**Discussion**

Single nucleotide polymorphisms are good indicators of evolutionary selection for different genomic regions and have already been employed to study natural selections on human miRNAs [28, 29]. In these studies, SNPs on functional regions such as pre-miRNAs especially seed-regions as well as miRNA binding site were very rare and much less than other conserved sequence motifs in 3’ UTR [28, 29]. And similar phenomena were also observed in this study that we found SNP density in pre-miRNAs were fewer than that in intergenic regions as well as exons, implying miRNAs have been subjected to stricter evolutionary pressure than intergenic regions and exons. This is consistent with the role of miRNAs as master regulators in plants. There are big differences between conserved miRNAs and non-conserved miRNAs, the so-called rice specific miRNAs or newly arising miRNAs. Generally, conserved miRNAs are conserved across different species and have identifiable binding sites on targets which are also conserved among species and they tend to target genes encoding transcriptional factors (TFs), while non-conserved miRNAs are commonly taken as new-comers with few if any identifiable binding sites in targets both by bioinformatics and degradome sequencing [20]. Our comparative analysis of SNPs fallen unto conserved miRNAs and non-conserved ones had deduced similar results, SNP density of conserved miRNA precursors was significantly less than that of non-conserved ones, showing the more important regulation role of conserved miRNAs would impose greater selection pressure on non-conserved miRNAs. Previous study conducted by Liu Q et al. found when less conserved miRNAs were excluded, pre-miRNAs accumulated much fewer SNPs, and this also confirmed the stricter purifying pressure on conserved miRNAs. Considering the different evolutionary processes of conserved and non-conserved miRNAs [30, 31] and the fact that by the common target prediction methods few identifiable targets are found for non-conserved miRNAs, the functioning mechanisms of miRNA towards miRNA binding sites of targets may be different. In our study, the comparison of positional SNP frequency distributions between conserved mature miRNAs and non-conserved mature miRNAs showed distinct rankings of SNP frequencies along the positions, which indicated different selection pressure distribution among the positions for conserved and non-conserved miRNAs. It was reported that there was coevolution of miRNAs and their cognate target genes [37, 55], and here in our study, correlation tests of positional SNP frequencies of conserved miRNAs and that of cognate targets showed moderate positive correlation with statistical significance, which provided molecular evidence for the coevolution of miRNAs and their cognate targets.

In plants, miRNAs serve as master regulator through high complementarity towards binding site of targets and previous researches revealed the different importance of different positions on target recognition and cleavage [32, 33, 34]. 5’ terminal nucleotide, which is position 1 on mature miRNA, determines which Argonaut protein to load for miRNA [35], and this layer of constraint on position 1 was reflected in this study as the lowest SNP frequency position both for conserved and non-conserved miRNAs. But unexpectedly, position 10 and 11, conventionally regarded as cleavage sites and were required to be perfect pairing to binding sites [37-39], were not even among the lowest SNP frequency positions (Fig. 5). And this implied selection constraints imposed other sites with lower SNP frequencies are stronger than the cleavage site constraint imposed on these two positions. Liu Q et al. had similar finding for position 1 and 10, except that position 11 was reported to have the lowest SNP frequency and the conflict was caused by the separate treating of conserved and non-conserved miRNAs. The separate treating of conserved miRNAs and non-conserved miRNAs would specify the general trend for the two class of miRNAs which have been reported to have different functioning mechanisms.

In order to search for biologically relevant target genes for miRNAs, generally ways such as 5’-RACE or degradome sequencing would be adopted. But for the abundant outcome of bioinformatic miRNA target prediction programs, expression correlation of miRNA and mRNA of cognate target genes was thought to be feasible way to search for those biologically relevant ones. In this study, degradome validated miRNA:target pairs were found not to be fully negatively correlated and in contrast, more interaction pairs were positively correlated than negative correlated pairs. Ming Wen et al. had found similar phenomenon, in whose study positively correlated interaction pairs prevailed [56]. And this may be caused by more complex mechanisms such as negative feedback loops (FBLs) and incoherent feedforward loops (FFLs) mentioned by Ming Wen et al. Also, the results showed that it’s not practical to use expression correlation for target screening.

No studies before had tried to adopt haplotype analysis to study the actual mutations caused by SNPs of miRNA-mediated regulations that happen to rice cultivars. In this study, haplotype analysis was extended to be combined complementarity pattern analysis (CCPA) and could help to study the polymorphisms of interactions between a family of miRNAs and their common target gene among different rice cultivars. And the afterward found complementarity recovery phenomenon of osa-miR818 family implied that less conserved miRNAs subject to low selection pressure and allow more flexible changes to the miRNA:target complementarity patterns.

Several studies showed the SNPs involved in miRNA-mediated regulation would cause apparent changes to plant phenotypes [21, 22]. While here in this study, genome-wide analysis of SNPs involved in miRNA-mediated regulations found 7 target genes carrying SNPs on their miRNA binding sites and two of them are promising in bringing big effects to miRNA’s regulation. One of them was located in cleavage site position 10 and the other brought up the free energy of binding by 6.575 kcal/mol. But unexpectedly, the comparison of relevant phenotypes of the SNP mutated rice cultivars and those of the reference pattern rice cultivars didn’t show distinct changes. And this implied that these plant phenotypes are controlled by more than one gene so that predicted changes in a single gene expression would not be reflected in the phenotype changes.

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