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Identification of novel miRNA--target pairs in rice (*Oryza sativa*) by a reversed approach

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Abstract

MicroRNAs (miRNAs) are crucial regulators of gene expression in plants. Here, we performed a comprehensive computational search for novel miRNA--target pairs in rice by a reversed approach. First, the potential cleavage sites in all the 67,392 cDNAs of rice were searched by degradome sequencing datasets. Then, the 'bait' sequences, which may be acted as the miRNAs binding sites in the targets, were generated by adding 10 nt sequences both upstream and downstream of the cleavage sites. Finally, the potential miRNA candidates were extracted by BLAST of these 'bait' sequences against the AGO1-associated sRNA sequencing data and were further validated considering the structure and expression characteristics of miRNA. As a result, 144 previously validated miRNA--target pairs and 7 novel miRNA candidates together with 15 mRNA targets were identified. According to the literature, some of these 15 corresponding targets play very important role in the stress signaling pathways in rice. Other interesting findings were the non-canonical slicing sites and higher accumulation isomiRs were identified in some of the previously validated miRNA--target pairs.

Keywords: microRNA--target pair, reversed framework, Argonaute 1 (AGO1), degradome, rice (*Oryza sativa*)
Abbreviations: miRNA_microRNA; sRNAs_small RNAs; siRNAs_small interfering RNAs; pri-miRNA_primary microRNA; pre-miRNA_precursor microRNA; HTS_high-throughput sequencing; U_uridine; DCL_Dicer-like; AGO_Argonaute; RISC_RNA-induced silencing complex; 5' RACE_ 5' rapid amplification of cDNA ends; RPM_reads per million; GEO_Gene Expression Omnibus; TIGR_the Institute for Genome Research; Cd_Cadmium; Al_aluminum.

Introduction

MicroRNAs (miRNAs) are ~21-nucleotide (nt) non-coding RNAs that regulate gene expression in most eukaryotes (Carthew and Sontheimer, 2009; Chen, 2009; Jones-Rhoades et al., 2006). From primary miRNA (pri-miRNA) to precursor miRNA (pre-miRNA), then to the miRNA/miRNA* duplex, Dicer-like 1 (DCL1)-mediated two-step cleavages in plants (Voinnet, 2009), or Drosha for the first step and Dicer for the second step in animals (Carthew and Sontheimer, 2009), are required. The mature miRNAs are then loaded into specific ARGONAUTE (AGO, normally AGO1 in plants)-associated RNA-induced silencing complexes (RISCs) to exert repressive regulatory roles through recognition of complementary binding sites on the target transcripts (Voinnet, 2009). The miRNAs bind to the target transcripts harboring highly complementary recognition sites, which serves a general feature of miRNA-guided cleavage actions in plants (Jones-Rhoades et al., 2006). Degradome sequencing, an innovative high-throughput method by combining the modified 5' rapid amplification of cDNA ends (5' RACE) with the next-generation sequencing technology, was developed for transcriptome-wide mapping of target cleavage sites in both plants and animals (Addo-Quaye et al., 2008; German et al, 2008; Gregory et al., 2008; Henderson et al., 2008; Shin et al., 2010; Thomson et al., 2011). These degradome sequencing data could be utilized to create target plots (t-plots) to extract reliable miRNA--target pairs from the prediction results (German et al., 2008), and hundreds of novel regulatory relationships between miRNAs and the targets have been unveiled by this approach (Addo-Quaye et al., 2009; Branscheid et al., 2011; Devers et al., 2011; German et al., 2009; Meng et al., 2010; Meng et al., 2011a; Meng et al., 2011b; Pantaleo et al., Wei et al., 2010;2010; Wu et al., 2009). By considering the spatio-temporal interactions between the miRNAs and the targets, it can't be expected to identify all the miRNA--target regulatory relationships in a few studies with limited tissues, developmental stages or treatments. Additionally, the weakly expressed miRNAs with their targets are more likely to be ignored when compared with those highly expressed miRNAs. So, we speculate that there are still a lot of novel miRNA--target pairs remaining to be identified. This hypothesis is supported by the increasing number of the miRNA registries in miRBase (Griffiths-Jones et al., 2008). Taking advantage of the high complementarity between the miRNAs and their targets, and the AGO1-enrichment of most plant miRNAs, we previously developed a reversed workflow for the identification of miRNA--target pairs (Shao et al., 2013). Using this method, the cleavage signal detected by degradome sequencing data on an mRNA could be used as the bait to extract the corresponding regulatory miRNA from AGO1-enriched sRNAs (Shao et al., 2013). In this study, by using the degradome sequencing data, we did a systemic search for the cleavage signals on all of the annotated rice transcripts. Then, the miRNA--target regulatory relationships were extracted by using the reversed approach introduced above. As a result, 144 previously validated miRNA--target pairs and

seven novel miRNA candidates together with 15 targets were identified.

Results and discussion

Non-canonical slicing sites and higher accumulation isomiRs were identified in some of the previously validated miRNA--target pairs by reversed framework

Four degradome sequencing datasets retrieved from GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/) were used to perform a large-scale identification of the specific cleavage signals on a total of 67,392 cDNAs in rice (TIGR, Release 7.0) (Yuan et al., 2003). The potential sRNA regulators responsible for these cleavage sites were extracted from three AGO1-associated sRNA sequencing datasets (see Table S1 for the details of the datasets) by using the reversed framework. As a result, 144 previously validated miRNA--target pairs were identified (Table S2). The canonical slicing site within a target binding site was usually mapped to 10^{th} to 11^{th} nt of the regulatory miRNA (Elbashir et al., 2001; Llave et al., 2002). But, here we found that 14 out of 144 previously validated miRNA--target pairs were not in this case (Table S2). The slicing sites of four pairs were mapped to 11^{th} to 12^{th} nt (such as osa-miR162a--LOC_OS03G02970.1), two pairs were mapped to 9^{th} to 10^{th} nt (such as osa-miR1425-5p--LOC_OS10G35640.1). The remaining eight pairs have two cleavage sites (one slicing site mapped to 10^{th} to 11^{th} nt and another site mapped to 9^{th} to 10^{th} nt or 11^{th} to 12^{th} nt; such as osa-miR408-3p--LOC_OS08G37670.1). Interestingly, some isomiRs rather than their miRNAs were identified by reversed framework because of their relatively abundant accumulation levels (Table S2, Table S3). For example, the accumulation level of iso-osa-miR528-5p (UGGAAGGGGCA-UGCAGAGGA) in AGO1 complexes was much higher than osa-miR528-5p (UGGAAGGGGCAUGCAGAGGAG) [1315 reads per million (RPM) vs. 165.72 RPM] (Fig 1). Thus, why were the lowly accumulated miRNA registered in miRBase? A plausible explanation is that the levels of certain miRNAs along with their isomiRs are variable in different conditions.

Identification of novel miRNA candidates and their targets by reversed framework

Also by using the reversed framework, seven novel miRNA candidates along with 15 targets were uncovered (Table 1). The sequence characteristics including 5' terminal compositions and sequence length distribution, both of which have a great impact on the sorting of sRNA into specific AGO proteins (Mi et al., 2008), of these miRNA candidates were analyzed. The results showed that they were predominantly 21 nt in length (6 out of 7) and started with 5' U (6 out of 7), which were similar to the AGO1-enriched miRNAs in plants (Mi et al., 2008). When we mapped the seven miRNA candidates to the rice genome to obtain the precursors (see details in Materials and Methods), we found that one of them could be mapped to the 3' arm of osa-MIR5788 registered in miRBase (Figs S1). In this regard, we designated it as osa-miR5788-3p. Secondary structure prediction showed that the pre-miRNAs of the remaining six miRNA candidates could form canonical "stem-loop" structures (Fig 2 and Figs S1). More supportively, we found that the miRNA candidates and the miRNA* candidates co-existed in the AGO1-associated sRNA sequencing data sets and nearly all of these miRNA/miRNA* candidate duplexes possess 2 nt 3'-overhangs in the stem-loop-structured precursors (Fig 2, Figs S1 and Figs S2), which were considered to be catalytic cleavage feature of the DCLs (Jones-Rhoades et al., 2006; Voinnet, 2009). These structure characteristics, as well as the sequence characteristics of these sRNA regulators indicated that they are potential miRNA candidates. For miRNA candidate 1, more than one precursor was found (Data S1). Thus, we could not tell from which precursors the sRNA was originated. Interestingly, two potential precursors of this miRNA candidate locate on the opposite strands of the highly overlapping regions on rice chromosome 1. In plant, small interfering RNAs (siRNAs) and miRNAs are the two major classes of endogenous sRNAs. The two sRNA classes have different biogenesis pathways. siRNAs are generated from double-stranded RNAs that are synthesized by RNA-dependent RNA polymerase (RDR) (Xie et al., 2004). These long double-stranded precursors are processed into siRNA by DCL2, DCL3 or DCL4 (Lippman and Martienssen, 2004). In contrast, miRNA are processed by DCL1 from imperfectly self-folded hairpin precursors (Park et al., 2002). To further confirm these identified sRNA regulators are the miRNA candidates but not siRNAs, analysis of DCL1 dependence was performed. The results showed nearly all of the miRNA candidates were repressed in *dcl1* mutant, but were not affected in the *dcl3* and the *rdr2* mutants (Table 2). These results support that the sRNAs identified in this study are likely to be miRNAs. Only one exceptional case was observed for miRNA candidate 3, which was repressed in both *dcl1* and *rdr2* (Table 2). Thus, the biogenesis pathway of miRNA candidate 3 might be different from that of most miRNAs. In plants, miRNAs should be incorporated into specific AGO proteins (normally AGO1) to perform regulation of specific targets (Voinnet, 2009). There are four AGO1 homologs (AGO1a, AGO1b, AGO1c and AGO1d) in rice. Wu et al. (2009) (Wu et al., 2009) found that three AGO1s (AGO1a, AGO1b, AGO1c) predominantly bound known miRNAs and had slicer activity. Most of the miRNAs were evenly distributed in the three AGO1 complexes, suggesting a redundant role for the AGO1s. However, a subset of miRNAs were specifically incorporated into or excluded from one of the AGO1s, suggesting functional specialization among the AGO1s (Wu et al., 2009). Here, we performed the AGO1 enrichment analysis of these miRNA candidates using the high-throughput sequencing (HTS) data of rice AGO1-associated sRNAs. The results showed that the seven miRNA candidates were enriched in one or more AGO1 homologs (Table 2, Table S3). Interestingly, miRNA candidate 2 was only associated with AGO1b, miRNA candidate 5 was enriched in AGO1c, and miRNA candidate 6, as well as osa-MIR5788-3p, was preferentially associated with AGO1a.

For the 15 targets of the seven miRNA candidates, the significant degradome-based cleavage signals were observed within the target binding sites predicted by miRU algorithm (Zhang, 2005) (Table 1, Fig 2 and Figs S3). However, the slicing sites of only 5 targets (LOC_Os03g10250.1, LOC_Os09g39910.1, LOC_Os09g39910.2, LOC_Os09g39910.3 and LOC_Os10g39970.1) could be mapped to 10^{th} - 11^{th} nt of the corresponding miRNA candidates. The offset slicing sites were detected for the other targets. By considering these shifted slicing sites in the targets were around the 10^{th} to 11^{th} nt and the similar phenomena had been observed in the previously validated targets of miRNAs, they were also considered to be the miRNA-mediated cleavages. According to the gene annotations provided by TIGR rice (release 7) (Yuan et al., 2003), miRNA candidate 4 targets a phytosulfokine receptor precursor (LOC_Os03g54900), which is involved in the salt and alkali stress response (Huang et al., 2003; Yang et al., 2010). LOC_Os04g53830.1 encoding a 3-beta hydroxysteroid dehydrogenase/isomerase is the target of osa-miR5788-3p candidate.

Table 1. Novel miRNA candidates and their targets identified by reversed framework.

Target_id	Cleavage signals detected by degradome sequencing data	extracted miRNA candidate id	extracted miRNA candidate sequence	Predicted binding site(s) on the target transcript	Target annotation
LOC_Os03g10250.1	19	osa-miRNA candidate1	UAAUAGAUGACGCCGUUGACU	8-28	expressed protein of unknown function
LOC_Os04g39600.1	1027/1028	osa-miRNA candidate2	CAAUGUAAGACUUUCUAGCAU	1016-1036	fasciclin domain containing protein, expressed
LOC_Os08g32500.1	155/156			144-164	nucleobase-ascorbate transporter, putative, expressed
LOC_Os05g02620.1	638	osa-miRNA candidate3	UCACAUGUUUGACCAUUCGUC	626-646	expressed protein of unknown function
LOC_Os03g48010.1	2709	osa-miRNA candidate4	UCCGUUCCAUAAUAUAAGAGA	2700-2720	exostosin, putative, expressed
LOC_Os03g54900.1	4491			4482-4502	phytosulfokine receptor precursor, putative, expressed
LOC_Os03g54900.2	4530			4521-4541	phytosulfokine receptor precursor, putative, expressed
LOC_Os09g39910.1	2283	osa-miRNA candidate5	UUAUAAGACUUUCUAGCAUU	2273-2292	ABC transporter, ATP-binding protein, putative, expressed
LOC_Os09g39910.2	2258			2248-2267	ABC transporter, ATP-binding protein, putative, expressed
LOC_Os09g39910.3	2345			2335-2354	ABC transporter, ATP-binding protein, putative, expressed
LOC_Os10g39970.1	963			953-972	cDNA harpin-induced protein 1 domain containing protein, expressed
LOC_Os09g07500.1	2108	osa-miRNA candidate6	UACAUCUCGCAACGAAAAAAA	2096-2116	expressed protein of unknown function
LOC_Os09g07500.2	2037			2025-2045	expressed protein of unknown function
LOC_Os09g07500.3	2747			2735-2755	expressed protein of unknown function
LOC_Os04g53830.1	1166	osa-miR5788-3p candidate	UAGUACUAGGAUAUGUCACAU	1156-1176	3-beta hydroxysteroid dehydrogenase/isomerase family protein, putative, expressed

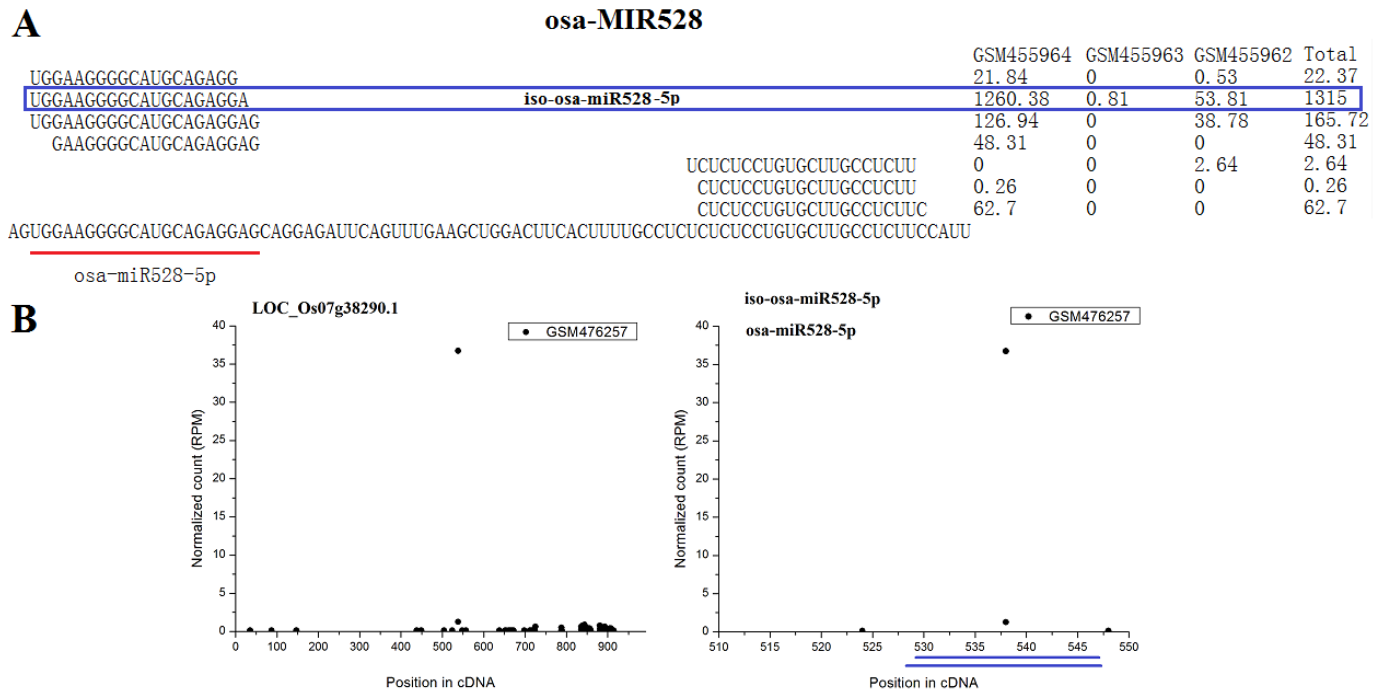

Fig 1. Highly expressed iso-osa-miR528-5p acting as the main regulator sRNA of LOC_Os07g38290.1.
(A) The short sequences of AGO1-enriched HTS data perfectly mapped to the pre-miRNA, osa-MIR528, along with their normalized read counts in RMP (Reads per million) are shown. The blue rectangular area includes iso-osa-miR528-5p sequence and its normalized read counts. The red line indicates the position of osa-miR528-5p. (B) Degradome sequencing data-based identification of the target of iso-osa-miR528-5p and osa-miR528-5p. The first panel depicts the degradome signals all along the target transcript, and the other panel provides detailed views of the cleavage signals within the regions surrounding the target recognition (denoted by blue horizontal lines). The transcript ID and the isomiR/miRNA are shown in the first and the other panel respectively. The *x* axes measure the positions of the signals along the transcripts, and the *y* axes measure the signal intensities based on normalized counts (in RPM, reads per million), allowing cross-library comparison.

Table 2. Dependence of novel miRNA candidates on DCL1 and their enrichments in Argonaute 1.

miRNA_candidate_ID	DCL1 dependent analysis				AGO1 enrichment analysis			
	GSM520640 _control	GSM520637_*dcl1*	GSM520638_*dcl3*	GSM520639_*rdr2*	GSM455965_control	GSM455962_AGO1a	GSM455963 _Ago1b	GSM455964 Ago1c
osa-miRNA candidate1	48.19	5.24	71.05	108.92	32.76	193.09	199.89	1046.08
osa-miRNA candidate2	0.79	0.22	0.61	1.77	1.24	0	62.57	0
osa-miRNA candidate3	4.54	1.75	13.44	1.55	3.47	23.48	76.38	7.97
osa-miRNA candidate4	3.75	0	4.89	7.08	6.95	12.66	1.63	14.65
osa-miRNA candidate5	3.16	0.87	4.89	3.32	2.73	2.37	0	12.59
osa-miRNA candidate6	0.59	0.22	0.61	1.11	0.25	2.11	0	0
osa-MIR5788-3p candidate	7.9	0.22	5.7	7.53	3.72	12.66	0.81	0

Note : The number means the normalized count (RPM)

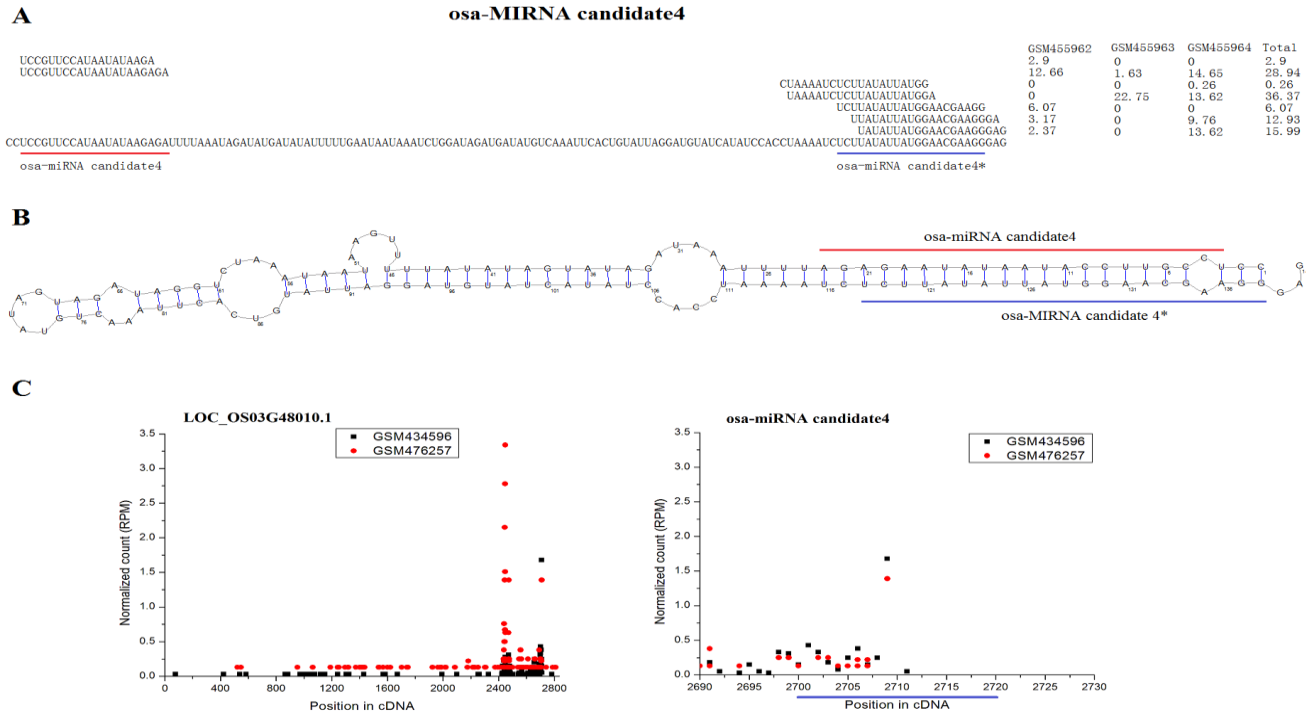

Fig 2. High-throughput sequencing (HTS) data- and structure-based identification of novel miRNA candidate 4--target. (A) The short sequences of AGO1-enriched HTS data perfectly mapped to the pre-miRNA, miRNA candidate 4, along with their normalized read counts in RPM (reads per million) are shown. The red and the blue lines indicate the position of miRNA candidate 4 and miRNA candidate 4*, respectively. (B) The structured miRNA candidate 4 predicted by RNAshapes. The red line indicates the position of miRNA candidate 4 and the blue one indicates the miRNA* positions. (C) Degradome sequencing data-based identification of the target of miRNA candidate 4. The first panel depicts the degradome signals all along the target transcript, and the other panel provides detailed views of the cleavage signals within the regions surrounding the target recognition (denoted by blue horizontal lines). The transcript ID and the miRNA candidate are shown in the first and the other panel respectively. The *x* axes measure the positions of the signals along the transcripts, and the *y* axes measure the signal intensities based on normalized counts (in RPM, reads per million), allowing cross-library comparison.

This target gene is induced in resistant rice plants after challenge by the pathogen *Rhizoctonia solani*, suggesting a defensive role of this gene in rice against attack by fungal (Lee et al., 2006). miRNA candidate 5 targets LOC_Os10g39970.1 encoding a hairpin-induced protein 1 domain containing protein, which is involved in biotic and abiotic stress responses and may also play roles in chalkiness formation of rice grains (Wei et al., 2010; Liu et al., 2010). Another target (LOC_Os09g39910) of miRNA candidate 5 encodes an ABC transporter, a protein related to direct stress resistance. ABC transporter has been reported to pump Cadmium (Cd) conjugated to glutathione into vacuoles, resulting in less accumulation of Cd in cells. It is also regulated by a Cd-responsive miRNA, miR192 (AUGAAUGUGGGCAAUGCUAGAA) (Ding et al., 2013). Besides, ABC transporter is also involved in response for cold, chromium (Cr) and aluminum (Al) stresses (Dubey et al., 2010; Lima et al., 2011; Venu et al., 2013). Taken together, these novel miRNA candidates mentioned above may play important roles in stress response in rice.

Materials and Methods

Data sources

The sRNA high-throughput sequencing (HTS) data sets, AGO1-associated sRNA sequencing data and the degradome sequencing data sets of *Oryza sativa* were retrieved from GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/) (Barrett et al., 2009). See Table S1 for the accession numbers and sequencing library information. In order to allow cross-library comparison, the normalized read count (in RPM, reads per million) of a short read from a specific library was calculated by dividing the raw count of this read by the total counts of the library, and then multiplied by 10^6. The miRNA and the pre-miRNA of *Oryza sativa* were downloaded from miRBase (release 20; http://www.mirbase.org/) (Griffiths-Jones et al., 2008). The cDNAs, full-length genomic sequences, and the gene annotations of *Oryza sativa* were retrieved from the FTP site of the rice genome annotation project established by the Institute for Genome Research (TIGR rice, Release 7; ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/) (Yuan et al., 2003).

Identification of novel miRNA candidates-targets by reversed framework

By taking advantage of the highly complementary between the miRNA and its target binding site, and the AGO1 –enrichment characteristic of the miRNA, we have developed a reversed framework for the identification of microRNA-target pairs in plants (Shao et al., 2013). In short, all the available 4 degradome sequencing datasets (GSM434596, GSM476257, GSM455938 and GSM455939) were included to do a comprehensive search for potential cleavage sites in all the 67,392 cDNAs of rice. Then, the 'bait' sequences, which may be acted as the miRNAs binding sites in the targets, were generated by adding 10 nt sequences both upstream and downstream of the cleavage sites. Third, the potential miRNA candidates were extracted by BLAST of these 'bait' sequences against the AGO1-associated sRNA sequencing data and were further filtered considering the length range and expression level of miRNAs. Subsequently, those extracted miRNA candidates were subjected to target prediction against the cleavage site-containing transcripts identified above using miRU algorithm (Zhang, 2005) and only the transcripts with the cleavage signals resided within 8-12nt region away from the 3'ends of the target binding sites predicted were retained as the potential miRNA-target pairs. After search of the miRBase (Griffiths-Jones et al., 2008), plant microRNA database (Zhang et al., 2010), and published literatures (Wei et al., 2010; Zheng et al., 2012), the previously validated miRNA-target pairs could be identified. The other potential miRNA candidates were retained for further validation to find the novel miRNA candidates.

Structure- and expression-based validation of these novel miRNA candidates

All the retained potential miRNA candidates mentioned above were mapped to the rice genome to obtain their loci information and the '1400 nt' fragments around these loci were extracted from genome. Then, the AGO1-enriched sRNA HTS datasets were used to gain the sRNA distribution patterns along the 1400nt sequences. According to our experiences, the pre-miRNA candidate is likely to possess two isolated sRNA clusters, i.e. the miRNA cluster centered on the sRNA locus and the miRNA* cluster. The potential pre-miRNAs containing both miRNA clusters and miRNA* clusters could be identified. They could be cut from 1400nt fragment and were then subjected to secondary structure-based validation by using RNAshapes (Steffen et al., 2006). The canonical 'stem-loop' structure served as the reference structure for determining whether the pre-miRNA candidates were valid. By considering the miRNA is processed by DCL1 and then loaded into AGO1 to perform its functions, the DCL1 dependence and AGO1 enrichment of these miRNA candidates were analyzed. The normalized read counts of the new miRNA candidates were obtained form the sRNAs HTS data sets of *dcl1* mutant, normal (control) sample, *dcl3* mutant and *rdr2* mutant of *Oryza sativa* individually to perform the DCL1 dependence analysis. It should be half or even less abundant in *dcl1* mutant than any other samples. For AGO1 enrichment analysis, the normalized read count of the new miRNA candidates should be two times or more abundant in either of the three AGO1 homologs (AGO1a, AGO1b, AGO1c) data set when compared to the control set.

Conclusions

Using the reversed approach, 7 novel miRNA candidates together with 15 corresponding mRNA targets were identified in rice (Oryza sativa). Additionally, we found some non-canonical slicing sites and higher accumulation isomiRs in some of the previously validated miRNA--target pairs. These findings could broaden the current understanding of miRNA gene mediated regulation in rice.

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