR166i	N < N9 < 9	HP	N < 9N < 9	LP
miR166k	N < N9 < 9	HP	N < 9N < 9	HP
miR166m	N < N9 ≈ 9	HP	N < 9N < 9	HP
*miR167a	N < N9 < 9	LP	N < 9 < 9N	AHP
*miR167d	N ≈ N9 < 9	LP	N < 9 < 9N	AHP
*miR168a	N > N9 ≈ 9	LP	N ≈ 9N > 9	HP
*miR168b	N9 < N ≈ 9	BLP	9 ≈ N < 9N	AHP
miR169h	N ≈ N9 ≈ 9	No variation.	N < 9N ≈ 9	HP
*miR171a	N < N9 ≈ 9	HP	N ≈ 9N < 9	LP
miR171b	N < N9 ≈ 9	Additive	N ≈ 9N < 9	LP
miR172a	N ≈ N9 < 9	LP	N ≈ 9N < 9	LP
miR319a	9 ≈ N > N9	BLP	9 ≈ N > 9N	BLP
miR396a	N ≈ N9 < 9	LP	N ≈ 9N < 9	LP
miR396c	N ≈ N9 < 9	LP	N ≈ 9N < 9	LP
miR396d	N < N9 ≈ 9	Additive	N ≈ 9N < 9	Additive
miR397a	N ≈ N9 ≈ 9	No variation.	N ≈ 9 < 9N	AHP
miR397b	N ≈ N9 ≈ 9	No variation.	N ≈ 9 < 9N	AHP
*miR399a	N ≈ N9 < 9	LP	N < 9N ≈ 9	HP
miR399d	N ≈ N9 < 9	LP	N < 9N ≈ 9	Additive
miR399e	N ≈ N9 < 9	LP	N < 9N ≈ 9	Additive
miR399h	N ≈ N9 < 9	LP	N < 9N ≈ 9	Additive
*miR399i	N ≈ N9 < 9	LP	N < 9N ≈ 9	HP
*miR399j	N ≈ N9 < 9	LP	N < 9N ≈ 9	HP
miR408	N ≈ N9 > 9	HP	N ≈ 9N > 9	Additive
*miR444b.1	N > N9 ≈ 9	BLP	N ≈ 9 < 9N	AHP
miR444b.2	N ≈ N9 < 9	LP	N < 9N ≈ 9	Additive
miR444f	N ≈ N9 < 9	LP	N < 9N ≈ 9	Additive
miR528	N ≈ N9 > 9	HP	N > 9N > 9	Additive
miR530	N ≈ N9 < 9	LP	N ≈ 9N ≈ 9	No variation
miR535	N > N9 > 9	Additive	9 < N < 9N	AHP

$P < 0.01$. AHP, above high-parent; BLP, below low-parent; HP, high parent; LP, low parent.

[a]* indicates miRNAs that shows opposite expression trends relative to mid parent value.

Table 3. miRNA, primer sequences designed for miRNAs stem-loop quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Universal	Reverse primer	GTGCAGGGTCCGAGGT
miR444b.1	MicroRNA sequence	UGUUGUCUCAAGCUUGCUGCC
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACggcagc
	Forward	GCGGCGTGTTGTCTCAAGCTTG
miR168b	MicroRNA sequence	AGGCUUGGUGCAGCUCGGGAA
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACttcccg
	Forward	CGGCGAGGCTTGGTGCAGC
miR168a	MicroRNA sequence	UCGCUUGGUGCAGAUCGGGAC
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgtcccg
	Forward	CGCGGTCGCTTGGTGCAG
miR156l	MicroRNA sequence	CGACAGAAGAGAGUGAGCAUA
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACtatgctc
	Forward	GCCGGGCGACAGAAGAGAGTG
miR156k	MicroRNA sequence	UGACAGAAGAGAGAGAGCACA
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACtgtgctc
	Forward	CGCCGGCTGACAGAAGAGAGAG
miR319a	MicroRNA sequence	UUGGACUGAAGGGUGCUCCC
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgggagc
	Forward	CGGCCGTTGGACTGAAGGGT
miR1432	MicroRNA sequence	AUCAGGAGAGAUGACACCGAC
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgtcggt
	Forward	GCCGCGCATCAGGAGAGATGAC
miR528	MicroRNA sequence	UGGAAGGGGCAUGCAGAGGAG
	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACctcctc
	Forward	GCCGTGGAAGGGGCATGC

RT, stem-loop RT primer.

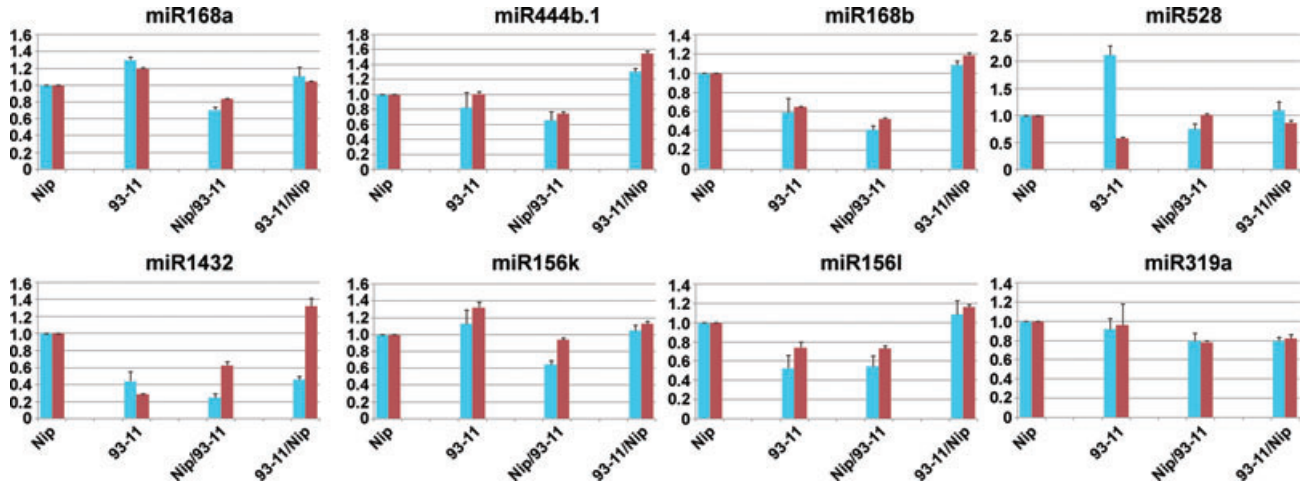

Figure 3. Validation of differentially expressed miRNAs via quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Blue bar, rice miRNA expression level identified by qRT-PCR; Red bar, rice miRNA expression level identified by miRNA microarray. y axis, miRNA relative expression level in four samples.

non-additive results. This discrepancy may be the outcome of different small RNA sample sizes in these two studies. Our study focused on 1141 sequences of miRNAs and highly expressed small RNAs, while He et al. (2010) investigated the small RNAs expression pattern on a genome-wide scale. It is likely that there are other small RNAs in the rice genome that exhibit an additive expression pattern, but these small RNAs might have been excluded from this study because not all small RNAs were under investigation. It is well accepted that non-additive effects are directly related to phenotypes linked to heterosis, and that both dominance and over-dominance can be non-additive with varying degrees of heterosis (Birchler et al. 2003; Hochholdinger and Hoecker 2007). This might indicate that miRNAs and the highly-expressed small RNAs play much more important roles in heterosis than previously thought. This also implies that although heterosis involves many genes, only a small fraction of all genes are involved in establishing it for a given trait.

Previous studies showed that all possible modes of action of protein-coding genes contribute to heterosis in maize (Swanson-Wagner et al. 2006) and rice (Zhang et al. 2008), including high parent, low parent, above high-parent and below low-parent. Here, we found that the same is true for small RNAs. Heterosis is the outcome of complex modes of action of individual genes. Our findings suggest that the presence of multiple modes of action might be a universal phenomenon of heterosis.

Since reciprocal hybrids share the same nuclear genetic background with no distinguishable phenotypic variations in terms of hybrid vigor performance, they may also share similar gene expression and epigenetic modification patterns. However, in another study, it was reported that transcript abundance and histone modifications (H3K4me3, H3K27me3) showed consistent additive/non-additive patterns, while DNA methylation exhibited inconsistent additive/non-additive patterns in reciprocal hybrids (He et al. 2010). In our study, we found 15 miRNAs that exhibited opposite expression patterns relative to mid-parent values in spite of the consistent overall expression patterns in the two hybrids. This discrepancy in variation may indicate cytoplasmic effects in reciprocal hybrids since they share the same nuclear genetic background. Also, this leads us to hypothesize that a large number of small RNAs function as a whole to regulate gene expression, but no single miRNA plays determining roles.

Computational predictions revealed that the targets of miRNAs studied here participate in multiple developmental pathways, including transcription regulatory activity, catalytic activity, and binding or transporter activity. Our investigation revealed that targets of differentially and non-differentially expressed miRNAs showed no obvious differences in their activities. Both groups of miRNA targets were involved in multiple pathways.

Although the research on the molecular mechanisms on heterosis has lasted for more than a century, not much work has been done to dissect the underlying function of small RNAs in heterosis. Here, we have described a microRNA microarray system to detect the expression of annotated rice miRNAs and highly expressed small RNAs in two rice subspecies and their reciprocal hybrids, and suggest that small RNAs might play critical roles in heterosis. The present study may provide more materials and evidence to unravel the molecular mechanisms of heterosis.

Materials and Methods

Plant growth and RNA isolation

Rice cultivars Nipponbare (*Oryza sativa* ssp. *japonica*) and 93-11 (*O. sativa* ssp. *indica*) and their reciprocal F1 hybrids (Nipponbare/93-11 and 93-11/Nipponbare) were used in this study. Seeds were grown in soil under 16:8 h light : dark (LD) conditions at 28°C in a greenhouse. After 4 weeks, seedling shoots at the four-leaf stage were harvested, frozen in liquid nitrogen, and stored at −80°C. Total RNA was extracted from the frozen tissue by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's instructions.

μParaflo™ MicroRNA microarray assay and data processing

A microarray assay was carried out using a service provider (LC Sciences, Texas, USA) with a probe set based on *O. sativa* Sanger version 11.0 and predicted probes. The assay started with 2 to 5 ug total RNA sample, which was size fractionated using a YM-100 Microcon centrifugal filter and the small RNAs (<300 nt) isolated were 3′ –extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining; two different tags were used for the two RNA samples in dual-sample experiments. Hybridization was carried out overnight on a μParaflo™ microfluidic chip using a micro-circulation pump (Atactic Technologies, Texas, USA). On the microfluidic chip, each detection probe consisted of a chemically modified nucleotide coding segment complementary to target microRNA (from miRBase, http://microrna.sanger.ac.uk/sequences/) or other RNA (control or customer defined sequences) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. The detection probes were made by *in situ* synthesis using PGR (photogenerated reagent) chemistry. The hybridization melting temperatures were balanced by chemical modifications of the detection probes. Hybridization used 100 μL 6×SSPE buffer (0.90 M NaCl, 60 mM Na_2HPO_4, 6 mM ethylenediaminetetraacetic acid (EDTA), pH 6.8) containing 25% formamide at 34°C. After hybridization

detection using fluorescence labeling using tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics, Maryland, USA). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression). For two color experiments, the ratio of the two sets of detected signals ($\log_2$ transformed, balanced) and *P*-values of the t-test were calculated; differentially detected signals were those with less than 0.01 *P*-values.

Validation of miRNA expression via qRT-PCR

Validation was carried out as described before (Varkonyi-Gasic et al. 2007). 100–300 ng total RNA was reverse transcribed with Superscript III reverse transcriptase (Invitrogen) using 0.5 μL 10 μM stem-loop RT primer in a 20 μL system. 2 μL of this system was used as a template to perform the real time PCR reaction with TaKaRa SYBR Premix ExTaq (TaKaRa, Shiga, Japan) as the reaction system. All real time PCR analyses were carried out on Eppendorf mastercycler realplex 4 (Hamburg, Germany). GAPDH was used as internal reference. GAPDH forward primer: GCCAGTTTCAACATCATCCC; GAPDH reverse primer: CAGTCAAATCAACGACAGAGACA.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Rice miRNA targets identified by computational prediction or experimental verification.

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