Comparative analysis of miRNA expression in *Oryza sativa* cultivar PTB33 and TN1

Amnat Phetrungnapha[1,*] and Panatda Jannoey[1]

[1]Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand 65000
[*]Corresponding author. E-mail: amnatp@nu.ac.th

ABSTRACT

It has been known that microRNAs (miRNAs) are involved in the regulation for the plant development and defense mechanism by regulating the expression of the target gene. In this study, by adopting next-generation sequencing, we profiled the miRNA expression profile from two rice cultivars, one susceptible (TN1) and another resistant to the brown planthopper (PTB33). Two small RNA libraries from *Oryza sativa* TN1 and PTB33 were constructed and sequenced. Bioinformatic analyses revealed that 66 unique osa-miRNAs were found to be differentially-expressed in PTB33 library, including 41 up-regulated and 25 down-regulated miRNAs. These differentially expressed osa-miRNAs have been reported to be involved in a variety of cellular processed in rice, especially immune response. By using stem-loop qRT-PCR, the expression of the representative miRNAs, including osa-miR393, osa-miR398 and osa-miR7695 was found to be up-regulated, corresponding to the sequencing data. Hence, the PTB33 cultivar-specific miRNAs, which might be involved in BPH disease resistance have been identified. This might be useful for biotechnological approaches for crop protection and development.

Keywords: rice, miRNA, small RNA library, stem-loop qRT-PCR

INTRODUCTION

Rice (*Oryza sativa*) is an important crop that is grown in Asia, Africa, and South and Central America. Over half of the global population consumes rice as the main food source. One of the major factors limiting rice production is the occurrence of diseases caused by microorganisms and insect pest. The most devastating fungal diseases of rice are rice blast and sheath blight caused by *Magnaporthe oryzae* and *Rhizoctonia solani*, respectively (Dean et al. 2005, Zheng et al. 2013). Bacterial diseases of rice are also a major bottleneck towards a sustainable productivity. The most important of these bacterial diseases are bacterial blight and bacterial leaf streak caused by *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively (Lee et al. 2005, Bogdanove et al. 2011). More recently, bacterial foot rot (*Dickeya zeae*, aka

Erwinia chrysanthemi pv. *zeae*) and bacterial panicle blight of rice (*Burkholderia glumae*) have emerged as important pathogens affecting global rice production (Baldrich and San Segundo 2016). Rice yields can be also severely compromised by the pest, especially the brown planthopper (BPH), *Nilaparvata lugens*. BPH damage rice directly through feeding and also by transmitting two viruses, rice ragged stunt virus and rice grassy stunt virus, resulting in "hopper burn symptom" (Xue et al. 2014). Therefore, to archive greater yields in rice production, the strategies used to minimize losses due to these diseases should be developed.

MicroRNAs (miRNAs) are small, endogenous, non-coding RNAs that post-transcriptionally regulate gene expression through translational repression or transcript degradation. miRNAs have been considered as fundamental regulators of cellular processes, such as cell cycle, cell growth, cell death, metabolism, development, and immune response (Jonas and Izaurralde 2015). In recent years, a significant progress has been made in the identification of miRNAs from rice. The first set of rice miRNAs experimentally identified was reported in 2004 (Wang et al. 2004). Later, more miRNAs, either conservative or novel in rice, were identified from rice shoot, root, in-florescence, panicle, calli, developing grains, and immature seeds. The relevance of distinct rice miRNAs in controlling traits of agronomic importance, such as tiller growth, early flowering, panicle, grain production, stress response, and plant immune response, is well demonstrated (Li et al. 2014, Zheng and Qu 2015). For instance, by exposing rice seedlings to drought or salt stress, Sunkar et al. identified 23 new miRNAs and 40 candidates (Sunkar et al. 2007). By analyzing 62 small RNA libraries that represent several tissues from control plants and those subjected to different environmental stress treatments, Jeong et al. reevaluated approximately 400 annotated miRNAs and found 76 new miRNAs that are responsive to water stress, nutrient stress, or temperature stress (Jeong et al. 2011). Li et al. identified seven miRNA families that are differentially responsive to oxidative stress and discovered 32 new rice miRNAs. In addition, miRNAs which are involved in rice immunity against the blast fungus, *Magnaporthe oryzae* have been identified (Li et al. 2014).

In this study, we are interested to identify the miRNAs related to disease resistance in rice, specifically BPH resistance. By comparing the miRNA expression profile of *Oryza sativa* cultivar PTB33 (BPH-resistance cultivar), with *O. sativa* cultivar TN1 (susceptible cultivar), the cultivar-specific miRNAs which might be involved in disease resistance have been identified.

METHODOLOGY

Plant materials and RNA isolation

Rice seeds were obtained from Phitsanulok Rice Research Center, Thailand. *Oryza sativa* cultivar Taichung Native 1 (TN1) and PTB33 were used as susceptible and resistant reference cultivars, respectively. Rice seedlings were prepared according to Jannoey et al. (2015). Briefly, rice seeds were soaked and grown in a seed tray containing soil from rice paddy fields for 2 weeks. Ten seedlings per row were separately grown for each cultivar. Total RNA extraction was done by using Direct-zol™ RNA MiniPrep (Zymo Research). Briefly, frozen rice seedlings were grinded in liquid nitrogen and 600 µl of RiboZol™ RNA Extraction Reagents (Amresco) was added. Then, the columns were washed with RNA wash buffer. Total RNA was eluted by DNase/RNase-Free water and stored at -80°C until used.

Small RNA library construction for high-throughput sequencing

Small RNA libraries prepared from TN1 and PTB33 rice seedlings were constructed, amplified and sequenced. Each library represented a pool of 5 rice seedlings. Briefly, total RNA was size-fractionated on a 15% tris-borate-EDTA (TBE) urea polyacrylamide gel to enrich for molecules of 15–30 nt. After 5′adapter and 3′adapter were ligated to small RNAs, RT-PCR was performed. Then PCR products were subsequently purified and used for clustering and sequencing by HiSeq4000 Illumina Genome Analyzer (Illumina Inc, CA, USA). The small RNA library construction and sequencing was performed by Beijing Genomics Institute, Shenzhen.

Bioinformatics analysis of sequencing data

For deep-sequencing reads produced by Illumina Genome Analyzer, low quality reads were filtered out to exclude those most likely to represent sequencing errors and 3'/5' adaptor sequence were subsequently trimmed into clean full length reads formatted into a non-redundant Fasta format. The occurrences of each unique sequence reads were counted as sequence tags (the number of reads for each tag reflects relative expression level) and only small RNA sequences of 18 to 30 nt were retained for further analysis. Next, the clean reads were perfectly mapped to BGI 9311 genome using the SOAP program. To identify known miRNA sequences, clean reads were mapped to *Oryza sativa* miRNA sequences in miRBase version 21.0 (http://www.mirbase.org). Sequences were also mapped to rRNA, snRNA, snoRNA and tRNA from GenBank (http://www.ncbi.nlm.nih.gov/genbank) and Sanger Rfam version 10.1 (ftp://ftp.sanger.ac.uk/pub/databases/Rfam), repeat-associated sequences in

Repbase RepeatMasker libraries (http://www.repeatmasker.org). To investigate the differential expression patterns of known miRNAs between TN1 and PTB33, the read count of each identified miRNA was normalized to transcripts per million (TPM) (Aanes, Winata et al. 2014) using the following formula: normalized expression=actual miRNA count/total count of clean reads×1000000. To minimize noise and improve accuracy, we only selected miRNAs with TPM ≥ 1 in at least one of the sample pair to be analyzed. The criteria to consider a miRNA to be cultivar-specific are $|\log_2$ fold change$| \geq 2$ and p-value ≤ 0.05.

Stem loop quantitative real-time PCR

cDNA synthesis was carried out using an SuperScript III reverse transcriptase (Invitrogen). Primers for stem-loop RT-PCR and gene-specific real-time PCR primers for miRNAs were designed according to Chen et al. (2005). Quantitative real-time PCR (qRT-PCR) was conducted with Exicycler™ 96 Real-Time PCR System (Bioneer) and Thunderbird® SYBR® qPCR Master Mix (Toyobo), according to the manufacture's protocols. All qRT-PCR experiments have been done in three biological and three technical replicates. Rice 18S rRNA was used as an internal control (forward primer 5′-ATAACTCGACGGATCGCAAG-3′; reverse primer 5′-CTTGGATGTGGTAGCCGTTT-3′). The differential expression of miRNAs was calculated using Ct and the $2^{-\Delta\Delta ct}$ formula (Schmittgen and Livak 2008). Finally, student t-test was performed for comparison between normal and treatments. A value of $p< 0.05$ was considered significant.

RESULTS AND DISCUSSION

Overview of deep sequencing datasets

In the present study, we carried out high-throughput sequencing of small RNA libraries in order to identify the cultivar-specific miRNAs in rice. *Oryza sativa* Taichung Native 1 (TN1) and PTB33 were used as susceptible and resistant reference cultivars, respectively. A small RNA library was constructed from each of the reference cultivars and sequenced by Illumina high-throughput sequencing technology. In total, 7,849,477 and 7,186,014 sequences were obtained from PTB33 and TN1, respectively, by eliminating the adaptor and low-quality tags. These reads represented 1,199,961 and 1,165,931 unique small RNA sequences, respectively, in the two libraries. The sequences from PTB33 and TN1 libraries were aligned to the rice genome BGI 9311 using SOAP2 program, and 96.5% and 97.3% of the total sRNA sequences, representing 98.1% and 97.6% of unique small RNAs, respectively, were found to match the

genome perfectly. Next, the fragments of repeat regions, tRNAs, rRNAs, protein coding regions, and snoRNAs were analyzed and removed those reads that mapped perfectly to the genome. Mapping distribution of the clean reads in PTB33 and TN1 small RNA libraries are shown in Table 1. All the sequences excluding noncoding RNAs, repeat and protein coding regions were then regarded as miRNAs for further analysis. The length of these small RNAs ranged from 20 nt to 24 nt. In both libraries, the 24 nt category was the most abundant small RNA followed by 22 nt and 21 nt (Fig.1). These were consistent with the typical lengths of plant mature small RNAs reported in other studies (Wang et al. 2004, Jeong et al. 2011, Li et al. 2014).

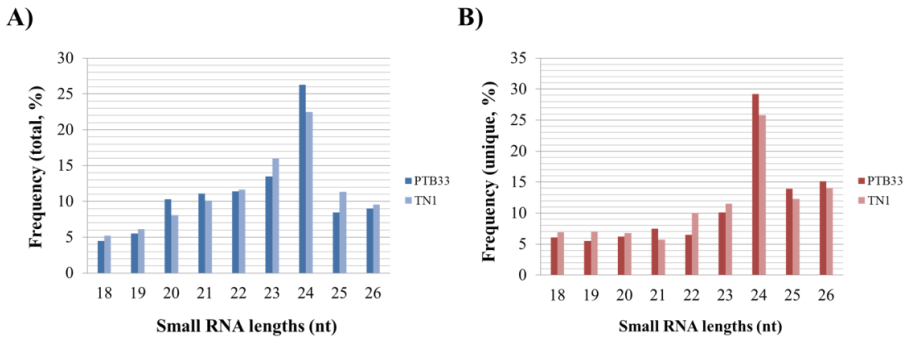

Fig.1. Length distribution of the total (A) and unique small RNA (B) in the PTB33 and TN1 libraries

Differential expression of known miRNAs

To annotate Oryza sativa miRNAs (osa-miRNAs), the reads of each library were mapped to the precursor and mature sequences from the rice miRNA database available in miRBase (release 21). As a result, a total of 1,342,395 reads and 1,422,831 from PTB33 and TN1 library were mapped to the osa-miRNAs, representing 3,284 and 2,332 unique osa-miRNAs, respectively (Table 1). The reads which could not be mapped to the rice miRNA database were considered as unannotated. The presence of the novel osa-miRNAs in PTB33 and TN1 libraries remains to be elucidated.

Table 1. Mapping distribution of the clean reads in small RNA libraries.

Category	PTB33		TN1	
	Unique	Reads	Unique	Reads
clean reads	1,199,961	7,849,477	1,165,931	7,186,014
9311 rice genome	1,177,162 (98.1%)	7,574,746 (96.5%)	1,137,948 (97.6%)	6,991,992 (97.3%)
exon_antisense	14,436 (1.2%)	47,102 (0.6%)	13,991 (1.2%)	74,016 (1%)
exon_sense	100,799 (8.4%)	204,107 (2.6%)	47,803 (4.1%)	129,348 (1.8%)
intron_antisense	15,612 (1.3%)	39,251 (0.5%)	15,157 (1.3%)	64,674 (0.9%)
intron_sense	24,372 (2%)	62,802 (0.8%)	19,821 (1.7%)	86,232 (1.2%)
miRNA	474 (0.04%)	342,395 (4.36%)	408 (0.0035%)	422,831 (5.87%)
rRNA	98,411 (8.2%)	3,703,754 (47.2%)	85,613 (7.3%)	3,364,199 (46.82%)
repeat	336,397 (28%)	761,475 (9.7%)	290,317 (24.9%)	876,694 (12.2%)
snRNA	1,459 (0.1%)	7,850 (0.1%)	700 (0.06%)	7,186 (0.1%)
snoRNA	1,343 (0.1%)	4,710 (0.1%)	1,632 (0.14%)	8,623 (0.1%)
tRNA	14,652 (1.2%)	573,069 (7.3%)	10,493 (0.9%)	657,520 (9.2%)
unannotated	589,196 (49.1%)	1,102,962 (14.1%)	678,572 (58.2%)	1,494,691 (20.8%)

PTB33 has been considered as a brown planthopper (BPH) resistant cultivar because it contains three BPH-resistant genes, including BPH 2, BPH 3 and BPH 9 (Jena and Kim 2010), whereas TN1 was considered as a susceptible cultivar. In this study, we were interested in comparison of miRNA expression in rice cultivar PTB33 and TN1. To this end, known miRNA expression profiles from the PTB33 library was compared with the TN1 library. Differential expression analysis revealed that 66 unique osa-miRNAs were found to be differentially-expressed in PTB33 library, including 41 up-regulated and 25 down-regulated miRNAs. These differentially expressed osa-miRNAs have been reported to be involved in a variety of cellular processed in rice. Osa-miR160a and osa-miR398b were highly expressed in PTB33 when compared with TN1 library. Li et al demonstrated that these two miRNAs play a crucial

role in blast disease resistance. Overexpression of miR160a or miR398b in transgenic rice results in increased H_2O_2 accumulation at the infection site and up-regulation of defence gene expression (i.e. PR1 and PR10) and enhanced resistance to the rice blast fungus, *Magnaporthe oryzae* (Li et al. 2014). Osa-miR7695 was also found to be highly expressed in the PTB33 library. It was up-regulated for 9.72 fold, compared with the TN1 library. Osa-miR7695 regulates the accumulation of an alternative spliced variant of the *Nramp* (Natural resistance-associated macrophage protein 6) gene which is involved in homeostasis of metal ions (Campo et al. 2013). Overexpression of miR7695 has been shown to confer resistance to infection by *M. oryzae* (Campo et al. 2013). In addition to osa-miRNAs mentioned above, the homologs of pathogen-responsive miRNAs in plants, including miR393, miR398, miR160a, and miR773 were found to be up-regulated in the PTB33, compared with the TN1 library. miR393 plays several roles in *Arabidopsis* defence reactions through regulation of the auxin pathway and modulation of PR1 exocytosis (Windels et al. 2014). MiR398 controls ROS production by targeting two Cu/Zn superoxide dismutase genes (CSD1 and CSD2) and a copper chaperone for superoxide dismutase, thus protecting plants against oxidative stress associated to pathogen infection (Khraiwesh et al. 2012). It is also known that miR160a functions as a positive regulator of PAMP-induced callose deposition, whereas miR398 and miR773 negatively regulate PAMP-induced callose deposition and hence disease resistance to *Pseudomonas syringae* (Harvey et al. 2011, Baldrich et al. 2015). Apart from immune-responsive miRNAs, we also identified some miRNAs which have been reported to be involved in plant development. The expression level of miR397 in PTB33 library was 5.87-fold higher than the one that found in TN1 library. Zhang et al reported that overexpression of miR397 improve rice yield by increasing grain size and promoting panicle branching (Mallory and Vaucheret 2010). In addition, we also found the miRNA which down-regulated in PTB33 library, compared with the TN1 library. For example, miR1425, miR166a, miR1863, and miR1870, were found to be involved in drought signaling in rice root (Bakhshi et al. 2016). Table 2 and 3 shows the top 10 miRNAs which up-regulated and down-regulated in PTB33 compared with TN1 library.

We selected three representative miRNAs, including osa-miR393, osa-miR398, and osa-miR7695 which were up-regulated in PTB33 library, compared with TN library for validating their expression (Fig.2.). By using stem-loop qRT-PCR, we found that these three selected miRNAs were up-regulated in the PTB33 library. The expression levels and patterns in the stem-loop qRT-PCR results correlated almost exactly with those from deep

Table 2 The top 10 up-regualted miRNAs in PTB33compared with TN1 library.

miRNAs	Sequence (5'→3')	Fold change
osa-miR398b	uguguucucaggucgccccug	12.50
osa-miR160a	ugccuggcucccuguaugcca	10.61
osa-miR7695	ugccuauguggcacgccacgugaa	9.72
osa-miR393	uccaaagggaucgcauugauc	9.01
osa-miR773	uugcuuccagcuuuugucuc	7.49
osa-miR397	ucauugagugcagcguugaug	5.87
osa-miR172b	ggaaucuugaugaugcugcau	5.49
osa-miR408-5p	cagggaugaggcagagcaugg	4.92
osa-miR827	uuagaugaccaucagcaaaca	4.87
osa-miR156a	ugacagaagagagugagcac	4.71

Table 3 The top 10 down-regulated miRNAs in PTB33 compared with TN1 library.

miRNAs	Sequence (5'→3')	Fold change
osa-miR1425	uaggauucaauccuugcugcu	-13.81
osa-miR166a	ucggaccaggcuucauuccc	-13.67
osa-miR1863	agcucugauaccauguuagauua	-13.11
osa-miR1870	ugcugaauuagaccuaguggcau	-12.04
osa-miR164d	uggagaagcagggcacgugcu	-11.78
osa-miR172a	agaaucuugaugaugcugcau	-10.14
osa-miR2867	ccaggacgugugggauggca	-9.92
osa-miR444d	gcaugaggcaacaacugcauu	-9.14
osa-miR1317	gaaaugaucuuggacgua	-8.46
osa-miR319a	acuggaugacgcgggagcuaa	-7.91

sequencing, confirming that the sequencing results are valid. To explore the role of differentially-expressed miRNAs in PTB33 library, the ratget mRNA will be further identified.

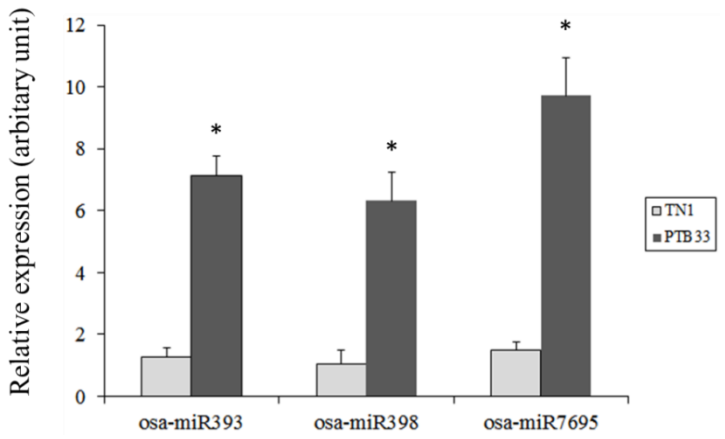

Fig.2. Validation of miRNA expression using stem-loop qRT-PCR. Three representative miRNAs, including osa-miR393, osa-miR398 and osa-miR7695 were chosen for qRT-PCR. Relative expression ratio was calculated by $2^{-\Delta\Delta ct}$ formula. The asterisks represent significant difference ($p < 0.05$).

CONCLUSION

In conclusion, by using differential expression analysis, the PTB33 cultivar-specific miRNAs which might be involved in BPH disease resistance have been identified. Further identification of the mRNA target will clarified the roles of these miRNAs in rice. This might be useful for biotechnological approaches for crop protection and development.

ACKNOWLEDGEMENT

This project is supported by National Research Council of Thailand (Grant Number: R2559B080). We greatly appreciate technical assistance of Ms.Thaneeya Nanthapodj (Mahidol University) for miRNA expression analysis.

REFERENCES

Aanes, H., C. Winata, L. F. Moen, O. Ostrup, S. Mathavan, P. Collas, T. Rognes and P. Alestrom (2014). *Normalization of RNA-sequencing data from samples with varying mRNA levels*. PLoS One 9(2): e89158.

Bakhshi, B., E. M. Fard, N. Nikpay, M. A. Ebrahimi, M. R. Bihamta, M. Mardi and G. H. Salekdeh (2016). *MicroRNA Signatures of Drought Signaling in Rice Root*. Plos One 11(6).

Baldrich, P., S. Campo, M. T. Wu, T. T. Liu, Y. L. Hsing and B. San Segundo (2015). *MicroRNA-mediated regulation of gene expression in the response of rice plants to fungal elicitors*. Rna Biology 12(8): 847-863.

Baldrich, P. and B. San Segundo (2016). *MicroRNAs in Rice Innate Immunity*. Rice (N Y) 9(1): 6.

Bogdanove, A. J., R. Koebnik, H. Lu, A. Furutani, S. V. Angiuoli, P. B. Patil, M. A. Van Sluys, R. P. Ryan, D. F. Meyer, S. W. Han, G. Aparna, M. Rajaram, A. L. Delcher, A. M. Phillippy, D. Puiu, M. C. Schatz, M. Shumway, D. D. Sommer, C. Trapnell, F. Benahmed, G. Dimitrov, R. Madupu, D. Radune, S. Sullivan, G. Jha, H. Ishihara, S. W. Lee, A. Pandey, V. Sharma, M. Sriariyanun, B. Szurek, C. M. Vera-Cruz, K. S. Dorman, P. C. Ronald, V. Verdier, J. M. Dow, R. V. Sonti, S. Tsuge, V. P. Brendel, P. D. Rabinowicz, J. E. Leach, F. F. White and S. L. Salzberg (2011). *Two new complete genome sequences offer insight into host and tissue specificity of plant pathogenic Xanthomonas spp*. J Bacteriol 193(19): 5450-5464.

Campo, S., C. Peris-Peris, C. Sire, A. B. Moreno, L. Donaire, M. Zytnicki, C. Notredame, C. Llave and B. San Segundo (2013). *Identification of a novel microRNA (miRNA) from rice that targets an alternatively spliced transcript of the Nramp6 (Natural resistance-associated macrophage protein 6) gene involved in pathogen resistance.* New Phytologist 199(1): 212-227.

Chen, C., D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Andersen, K. Q. Lao, K. J. Livak and K. J. Guegler (2005). *Real-time quantification of microRNAs by stem-loop RT-PCR.* Nucleic Acids Res 33(20): e179.

Dean, R. A., N. J. Talbot, D. J. Ebbole, M. L. Farman, T. K. Mitchell, M. J. Orbach, M. Thon, R. Kulkarni, J. R. Xu, H. Pan, N. D. Read, Y. H. Lee, I. Carbone, D. Brown, Y. Y. Oh, N. Donofrio, J. S. Jeong, D. M. Soanes, S. Djonovic, E. Kolomiets, C. Rehmeyer, W. Li, M. Harding, S. Kim, M. H. Lebrun, H. Bohnert, S. Coughlan, J. Butler, S. Calvo, L. J. Ma, R. Nicol, S. Purcell, C. Nusbaum, J. E. Galagan and B. W. Birren (2005). *The genome sequence of the rice blast fungus Magnaporthe grisea*. Nature 434(7036): 980-986.

Harvey, J. J. W., M. G. Lewsey, K. Patel, J. Westwood, S. Heimstadt, J. P. Carr and D. C. Baulcombe (2011). *An Antiviral Defense Role of AGO2 in Plants*. Plos One 6(1).

Jannoey, P., Pongprasert, W., Lumyong, S., Roytrakul, S. and Nomura, M. (2015). *Comparative proteomic analysis of two rice cultivars (Oryza sativa L.) contrasting in Brown Planthopper (BPH) stress resistance.* 8(2): 96-105.

Jena, K. K. and S. M. Kim (2010). *Current Status of Brown Planthopper (BPH) Resistance and Genetics.* Rice 3(2-3): 161-171.

Jeong, D. H., S. Park, J. Zhai, S. G. Gurazada, E. De Paoli, B. C. Meyers and P. J. Green (2011). *Massive analysis of rice small RNAs: mechanistic implications of regulated microRNAs and variants for differential target RNA cleavage*. Plant Cell 23(12): 4185-4207.

Jonas, S. and E. Izaurralde (2015). *Towards a molecular understanding of microRNA-mediated gene silencing*. Nat Rev Genet 16(7): 421-433.

Khraiwesh, B., J. K. Zhu and J. H. Zhu (2012). *Role of miRNAs and siRNAs in biotic and abiotic stress responses of plants.* Biochimica Et Biophysica Acta-Gene Regulatory Mechanisms 1819(2): 137-148.

Lee, B. M., Y. J. Park, D. S. Park, H. W. Kang, J. G. Kim, E. S. Song, I. C. Park, U. H. Yoon, J. H. Hahn, B. S. Koo, G. B. Lee, H. Kim, H. S. Park, K. O. Yoon, J. H. Kim, C. H. Jung, N. H. Koh, J. S. Seo and S. J. Go (2005). *The genome sequence of Xanthomonas oryzae pathovar oryzae KACC10331, the bacterial blight pathogen of rice*. Nucleic Acids Res 33(2): 577-586.

Li, Y., Y. G. Lu, Y. Shi, L. Wu, Y. J. Xu, F. Huang, X. Y. Guo, Y. Zhang, J. Fan, J. Q. Zhao, H. Y. Zhang, P. Z. Xu, J. M. Zhou, X. J. Wu, P. R. Wang and W. M. Wang (2014). *Multiple rice microRNAs are involved in immunity against the blast fungus Magnaporthe oryzae*. Plant Physiol 164(2): 1077-1092.

Mallory, A. and H. Vaucheret (2010). *Form, Function, and Regulation of ARGONAUTE Proteins*. Plant Cell 22(12): 3879-3889.

Schmittgen, T. D. and K. J. Livak (2008). *Analyzing real-time PCR data by the comparative C(T) method.* Nat Protoc 3(6): 1101-1108.

Sunkar, R., V. Chinnusamy, J. Zhu and J. K. Zhu (2007). *Small RNAs as big players in plant abiotic stress responses and nutrient deprivation*. Trends Plant Sci 12(7): 301-309.

Wang, J. F., H. Zhou, Y. Q. Chen, Q. J. Luo and L. H. Qu (2004). *Identification of 20 microRNAs from Oryza sativa.* Nucleic Acids Res 32(5): 1688-1695.

Windels, D., D. Bielewicz, M. Ebneter, A. Jarmolowski, Z. Szweykowska-Kulinska and F. Vazquez (2014). *miR393 Is Required for Production of Proper Auxin Signalling Outputs*. Plos One 9(4).

Xue, J., X. Zhou, C. X. Zhang, L. L. Yu, H. W. Fan, Z. Wang, H. J. Xu, Y. Xi, Z. R. Zhu, W. W. Zhou, P. L. Pan, B. L. Li, J. K. Colbourne, H. Noda, Y. Suetsugu, T. Kobayashi, Y. Zheng, S. Liu, R. Zhang, Y. Liu, Y. D. Luo, D. M. Fang, Y. Chen, D. L. Zhan, X. D. Lv, Y. Cai, Z. B. Wang, H. J. Huang, R. L. Cheng, X. C. Zhang, Y. H. Lou, B. Yu, J. C. Zhuo, Y. X. Ye, W. Q. Zhang, Z. C. Shen, H. M. Yang, J. Wang, J. Wang, Y. Y. Bao and J. A. Cheng (2014). *Genomes of the rice pest brown planthopper and its endosymbionts reveal complex complementary contributions for host adaptation*. Genome Biol 15(12): 521.

Zheng, A., R. Lin, D. Zhang, P. Qin, L. Xu, P. Ai, L. Ding, Y. Wang, Y. Chen, Y. Liu, Z. Sun, H. Feng, X. Liang, R. Fu, C. Tang, Q. Li, J. Zhang, Z. Xie, Q. Deng, S. Li, S. Wang, J. Zhu, L. Wang, H. Liu and P. Li (2013). *The evolution and pathogenic mechanisms of the rice sheath blight pathogen*. Nat Commun 4: 1424.

Zheng, L. L. and L. H. Qu (2015). *Application of microRNA gene resources in the improvement of agronomic traits in rice.* Plant Biotechnol J 13(3): 329-336.