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Transcriptome-wide identification of microRNA targets in rice

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SUMMARY

MicroRNA (miRNA)-guided target RNA expression is vital for a wide variety of biological processes in eukaryotes. Currently, miRBase (version 13) lists 142 and 353 miRNAs from Arabidopsis and rice (*Oryza sativa*), respectively. The integration of miRNAs in diverse biological networks relies upon the confirmation of their RNA targets. In contrast with the well-characterized miRNA targets that are cleaved in Arabidopsis, only a few such targets have been confirmed in rice. To identify small RNA targets in rice, we applied the 'degradome sequencing' approach, which globally identifies the remnants of small RNA-directed target cleavage by sequencing the 5′ ends of uncapped RNAs. One hundred and sixty targets of 53 miRNA families (24 conserved and 29 rice-specific) and five targets of TAS3-small interfering RNAs (siRNAs) were identified. Surprisingly, an additional conserved target for miR398, which has not been reported so far, has been validated. Besides conserved homologous transcripts, 23 non-conserved genes for nine conserved miRNAs and 56 genes for 29 rice-specific miRNAs were also identified as targets. Besides miRNA targets, the rice degradome contained fragments derived from *MIRNA* precursors. A closer inspection of these fragments revealed a unique pattern distinct from siRNA-producing loci. This attribute can serve as one of the ancillary criteria for separating miRNAs from siRNAs in plants.

Keywords: degradome, miRNAs, miRNA targets, post-transcriptional gene regulation, rice.

INTRODUCTION

MicroRNA (miRNA)-guided post-transcriptional gene regulation constitutes one of the most conserved and well-characterized gene regulatory mechanisms; it is important for development, stress responses and a myriad of other biological processes in eukaryotes (Jones-Rhoades *et al.*, 2006; Mallory and Vaucheret, 2006; Sunkar *et al.*, 2007; Axtell, 2008; Axtell and Bowman, 2008; Shukla *et al.*, 2008; Voinnet, 2009). Therefore, identification of miRNAs in diverse species has been a major focus in recent years. Arabidopsis and rice (*Oryza sativa*) have been focused on as representatives of monocotyledonous and dicotyledonous plants, respectively. The published reports as well as publicly accessible small RNA datasets from different plant species suggests that small RNAs in plants are highly complex and abundant (Fahlgren *et al.*, 2007; Nobuta *et al.*, 2007; Meyers *et al.*, 2008). The complexity is not only reflected in endogenous small interfering RNAs (siRNAs) but also in the miRNA component, as many lineage-specific or species-specific miRNAs have been identified in Arabidopsis, rice, poplar (*Populus trichocarpa*), tomato (*Solanum lycopersicum*) and *Medicago truncatula* (Lu *et al.*, 2005a,b, 2006, 2008a,b; Sunkar *et al.*, 2005, 2008; Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007; Moxon *et al.*, 2008; Sunkar and Jagadeeswaran, 2008; Szittya *et al.*, 2008; Jagadeeswaran *et al.*, 2009b).

Conserved plant miRNAs regulate homologous targets at identical target sites in every species in which they exist (Axtell, 2008; Axtell and Bowman, 2008), and such targets can be predicted easily (Rhoades *et al.*, 2002). As well as conserved homologous transcripts as targets, non-conserved targets were identified for miR168 (the putative Zn-finger family transcript At3g58030) and miR159 (a Cu/Zn superoxide dismutase transcript At5g18100-*CSD3*) in Arabidopsis (German *et al.*, 2008). These findings suggested

that conserved miRNAs are also regulating non-conserved targets in Arabidopsis (German *et al.*, 2008). By contrast, it is also known that not all predicted non-conserved targets are genuine miRNA targets. For instance, At2g28780 transcript has only two mismatches within the target site for miR395, yet evidence for cleavage could not be found (Kawashima *et al.*, 2009). Although translational repression in the absence of slicing, or a very low level of slicing, cannot be ruled out, potential false-positive predictions like this emphasize the importance of experimental detection of miRNA targets (Kawashima *et al.*, 2009).

Plant miRNAs have been largely implicated in degradation of their RNA targets by slicing precisely between the 10th and 11th nucleotides (nt) from the 5′ end of the miRNA. The resulting 3′ fragment of the target RNA possesses a monophosphate at its 5′ end. This biochemical property has been exploited to validate mRNA transcripts targeted by miRNAs (Llave *et al.*, 2002), and isolation of such fragments is currently the method of choice for validating miRNA targets that are sliced. However, a major limitation is that every single predicted gene has to be verified independently; thus one-at-a-time isolation of target cleavage remnants is laborious, time-consuming and costly. To overcome this limitation, a high-throughput sequencing method that can identify small RNA target transcripts at a global scale has been developed (Addo-Quaye *et al.*, 2008; German *et al.*, 2008; Gregory *et al.*, 2008). Application of this method resulted in the validation of several miRNA targets in Arabidopsis that had evaded predictions due to weak complementarity with their corresponding miRNAs (Addo-Quaye *et al.*, 2008; German *et al.*, 2008).

Rice occupies a prominent position among the cereals because one-third of the world's population depends on rice for more than 50% of their caloric intake (Khush, 1997). Rice has also emerged as an important model system in cereal genomics and biotechnology. High-throughput sequencing technologies such as massively parallel signature sequencing (MPSS), 454 and sequencing-by-synthesis (SBS) have enabled the identification of a large number of miRNAs in rice. Currently, miRBase lists 353 miRNAs forming 142 miRNA families in rice. The extent of miRNA-directed post-transcriptional gene regulation in any organism can only be fully realized by identifying not only the miRNA component but also the set of their RNA targets. By contrast with the well-characterized miRNA targets in Arabidopsis, only a small number of miRNA targets have been confirmed in rice (Sunkar *et al.*, 2005; Luo *et al.*, 2006; Heisel *et al.*, 2008; Lacombe *et al.*, 2008; Lu *et al.*, 2008a; Zhu *et al.*, 2008). To identify transcriptome-wide small RNA targets in rice, we generated a cDNA library from polyadenylated transcripts harboring a 5′-monophosphate isolated from 3-week-old rice (*O. sativa* spp *japonica* cv. Nipponbare) seedlings. The library was sequenced using SBS sequencing technology and the dataset was computationally analyzed. A total of 11 552 007 unique signatures that could be mapped to the rice transcriptome were obtained. A total of 160 target genes were confirmed as miRNA targets, which included a new conserved target for miR398, 23 non-conserved targets for nine conserved miRNAs and 56 targets for the 29 rice-specific miRNAs. Additionally, five transcripts were identified as targets of TAS3-siRNA in rice. Another interesting observation that emerged from this study is that the processing remnants of primary *MIRNA* transcripts show a unique cleavage pattern, which can be used to distinguish between miRNA loci and endogenous siRNA loci.

RESULTS

Library generation, sequencing and sequence analysis

We applied a recently developed high-throughput experimental approach that can identify small RNA targets at a global level to rice (Addo-Quaye *et al.*, 2008; German *et al.*, 2008). We obtained a total of 39 376 422 short sequencing reads representing the 5′ ends of uncapped, poly-adenylated RNAs. After initial processing and removal of duplications, 15 454 006 unique 20-nt signatures were obtained (Table 1). Of these, 11 552 007 unique signatures representing ~75% of the total unique signatures could be mapped to the rice genome (Table 1). A small portion (0.5%) of the unique signatures could also be mapped to the rice chloroplast or mitochondrial genome (Table 1). Using a BLASTN search against the Rfam database, the structural RNAs [rRNAs, tRNA, small nuclear RNA (snRNA) and small nucleolar RNA (snoRNA)] represented by 0.13% of our unique dataset were removed (Table 1). Similarly, the signatures corresponding to repeats/transposons were also removed from the unique dataset by performing searches against the repeat database (http://www.girinst.org/server/RepBase/). The remaining sequences were mapped to the rice transcriptome. This analysis revealed that 92.2% of the genome-mapped unique

Table 1 Summary of the reads analysis

Reads category	Total reads	Unique reads
Reads	39 376 422	15 454 006
Reads mapped to CDS	15 469 840	6 078 329
Reads mapped to introns	2 248 505	945 965
Reads mapped to UTRs	13 982 871	3 630 702
Reads mapped to 5′-UTRs	10 929 758	353 798
Reads mapped to 3′-UTRs	3 142 372	3 304 284
Reads mapped 1 kb upstream to the CDS	4 498 883	864 245
Reads mapped to the pre-miRNAs	42 915	7000
Reads mapped to repeats	721 695	161 030
Reads mapped to rRNAs and tRNAs	170 563	15 111
Reads mapped to the genome	27 610 802	11 552 007
Reads mapped to the intergenic region	5 610 858	725 439
Reads mapped to chl/mit genome	3 169 327	71 426

UTR, untranslated region; CDS, coding sequence; miRNA, microRNA; chl, chloroplast; mit, mitochondria.

reads could be mapped to the sense strands of annotated transcripts either in their coding regions (CDS) or their untranslated regions (UTRs). Unexpectedly, 945 965 unique tags (8.1% of the total genome matching unique reads) could be mapped to introns. Similarly, a significant proportion of the unique reads (725 439 unique reads constituting 6.2% of the genome matching reads) could only be mapped to the intergenic regions. The reads mapped to the intergenic regions could be derived from the transcripts from unannotated genomic regions. Interestingly, 7000 unique reads represented by 42 915 tags could be mapped to the reported rice *MIRNA* precursors (Table 1), possibly representing processing intermediates, remnants or random degradation products. Approximately 25% of the unique sequences did not match to the rice nuclear or organellar sequence (Table 1). About 4.5% of the unique sequences mapped to the sense strand of one or more transcripts but not to the genome, suggesting that these were reads which spanned splice junctions. Approximately 15% of the unique reads did not match the genome perfectly but could be mapped to the genome with one mismatch, suggesting that sequencing errors accounted for many of the non-mapped reads. Another source of noise in this library was reads comprising polyA fragments: 2.5% of the unique sequences ended with four or more A residues. These were probably highly degraded mRNA fragments whose 5′ ends were in or near the polyA tail. Previous degradome analyses have reported similar findings (German *et al.*, 2008).

Abundantly captured signatures are conserved miRNA targets

A characteristic feature of miRNA-guided slicing is that the cleavage takes place precisely between the 10th and 11th nt from the 5′ end of miRNA in the complementary region of the target transcript. Therefore, sliced RNA targets should have distinct peaks of degradome sequence tags at the predicted cleavage site relative to other regions of the transcript (Addo-Quaye *et al.*, 2008; German *et al.*, 2008). The *CleaveLand* pipeline (Addo-Quaye *et al.*, 2009a) was used to find sliced miRNA targets in rice. Among the small RNA targets, the signatures associated with the conserved miRNA targets are the most abundantly represented (Table 2). Abundance of the signatures for each of the target transcripts was plotted as a function of its position in the transcript (Figure 1). We used absolute numbers in plotting the cleavages on target mRNAs, which were referred to as 'target plots' (t-plots) by German *et al.* (2008). Based on the signature abundance at the target sites and all along the length of the transcripts, the target transcripts have been categorized into three classes (class I, II or III) as reported previously for Arabidopsis and *Physcomitrella patens* (Addo-Quaye *et al.*, 2008, 2009b). Class I miRNA-guided cleavage remnants are the most abundantly recovered species (Figure 1a). A total of 103 targets fall into this category (Tables 2 and 3, Figure 1a, Table S1 and Figure S1 in Supporting Information). In the class II category, the cleavage abundance was higher than the median on the transcript, but below the maximum. Twenty-three targets were found to belong to this category (Figures 1b and S2 and Tables 2, 3 and S1). Class III encompasses all other transcripts with more than one read matching the 5′ end of a slicing remnant. As shown in Figures 1c and S3 and Table S1, 34 transcripts fall into this category. Small RNA targets are not the most abundant signatures in the library (Table 1). The tag abundance associated with several transcripts that are not targeted by known small RNAs are even more abundant, suggesting a high rate of turnover of these transcripts through uncapping and 5′–3′ exosome-mediated pathways.

Validation of additional conserved and non-conserved targets for conserved miRNAs

It has been shown that miR398 targets two Cu/Zn superoxide dismutases (Cu/Zn SODs) and this is highly conserved among dicots and monocots (Sunkar *et al.*, 2006; Dugas and Bartel, 2008; Sunkar and Jagadeeswaran, 2008). In addition to these two Cu/Zn SODs, sequencing of sliced ends also found another target, Os04g48410 (a copper chaperone for Cu/Zn superoxide dismutase, *CCS1*), for miR398 (Table 2). To independently test whether Os04g48410 is a genuine target for miR398, we also isolated slicing remnants using a gene-specific 5′-rapid amplification of cDNA ends (RACE) assay (Figure 2a). Interestingly, the target site for miR398 in Os04g48410 homologs of diverse plant species is highly conserved (Figure 2b). Using a 5′-RACE assay, we validated the Os04g48410 homolog in Arabidopsis (At1g12520) as a genuine target for miR398 in Arabidopsis (data not shown). Os04g48410 encodes for a rice homolog of copper chaperone, a key factor integrating copper into Cu/Zn SODs in budding yeast and mammals (Chu *et al.*, 2005). To provide direct evidence that miR398 indeed targets *CCS1* transcript, we infiltrated *Agrobacterium tumefaciens* harboring *Ath-MIR398a* primary transcript and Arabidopsis *CCS1* into *Nicotiana benthamiana* leaves for transient co-expression analysis. We confirmed the expression of miR398 in mock, *CCS1* or miR398 or miR398 and *CCS1* infiltrated leaves. The detection of miR398 in mock and *CCS1* infiltrated *N. benthamiana* leaves is not surprising given the fact that miR398 is highly conserved and the endogenous miR398 could be easily detected (Figure 2c). However, miR398 accumulation is much greater in leaves infiltrated with miR398 or miR398 and *CCS1* constructs relative to the mock or *CCS1* infiltrated leaves, as expected (Figure 2c). *CCS1* transcript has been detected in samples infiltrated with *CCS1* or *CCS1* and miR398 constructs, but not in mock or miR398 infiltrated leaves (Figure 2c). *CCS1* levels were substantially reduced in the leaves when miR398 and *CCS1* were co-expressed and the *CCS1* levels were much greater in leaves in which *CCS1* was expressed alone (Figure 2c). These results suggested

Table 2 Conserved rice microRNA (miRNA) targets identified by degradome sequencing

miRNA	Target gene (class)	Location of the target site (score)	Percentage cleavage at the expected site	Estimated reads at the cleavage site (tpb)	Gene annotation
miR156	*Os01g69830* (I)	CDS (0)	3.8	1958.1	SBP domain containing protein
miR156	*Os02g04680* (I)	CDS (2)	8.1	3585.6	SBP domain containing protein
miR156	*Os06g45310* (I)	CDS (1)	8.2	3661.9	SBP domain containing protein
miR156	*Os07g32170* (III)	CDS (2)	10.8	1678.4	SBP domain containing protein
miR156	*Os11g30370* (I)	CDS (0)	45.2	1805.5	SBP domain containing protein
miR156	*Os02g07780* (II)	3′-UTR (1)	4.5	1195.2	SBP domain containing protein
miR156	*Os08g39890* (III)	CDS (0)	2.7	279.7	SBP domain containing protein
miR156	*Os06g49010* (III)	CDS (1)	0.5	228.9	SBP domain containing protein
miR156	*Os09g32944* (II)	CDS (0)	3.3	50.9	SBP domain containing protein
miR156	*Os09g31438* (III)	CDS (0)	0.4	25.4	SBP domain containing protein
miR159	*Os01g59660* (I)	CDS (3)	30.4	35373.1	MYB family transcription factor
miR159	*Os06g40330* (II)	CDS (3)	9.4	1627.5	MYB family transcription factor
miR160	*Os06g47150* (I)	CDS (1)	61.8	115286.8	Auxin response factor
miR160	*Os10g33940* (I)	CDS (1)	67.7	115286.8	Auxin response factor
miR160	*Os02g41800* (II)	CDS (1)	4.7	1042.6	Auxin response factor
miR160	*Os04g43910* (II)	CDS (1)	6.0	813.8	Auxin response factor
miR162	*Os03g02970* (I)	CDS (2)	13.1	3890.8	Endoribonuclease Dicer
miR164	*Os06g46270* (I)	CDS (1)	19.5	2568.4	NAC domain-containing protein
miR164	*Os08g10080* (I)	CDS (2.5)	12.4	1042.6	NAC domain-containing protein
miR164	*Os12g41680* (I)	CDS (1)	56.0	32677.5	NAC domain-containing protein
miR164	*Os02g36880* (III)	CDS (3)	0.3	25.4	No apical meristem protein
miR164	*Os04g38720* (III)	CDS (3)	0.2	25.4	No apical meristem protein
miR166	*Os03g43930* (I)	CDS (2.5)	8.8	3839.9	START domain containing protein
miR166	*Os10g33960* (I)	CDS (2.5)	15.4	18792.8	START domain containing protein
miR166	*Os03g01890* (I)	CDS (2.5)	45.1	39441.9	Rolled leaf-1
miR166	*Os12g41860* (I)	CDS (2.5)	13.9	3839.9	START domain containing protein
miR167	*Os04g57610* (II)	CDS (4)	1.7	2110.7	Auxin response factor
miR167	*Os02g06910* (I)	CDS (5)	9.7	6967.8	Auxin response factor
miR167	*Os06g46410* (I)	CDS (5)	23.5	1055.0	Auxin response factor
miR167	*Os12g41950* (I)	CDS (5)	7.3	1805.5	Auxin response factor
miR168	*Os04g47870* (II)	CDS (1)	0.5	2568.4	PINHEAD protein
miR168	*Os02g45070* (III)	CDS (0)	0.5	966.3	PINHEAD protein
miR168	*Os06g51310* (III)	CDS (6)	0.25	76.3	PINHEAD protein
miR168	*Os02g58490* (III)	CDS (5)	1.6	762.9	PINHEAD protein
miR169	*Os02g53620* (I)	3′-UTR (2)	35.3	6967.8	Nuclear transcription factor Y subunit
miR169	*Os03g07880* (I)	3′-UTR (3)	53.5	51648.3	Nuclear transcription factor Y subunit
miR169	*Os03g29760* (I)	3′-UTR (3)	21.1	16351.5	Nuclear transcription factor Y subunit
miR169	*Os03g44540* (I)	3′-UTR (3)	21.0	12715.0	Nuclear transcription factor Y subunit
miR169	*Os03g48970* (I)	3′-UTR (3)	23.6	18843.6	Nuclear transcription factor Y subunit
miR169	*Os07g06470* (I)	3′-UTR (2)	55.3	2314.1	Nuclear transcription factor Y subunit
miR169	*Os07g41720* (I)	3′-UTR (3)	35.5	9078.5	Nuclear transcription factor Y subunit
miR169	*Os12g42400* (I)	3′-UTR (3)	19.1	5467.4	Nuclear transcription factor Y subunit
miR171	*Os02g44360* (I)	CDS (0)	5.4	1042.6	SCARECROW gene regulator
miR171	*Os04g46860* (I)	CDS (0)	8.0	2237.8	SCARECROW gene regulator
miR171	*Os02g44370* (III)	CDS (0)	1.1	356.0	SCARECROW gene regulator
miR172	*Os05g03040* (I)	CDS (2)	27.3	38043.2	AP2 domain containing protein
miR172	*Os07g13170* (III)	CDS (2)	1.2	381.4	AP2 domain containing protein
miR172	*Os04g55560* (I)	CDS (3)	21.1	2026.0	AP2 domain containing protein
miR172	*Os03g60430* (III)	CDS (2)	0.2	50.9	AP2 domain containing protein
miR319a	*Os07g05720* (III)	CDS (2)	0.2	25.4	TCP family transcription factor
miR390	EU293144 (I)		16.7	761.8	TAS3a
miR390	AU100890 (I)		49.3	2793.6	TAS3b
miR390	CA765877 (I)		35.4	963.5	TAS3c
miR393	*Os05g05800* (I)	CDS (1)	54.6	68660.9	Transport inhibitor response 1 protein
miR393	*Os04g32460* (I)	CDS (1)	7.9	4984.3	Transport inhibitor response 1 protein
miR394	*Os01g69940* (I)	CDS (0)	23.1	7120.4	F-box domain containing protein
miR395	*Os06g05160* (II)	CDS (6)	2.3	279.4	Sulfate transporter 3.4

Table 2 (*Continued*)

miRNA	Target gene (class)	Location of the target site (score)	Percentage cleavage at the expected site	Estimated reads at the cleavage site (tpb)	Gene annotation
miR396	*Os02g47280* (I)	CDS (1)	21.7	432.3	Growth-regulating factor
miR396	*Os03g47140* (I)	CDS (1)	39.1	1729.2	Growth-regulating factor
miR396	*Os12g29980* (I)	CDS (3)	22.1	1754.7	Growth-regulating factor
miR396	*Os04g51190* (III)	CDS (1)	1.5	406.9	Growth-regulating factor
miR396	*Os11g35030* (I)	CDS (4)	26.0	2517.6	Growth-regulating factor
miR396	*Os06g02560* (I)	CDS (1)	50.4	15919.2	Growth-regulating factor
miR396	*Os02g45570* (I)	CDS (1)	8.5	101.7	Growth regulating factor
miR396	*Os02g53690* (III)	CDS (1)	0.7	25.4	Growth regulating factor
miR396	*Os12g29980* (I)	CDS (3)	22.1	1754.7	Growth-regulating factor
miR396	*Os04g48510* (III)	CDS (5)	16.7	25.4	Growth-regulating factor
miR396	*Os03g51970* (III)	CDS (3)	0.42	50.9	Growth-regulating factor
miR397	*Os11g48060* (III)	CDS (2)	0.3	25.4	Laccase
miR398	*Os07g46990* (I)	5′-UTR (3)	8.1	16173.5	Copper/zinc superoxide dismutase 2
miR398	*Os03g22810* (III)	CDS (5.5)	0.3	610.3	Copper/zinc superoxide dismutase
miR398	*Os04g48410* (II)	CDS (6)	1.3	432.3	Copper chaperone for SOD
miR399	*Os05g48390* (III)	5′-UTR (0)	0.04	50.9	Ubiquitin conjugating enzyme
miR408	*Os02g52180* (I)	3′-UTR (4)	18.5	279.7	Plastocyanin-like protein
miR408	*Os04g46130* (I)	3′-UTR (4.5)	15.4	1322.4	Blue copper protein precursor
miR408	*Os06g11490* (I)	3′-UTR (4)	10.1	4246.8	Blue copper protein precursor
miR408	*Os08g37670* (I)	3′-UTR (3)	26.9	60294.5	Blue copper protein precursor
miR444a.2	*Os08g33488* (I)	CDS (0)	44.8	991.8	MADS-box transcription factor 23
miR444b.2	*Os02g36924* (I)	CDS (0)	60.6	38933.3	MADS-box transcription factor 27a
miR444b.2	*Os04g38780* (I)	CDS (0)	62.1	1271.5	MADS-box transcription factor 27b
miR444d.3	*Os02g49840* (I)	CDS (0)	24.5	7069.5	MADS-box transcription factor 57
Tas3a-siRNA	*Os05g48870* (I)	CDS (1)	11.8	31059.2	Auxin response factor
Tas3a-siRNA	*Os01g48060* (I)	CDS (0)	4.6	1599.9	Auxin response factor
Tas3b-siRNA	*Os01g54990* (I)	CDS (1)	3.9	2184.0	Auxin response factor
Tas3a-siRNA	*Os05g43920* (I)	CDS (1)	3.5	634.9	Auxin response factor
Tas3a-siRNA	*Os01g70270* (I)	CDS (1)	2.7	7999.7	Auxin response factor

tpb, transcripts per billion; CDS, coding sequence; UTR, untranslated region; siRNA, small interfering RNA.

that miR398 directs degradation of the *CCS1* transcript and is therefore a target for miR398. Identification of *CCS1* as another conserved target for miR398 with 5.5 mismatches illustrates the fact that current computational target predictions with a cut-off of 3 or 3.5 mismatches may have underestimated the number of conserved targets that conserved miRNAs are regulating in plants. These results suggest a scope for finding additional conserved targets for conserved miRNAs in plants.

Conserved miRNAs are targeting conserved homologous genes in diverse plant species. In fact this attribute is one of the criteria in predicting miRNA targets with high confidence in diverse plant species (Jones-Rhoades *et al.*, 2006; Axtell, 2008). Nevertheless, it was shown recently that some of the conserved miRNAs, such as miR168 and miR159 in Arabidopsis, are regulating diverse gene families, besides the well-conserved target gene family (German *et al.*, 2008). For instance, besides *AGO1*, miR168 has been found to target *At3g58030*, a gene coding for Zn-finger family protein (German *et al.*, 2008). Similarly, miR159 is targeting *Cu/Zn SOD* (*At5g18100-CSD3*), in addition to the well-conserved *MYB* family (German *et al.*, 2008). These findings suggest that at least some conserved miRNAs are regulating non-conserved targets in addition to the well-documented conserved targets. A total of 23 non-conserved transcripts were found as targets for nine conserved miRNA families (miR156, miR164, miR166, miR167, miR171, miR172, miR393, miR396 and miR444) in rice (Table 3, Figure 3a–f) based on the tag abundances at the complementary sites. For example, besides four MADS-box genes, miR444b.1 is potentially targeting other genes with homology to WD-domain-containing proteins, DNA-binding proteins and leucine-rich repeat (LRR) kinases (Table 3, Figure 3e,f).

Of these 23 non-conserved targets, we attempted to independently isolate cleavage remnants using gene-specific 5′-RACE. Of the nine, remnants consistent with miRNA-directed cleavage for six transcripts were readily identified (Figure 3a–f) as genuine non-conserved targets. For the remaining three transcripts, fragments consistent with miRNA-directed cleavage could not be found because the fragments could not be amplified, which could be due to their low-level expression in seedlings. These findings suggest that many of the non-conserved targets listed in Table 3 are likely to be bona fide targets for conserved

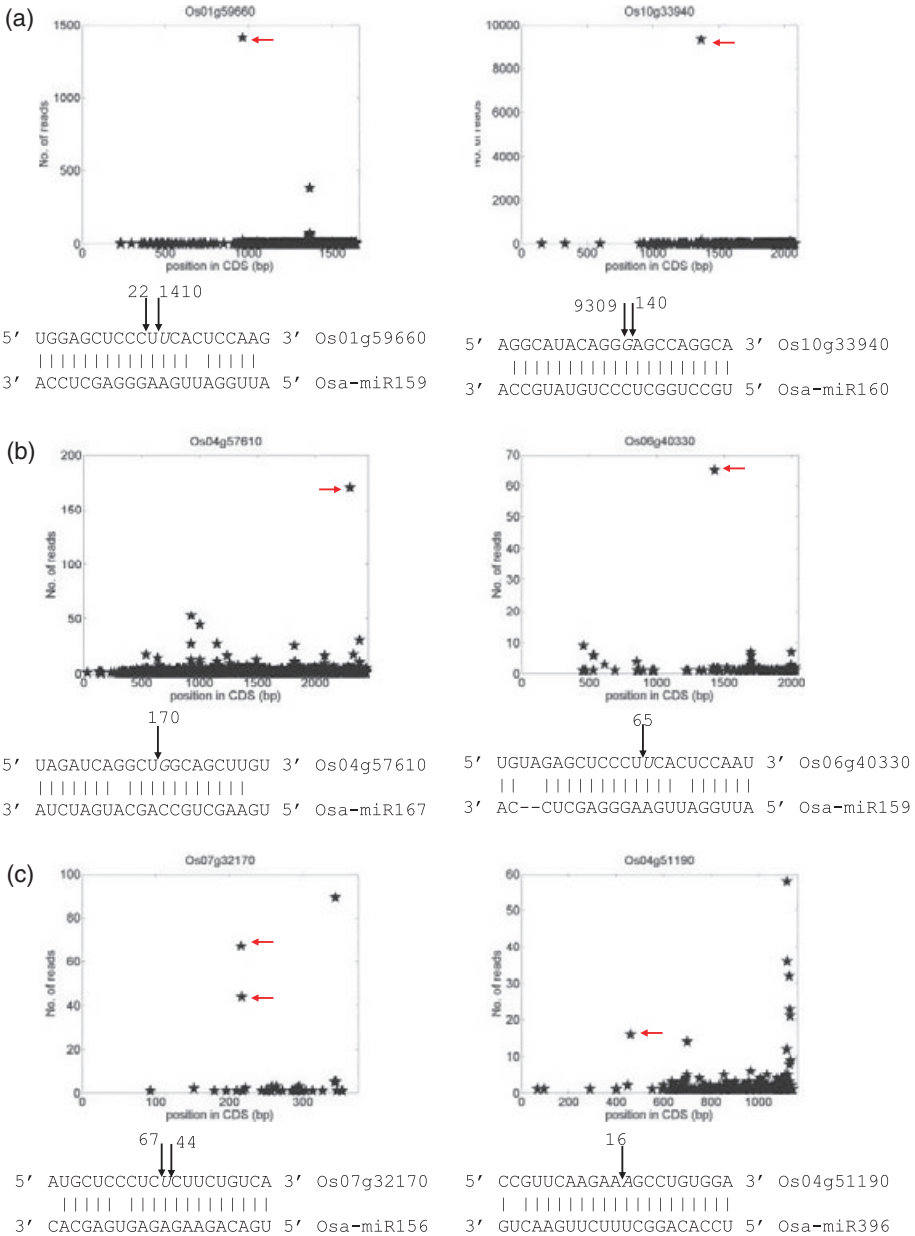

Figure 1. Confirmed microRNA (miRNA) targets using degradome sequencing are presented in the form of target plots (t-plots).
We used absolute numbers in plotting the cleavages on target mRNAs, which were referred to as 'target plots' (t-plots) by German *et al.* (2008). Signature abundance throughout the length of the indicated transcripts is shown. Representative t-plots for class I (a), class II (b), and class III (c) categories are shown. Arrows indicate signatures consistent with miRNA-directed cleavage. miRNA:mRNA alignments along with the detected cleavage frequencies (absolute numbers) are shown. The italicized nucleotide on the target transcript from the 3′ end indicates the cleavage site detected in the degradome.

miRNAs in rice. Together, these findings demonstrate that deeply conserved miRNAs can also regulate non-conserved targets, besides their conserved targets.

Identification of rice-specific miRNA targets

Studies in diverse plant species have annotated a large number species-specific miRNAs that are typically expressed at low abundance (Sunkar *et al.*, 2005, 2008; Lu *et al.*, 2006, 2008b; Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007; Jagadeeswaran *et al.*, 2009b). Target genes for many of these species-specific miRNAs are difficult to predict and often cannot be validated; thus many species-specific miRNAs have been suggested to be non-functional (Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007). We identified at least 56 genes as targets for 29 rice-specific miRNAs in this study (Table S1). This includes 11 genes confirmed as targets for four different rice-specific miRNAs (miR820, miR827, miR1425 and miR1428-3p) which have been reported recently (Luo *et al.*, 2006; Guo *et al.*, 2008; Heisel *et al.*, 2008; Lacombe *et al.*, 2008; Lu *et al.*, 2008a; Zhu *et al.*,

Table 3 Non-conserved rice microRNA (miRNA) targets identified by degradome sequencing

miRNA	Target gene (class)[a]	Location of the target site (score)	Percentage of cleavage at the expected site	Estimated reads at the cleavage site (tpb)	Gene annotation
miR156	*Os05g48800* (III)	CDS (6)	0.1	152.6	Drought induced 19 protein
miR156	*Os07g36500* (III)	3′-UTR (4.5)	1.3	254.3	Core histone H2A/H2B/H3/H4 domain containing protein
miR164	*Os03g50040* (I)	3′-UTR (7)	5.8	1373.2	Phytanoyl-CoA dioxygenase
miR164	*Os05g39650* (I)	3′-UTR (7)	7.0	1373.2	Phytanoyl-CoA dioxygenase
miR166	*Os03g01890* (I)	CDS (2.5)	45.1	39441.9	Hypothetical protein
miR167	***Os06g03830*** (I)	CDS (6)	40.4	1168.2	Retinol dehydrogenase 14
miR167	*Os07g33790* (I)	3′-UTR (3.5)	4.0	1118.9	Glutamate receptor 3.4 precursor
miR167	*Os07g29820* (II)	CDS (4)	1.4	661.2	NBS-LRR disease resistance protein
miR171	***Os04g39864*** (I)	3′-UTR (7)	4.1	381.4	Beta-glucosidase homologue
miR172	*Os08g39630* (I)	3′-UTR (3)	9.1	6128.6	Helix-loop-helix DNA-binding domain containing protein
miR172	*Os01g04550* (I)	3′-UTR (7)	6.4	534.0	Expressed protein
miR393	*Os03g36080* (II)	CDS (3)	1.7	432.3	Expressed protein
miR393	***Os04g58734*** (III)	5′-UTR (2.5)	1.4	330.6	Expressed protein
miR396	*Os01g32750* (III)	CDS (3)	0.2	50.9	TBP-associated 59 kDa subunit
miR396	***Os11g35030*** (I)	CDS (4)	26	2517.6	Expressed protein
miR396	*Os01g44230* (III)	CDS (3.5)	0.3	254.3	Transcription factor X1
miR396	*Os06g29430* (I)	CDS (7)	4.2	584.9	Cytidine and deoxycytidylate deaminase zinc-binding region family protein
miR444	*Os02g49090* (I)	CDS (4)	8.9	5009.7	WD domain, G-beta repeat domain containing protein
miR444	***Os03g63750*** (1)	CDS (3.5)	1.4	610.3	HSF-type transcription factor
miR444	*Os05g08410* (I)	CDS (4)	3.2	279.7	ATP10 protein
miR444a.2	*Os08g33479* (III)	CDS (1)	0.1	305.2	Expressed protein
miR444b.1	***Os02g13420*** (I)	CDS (7)	29.4	495.9	LRR protein kinase
miR444b.2	*Os03g54084* (II)	CDS (3.5)	0.3	254.3	Phytochrome C

tpb, transcripts per billion; CDS, coding sequence; UTR, untranslated region.
[a]Bold entries were independently confirmed using gene-specific 5′-rapid amplification of cDNA ends (5′-RACE).

2008). Indeed, a transcript (Os06g06050) coding for a putative F-box protein has been validated as a genuine target for miR528 in rice using gene-specific 5′-RACE (Figure 3g). We hypothesize that the observed lower percentage cleavage with respect to some of the non-conserved targets could be due to the lower abundance of the target transcript or corresponding miRNA in rice seedlings or inefficient cleavage directed by the miRNA.

Regulation of mRNA targets by multiple miRNAs

Different miRNA isoforms belonging to one miRNA family such as miR444 could potentially target two distinct locations or one location but in a partially overlapping manner (Figure 4). Validating such overlapping sites using a 5′-RACE assay cannot be quantitative in terms of which of those miRNAs guide the target mRNA cleavages. High-throughput assays such as this can potentially clarify some of those issues. The miR444 family is represented by several variants (miR444a–e, b.2, c.2) (Sunkar *et al.*, 2005, 2008; Lu *et al.*, 2008a), and the target sites for these variants are distinct as well as overlapping (Figure 4). This study confirms that each of the four *MADS*-box mRNAs is subjected to cleavages at multiple sites, because these sites are highly complementary to different miR444 variants. However, the cleavage frequency differed among the different target sites, and one target site is preferentially cleaved relative to other target site(s) (Figure 4a–d). As shown in Figure 4a, the cleavage frequency for Os02g36924 is most abundant at one position (C1) potentially targeted by miR444b.2/c.2, whereas miR444b.1 targeting site (C2) had far fewer observed cleavages. Another target, Os02g49840, possesses three perfectly complementary sites to members of the miR444 family (Figure 4d). All three sites were cleaved although the cleavage frequency varied among them. These results revealed that the miR444 variants collectively silence the MADS-box genes *in vivo* in a combinatorial manner and that many miR444 variants are functional.

Cleavage analyses with respect to certain target transcripts targeted by two independent (distinct) miRNAs are also worth mentioning here. For instance, miR156 and miR529b in rice share significant sequence homology (out of 16 overlapping nucleotides, 14 are identical). The major difference is that miR156 has an additional 4 nt at its 5′ end and as such is shorter by 4 nt at the 3′ end (Figure 5a).

Because of high sequence similarity, both miRNAs are predicted to target the same site, offset by 4 nt, on several members of the *Squamosa Promoter Binding* (*SBP*) gene family (Figure 5b). However, our analysis indicated that the detectable cleavages were guided by miR156 but not by miR529 (Figure 5b). This is true for three other target genes (*Os01g69830*, *Os02g04680* and *Os02g07780*) and suggests that miR529 plays little to no role in regulating *SBP* transcripts in rice seedlings. Consistent with this observation, miR529 expression could not be detected in rice seedlings using up to 50 μg of total RNA (data not shown), while miR156 appears to be abundantly expressed (see below, Figure 7a). Similarly, miR169 targets the 3′ UTRs of eight *nuclear transcription factor Y* (*NFY*) mRNAs (Table 2). Interestingly, miR1433* also has high complementarity to the same sites on these eight *NFY* transcripts (Figure 5c). To determine whether miR169 or miR1433* or both are guiding the cleavages on these transcripts, we performed gene-specific 5′-RACE for two of these transcripts (Os03g07880 and Os03g44540). Analysis of the cleavages unambiguously confirmed that miR169 is guiding these cleavages *in vivo*, but not miR1433* (Figure 5c).

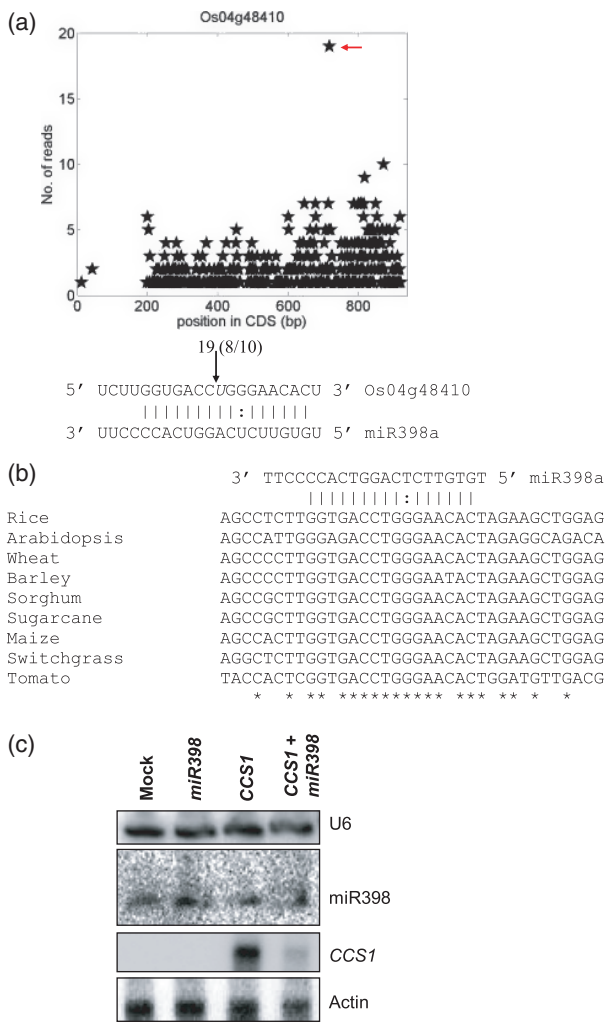

Figure 2. Identification of *CCS1* as a conserved target for miR398.
The italicized nucleotide on the target transcript from the 3′ end indicates the cleavage site detected in the degradome.
(a) Target plot (t-plot) and alignment of confirmed target (*Os04g48410*, *CCS1*) for miR398. Absolute numbers of signature sequences are indicated. The arrow indicates signatures consistent with miRNA-directed cleavage. Cleavage frequency as determined by gene-specific 5′-rapid amplification of cDNA ends (5′-RACE) at the indicated position is shown in parentheses. Primer sequences used for 5′-RACE are provided in Table S3.
(b) Conserved miR398 target sites on *CCS1* homologs of *Os04g48410* in dicots and monocots. *CCS1* sequences complementary to miR398 were shown from Arabidopsis (At1g12520), sorghum (CN144414), wheat (CJ893771), maize (CF635464), switchgrass (FL736093), barley (CK566086), sugarcane (CA254377) and tomato (FS180542).
(c) The RNA blots for the indicated targets after transient co-expression analysis of Arabidopsis *MIR398* and *CCS1* transcripts in *Nicotiana benthamina* leaves.

Signatures associated with the *TAS3* precursors and TAS3-siRNA targeting *ARF* genes in rice

The rice genome contains three *TAS3* loci, all of which have two potential target sites for miR390 (Lu *et al.*, 2008a). As in other flowering plants, the 3′ target site is highly complementary to miR390 whereas the 5′ target site has conserved mismatches in the central region of the miR390 target site. To determine the frequency of cleavage events on these three transcripts, we searched for the signature sequences, both at the 5′ and 3′ target sites corresponding to miR390. Cleaved signatures were detected corresponding to the 3′ site but not to the 5′ site (Figure 6a). This is consistent with previous reports that the conserved 5′ target site with central mismatches is resistant to cleavage (Axtell *et al.*, 2006; Montgomery *et al.*, 2008).

Five auxin response factors (*ARF2-Os01g70270*; two *ARF3* homologs, *Os05g43920* and *Os01g54990*; two *ARF4* homologs, *Os01g48060* and *Os05g48870*) have been predicted as TAS3-siRNA targets in rice. Whether TAS3-siRNAs actually direct slicing of these *ARF*s has not been reported. In their open reading frames (ORFs), each of the two *ARF3* and *ARF4* homologs possesses two complementary sites (5′ and 3′), whereas the *ARF2* homolog (*Os01g70270*) appears to have only a single target site for TAS3-siRNAs (Figure 6b). Cleaved signatures associated with these *ARF* transcripts were found in our library (Table 2, Figure 6b). Two peaks were observed as expected in the case of *ARF3* and *ARF4* target transcripts. Interestingly, in the four transcripts that possess dual target sites for TAS3-siRNAs, the signature associated with the second (3′) site is far greater than the first (5′) site. The differential cleavage frequency between these two target sites on the same transcript does not correlate with major differences in complementarity (Figure 6b). The relatively low cleavage frequency at the 5′ site could simply be due to the fact that the 5′ site signatures cannot be captured as completely as the 3′ site, because reverse transcription proceeded from the polyA tail of the transcripts in our experiments.

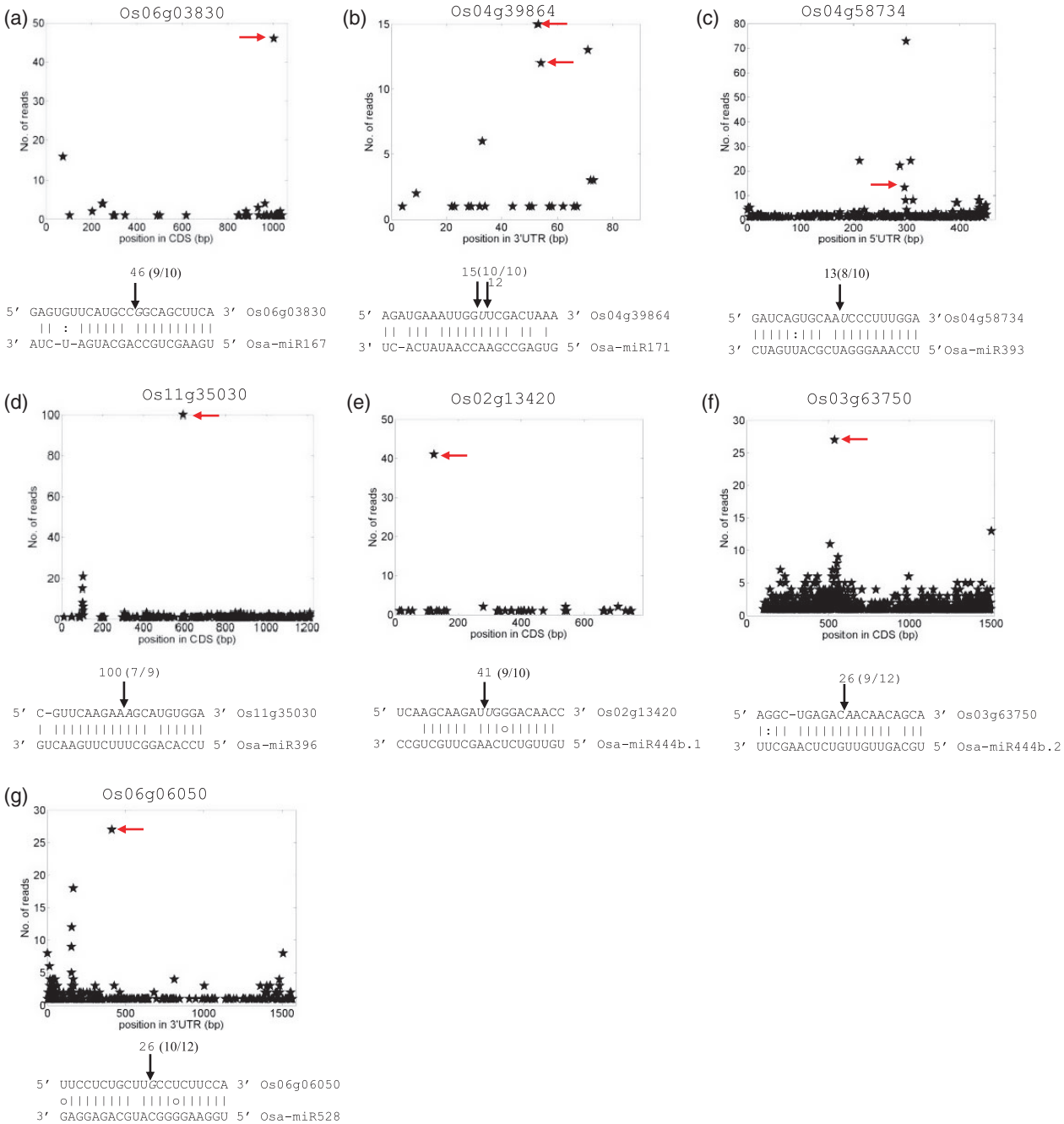

Figure 3. Identification of non-conserved targets for conserved rice microRNAs (miRNAs).
The italicized nucleotide on the target transcript from the 3′ end indicates the cleavage site detected in the degradome. Primer sequences used for 5′-rapid amplification of cDNA ends (5′-RACE) are provided in Table S3.
(a) Target plots (t-plot) and alignment of confirmed non-conserved target (*Os06g03830*) for miR167 in rice. Absolute numbers of signature sequences are indicated. Arrow indicates signatures consistent with miRNA-directed cleavage. Cleavage frequency as determined by gene-specific 5′-RACE at the indicated position is shown in parentheses.
(b) As in (a) for the regulation of the non-conserved target *Os04g39864* by the conserved miRNA, miR171.
(c) As in (a) for the regulation of the non-conserved target *Os04g58734* by the conserved miRNA, miR393.
(d) As in (a) for the regulation of the non-conserved target *Os11g35030* by the conserved miRNA, miR396.
(e, f) As in (a) for the regulation of the non-conserved targets *Os02g13420* and *Os03g63750* by two variants of the conserved miRNA, miR444.
(g) As in (a) for the regulation of *Os06g06050* by the non-conserved miRNA, miR528.

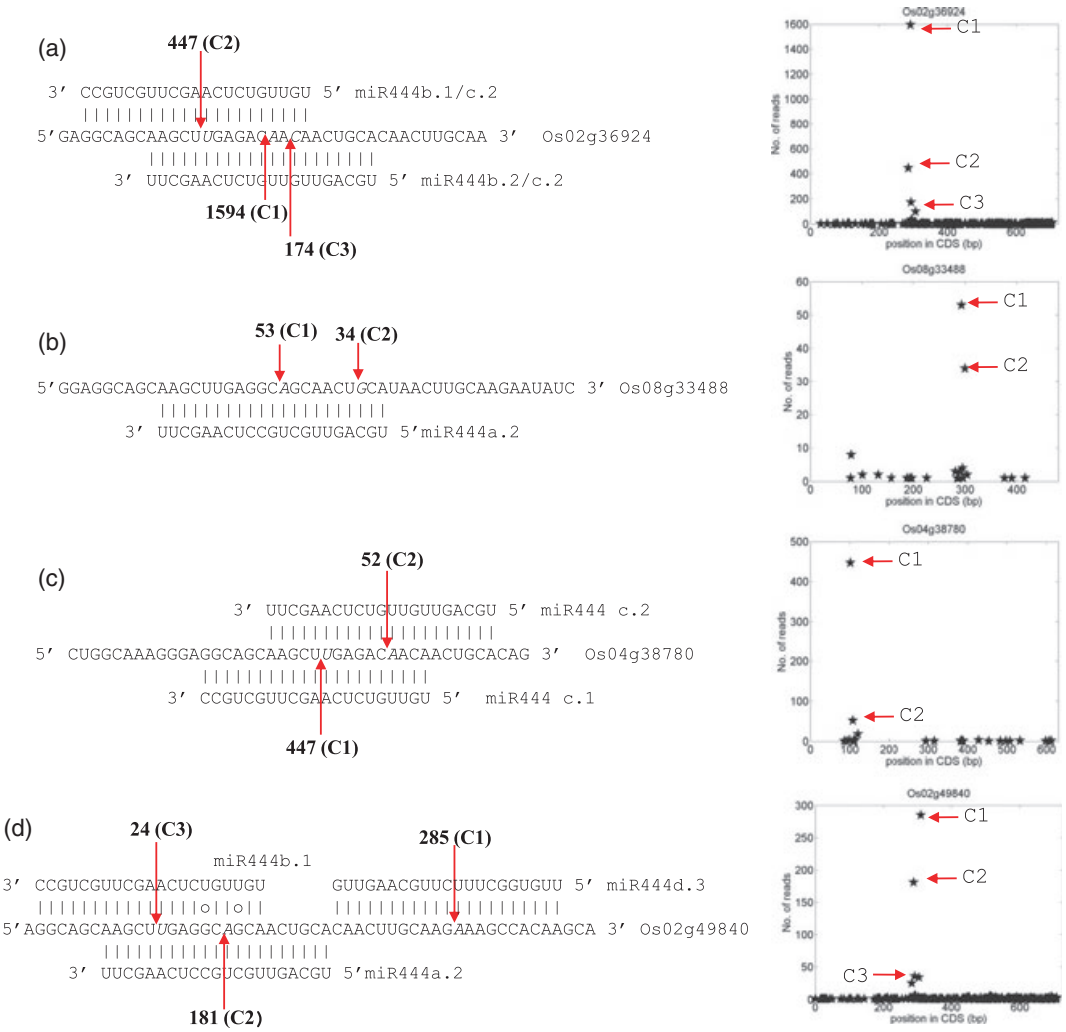

Figure 4. Regulation of four *MADS*-box genes by miR444 variants in rice.
The italicized nucleotide on the target transcript from the 3′ end indicates the cleavage site detected in the degradome.
MicroRNA (miRNA):mRNA alignments and target plots (t-plots) for the four *MADS*-box transcripts. The detected cleavage frequencies (absolute numbers) are shown. Arrows indicate signatures consistent with miRNA-directed cleavage. Cleavage frequency at different indicated positions is shown as C1, C2 and C3.
(a) *Os02g36924* aligned with miR444b.1/c.2 and miR444b.2/c.2.
(b) *Os08g33488* aligned with miR444a.2.
(c) *Os04g38780* aligned with miR444c.2 and miR444c.1.
(d) *Os02g49840* aligned with miR444a.2 and miR444d.3.

There are no clear correlations between the miRNA level and the cleavage abundance of its target transcript

Most conserved miRNAs regulate multiple transcripts belonging to the same gene family (Jones-Rhoades *et al.*, 2006; Mallory and Vaucheret, 2006). We found remarkable differences in tag abundance on the transcripts targeted by different miRNA families. For example, cleavage abundance associated with the *ARF*s (*Os06g47150*) targeted by miR160 was the most abundant [115 286 transcripts per billion (tpb)], while an *SBP* transcript (*Os08g39890*) targeted by miR156 was much lower (3661 tpb) (Table 2). Such huge disparities in cleavage abundance between different transcripts targeted by different miRNA families could be attributed either to the differential miRNA abundance or target transcript abundance in the analyzed sample or to different overlaps of expression between miRNA and transcript at the tissue or cellular levels. Small RNA blot analysis for miR156, miR159, miR160, miR169 and miR172 revealed only minor differences in their expression levels in rice seedlings (Figure 7a) and this is unlikely to account for the huge differences in cleavage abundance of their target transcripts. The relative abundance of their corresponding target transcripts was assessed using quantitative real-time PCR and the difference in abundance among these targets does not support the differences in cleavage abundance

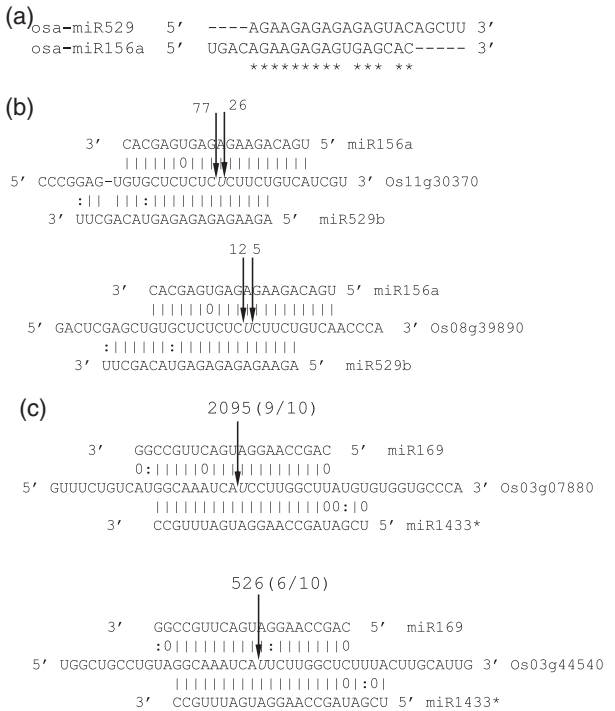

Figure 5. Cleavage abundance on target transcripts harboring overlapping complementary sites for two distinct microRNAs (miRNAs).
The italicized nucleotide on the target transcript from the 3′ end indicates the cleavage site detected in the degradome.
(a) miR156 and miR529 sequence alignments.
(b) An *SBP* transcript aligned with miR156 and miR529 with cleavage frequencies shown.
(c) miR169 and miR1433*. The observed cleavage frequency at the indicated position is shown. The numbers in parenthesis represent the cleavage frequencies obtained using a gene-specific 5′-rapid amplification of cDNA ends (5′-RACE) assay.

(Figure 7b). Thus, comparisons between the miRNA and target transcript abundance did not reveal a clear relationship between them. Consistent with our observation, no clear negative correlations between the miRNA abundance and the cleavage frequency of target transcripts in Arabidopsis has been reported (Jiao *et al.*, 2008).

Differential cleavage frequencies for targets of the same miRNA

Within a related family of targets, we found that some have much higher levels of slicing remnants than others. A few examples are *ARF*s, *MYB* mRNAs, *TIR1* homologs and *NAC* transcripts that are targeted by different miRNA families. Cleavage frequencies associated with the two *ARF*s (*Os06g47150* and *Os10g33940*) are the most abundant, whereas two other *ARF*s (*Os02g41800* and *Os04g43910*) are extremely low (Table 2), although all these four genes are targeted by miR160. Similarly, two *TIR1* homologs and two *MYB* transcripts had remarkable differences in their cleavage frequencies (Table 2). The differential cleavage frequency at the expected target site within a related family of transcripts could be due to the differential target transcript abundance in the tissue or due to differences in complementarity (high or low) between the miRNA and its target transcript. The latter appears to be the case for *MYB* transcripts targeted by miR159 (Figure 1). However, there were no differences with respect to complementarity between the miR156, miR160 and miR393 and their respective RNA targets belonging to related gene families. Quantitative real-time PCR data (Figure 7b) was used to examine whether differential abundance of the target transcript is the cause. The lowest and highest cleavage frequencies of *ARF* and *SBP* genes appear to be correlated with their abundance (Figure 7b). However, no such correlations were observed for *MYB* transcripts targeted by miR159 (Figure 7b). These mixed results suggest that several attributes, including target RNA abundance, complementarity and probably as yet unidentified factors, contribute to the differential cleavage frequencies within a related family of miRNA targets.

miR156 and miR172 have been shown to regulate their target genes (*SBP*s and *AP2*) predominantly by inhibiting the translation of target genes, and transcript cleavage appears to be a minor mode of regulation (Aukerman and Sakai, 2003; Chen, 2004; Schwab *et al.*, 2005; Gandikota *et al.*, 2007). We detected lower cleavage frequencies on *SBP*s as well as *AP2* genes (Table 2). In contrast, the abundance of miR156 and miR172 is very similar to that of some of the very abundantly expressed miRNAs such as miR159 and miR160 (Figure 7a), but they resulted in a small cleavage frequency on *SPB* and *AP2* transcripts. On the other hand the transcript abundance as determined by real-time PCR suggests a moderate abundance of *SBP* genes in rice seedlings (Figure 7b). On the basis of observed lower cleavage frequency on these two target gene families but abundant expression of miR156 and miR172, we speculate that the translational repression could be the predominant mode of regulation in rice, just like in Arabidopsis.

MIRNA precursor degradome patterns can distinguish primary (pri)-miRNA transcripts from siRNA-generating transcripts

The approach used here can also identify signatures associated with Dicer activity on *MIRNA* precursors because, like AGO-catalyzed slicing, dicing leaves a fragment with a 5′-monophosphate, which is competent for ligation with an RNA adapter. A total of 42 915 degradome reads perfectly mapped to the 325 (conserved and non-conserved) unique *MIRNA* precursors (Table 1). Interestingly, the detected cleavages on the conserved *MIRNA* precursors showed a unique pattern (Figures 8 and S4, Table S2). Observed cleavages on conserved *MIRNA* precursors were classified as one of the following six types: (i) at the beginning of miRNA (5′ end of the mature miRNA), (ii) at the end of

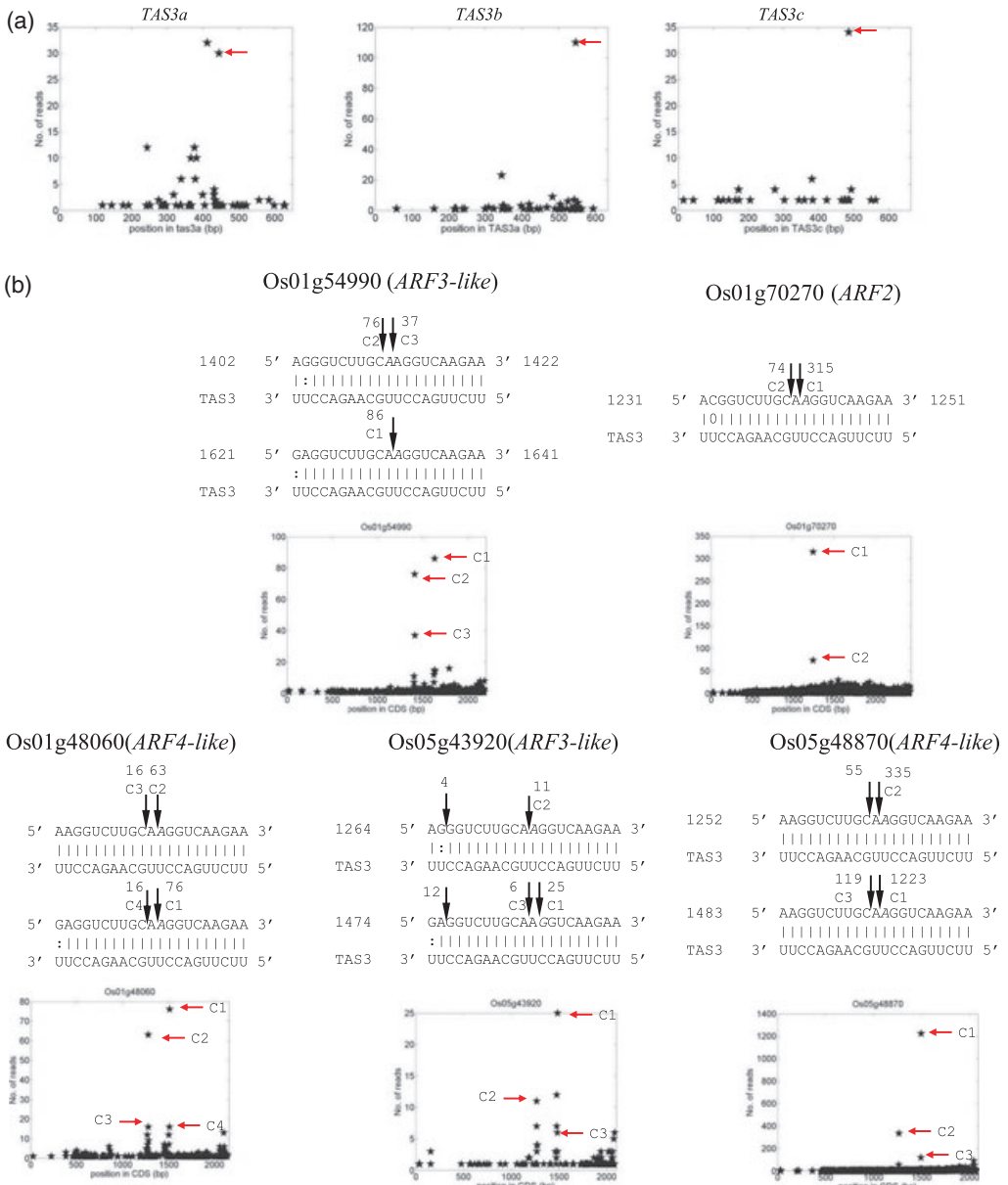

Figure 6. Observed cleavage abundance on *TAS3*-siRNA (small interfering RNA) precursors and their targets in rice.
The italicized nucleotide on the target transcript from the 3′ end indicates the cleavage site detected in the degradome.
(a) Cleavage abundance on *TAS3*-siRNA precursors.
(b) Alignment of TAS3-siRNA and its target *ARF* transcripts along with target plots (t-plots) for *ARF* transcripts. Cleavage frequency at different indicated positions is shown as C1, C2 and C3.

miRNA (3′ end of the mature miRNA), (iii) beginning of miRNA star, (iv) at the end of miRNA star, (v) middle of miRNA/miRNA*, and (vi) random cleavages on the precursor besides the expected cleavages at the beginning/end of miRNA/miRNA*. The first four types potentially represent Dicer-guided cleavages, whereas the latter two types represent AGO-mediated cleavages or other types of cleavage that occurred by chance in the middle of miRNA or miRNA*. These observations are similar to what has been reported for Arabidopsis and *P. patens* (German *et al.*, 2008; Addo-Quaye *et al.*, 2009b) and suggest that processing of *MIRNA* precursors in rice is similar to in Arabidopsis. Interestingly, two-thirds of cleavage events could be mapped precisely at the beginning of the mature miRNA (5′ end of the mature miRNA) (Table S2). Mapping the cleaved fragments to the miRNA pri-transcripts yielded interesting insights into the regulation of miRNA abundance in Arabidopsis (German *et al.*, 2008). Signatures indicating a cleavage event in the middle of either miRNA or miRNA* were poorly represented in our sequences (Table S2),

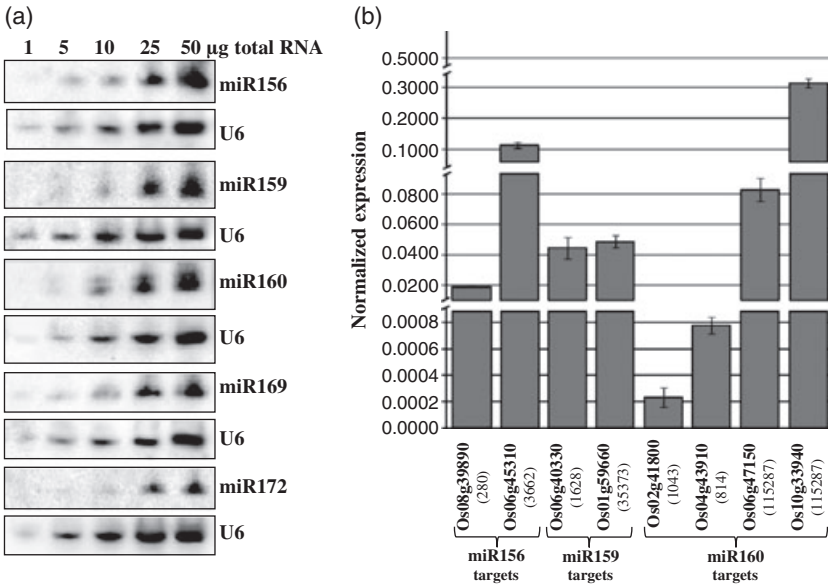

Figure 7. Determination of relative expression abundance of microRNAs (miRNAs) and their corresponding target genes in rice seedlings.
(a) Expression levels of miR156, miR159, miR160, miR169 and miR172 as determined by using varying amounts of total RNA.
(b) Expression levels of different members of *SBP*, *MYB* and *ARF* gene families targeted by miR156, miR159 and miR160, respectively as determined by real-time PCR. The amplified fragment spans the miRNA target site and thus measures accumulation of the unsliced mRNA transcript.

indicating that self-regulation of *MIRNA* precursors by the miRNA or miRNA* is not widespread in rice.

Based on resemblances of the cleavage pattern between conserved and non-conserved *MIRNA* precursors, 35 non-conserved miRNAs qualify for annotation of miRNAs in rice (Table S2, Figure S4). Intriguingly, the processing of several non-conserved pri-miRNA transcripts in rice does not resemble typical miRNA processing as discussed above, consequently their designation as miRNAs should be reconsidered (Figure S5). Consistent with our results, Lu *et al.* (2008a) also suggested that several small RNAs, including miR439, miR442, miR445, miR812, miR813, miR815, miR818 and miR819 are not miRNAs, based on cloning small RNAs from both the strands, which is typical of siRNA loci but not *MIRNA*s. Some other annotated rice miRNAs, such as miR441, miR808, miR809, miR810, miR1847, miR1871, miR1875 and miR2123, also appear to be border-line examples (Figure S6) and confident annotation of these as miRNAs warrants their expression analysis using a rice *dcl1* mutant. Thus, the dicing events on *MIRNA* precursor could be considered as ancillary criteria for annotation of miRNAs (Meyers *et al.*, 2008).

DISCUSSION

Most of the rice transcriptome exists in an uncapped form

In a cell, the steady-state level of mRNA is dependent on the rate of its synthesis and degradation; thus understanding mRNA degradation is as important as studying the transcriptional regulation. It is well recognized that there are multiple ways through which an mRNA is degraded. One important mode relevant to this study is de-capping followed by subsequent digestion by a 5′–3′ exonuclease activity. Such uncapped transcripts could be potentially cloned using the protocol described in this study and elsewhere (Addo-Quaye *et al.*, 2008; Jiao *et al.*, 2008). A total of 11 552 007 unique signatures perfectly matching with the rice genome corresponding to 51 682 annotated genomic loci have been identified in this study. Intriguingly, only a small fraction of these are clearly small RNA targets, implying that the vast majority of them are other types of RNA turnover products. Currently, the TIGR rice database has 52 678 annotated unique transcripts. Our study suggests that 51 682 of these (98%) exist in uncapped form. These observations suggest that overall transcriptome abundance in rice is dynamically regulated through uncapping and a 5′–3′ exosome-mediated turnover pathway. A similar study in Arabidopsis has uncovered uncapped transcripts for more than 90% of the expressed genes, suggesting that the majority of the mRNAs exist in uncapped form (Jiao *et al.*, 2008).

Conserved and non-conserved targets for the conserved miRNAs in rice

Identification of a putative copper chaperone as target for the conserved miR398, in addition to the previously known Cu/Zn SODs, resembles the situation with miR395 and miR399, both of which can target two diverse gene families that fall into one biochemical pathway (Allen *et al.*, 2005). Validation of a Cu chaperone (*CCS1*) as another conserved target for miR398 illustrates the fact that the identification of all conserved targets for conserved miRNAs is still incomplete and there may be additional targets with poor complementarity which have (so far) escaped identification. This unbiased experimental approach is well-suited to finding such targets, provided that they are sliced at appreciable levels.

Computational analysis of the dataset confirmed 160 mRNAs as genuine targets for rice miRNAs. If a conserved

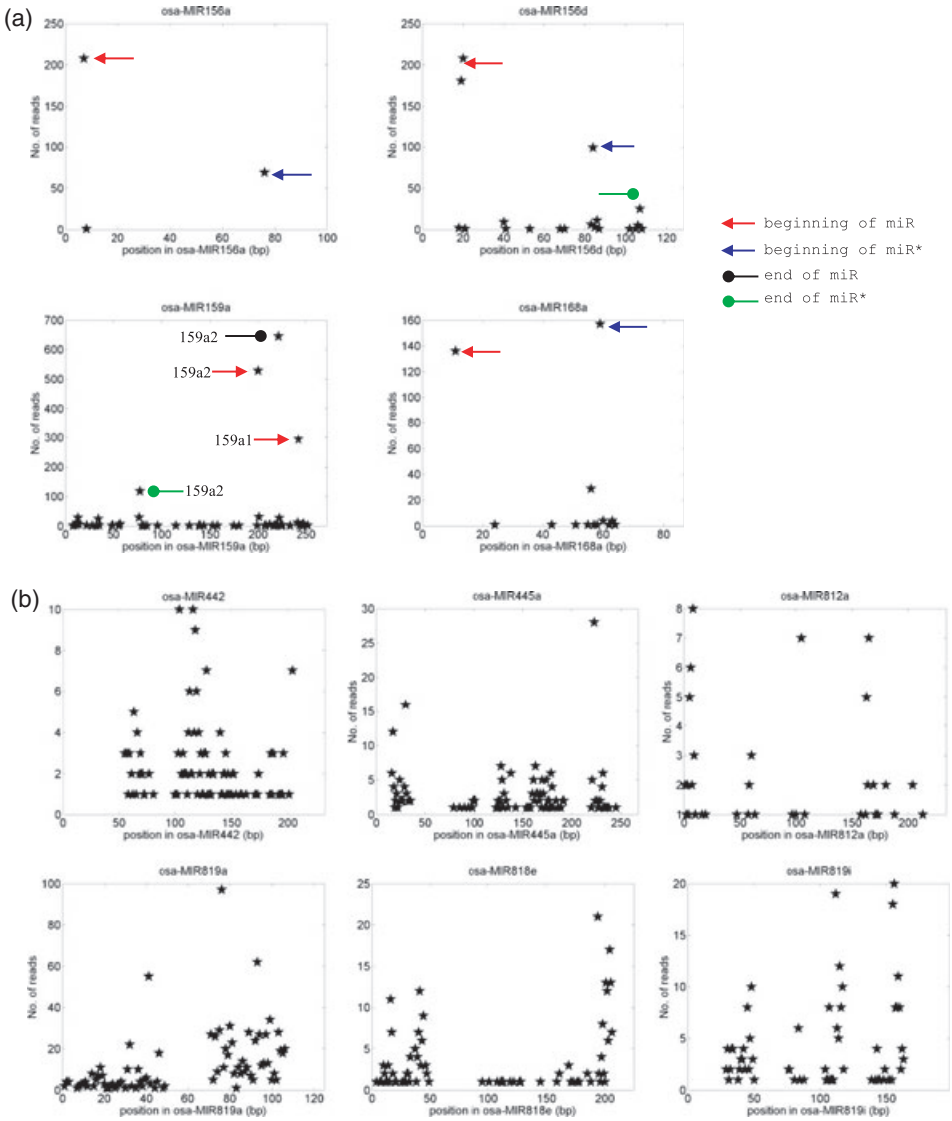

Figure 8. Degradome signature abundance corresponding to annotated *MIRNA* loci.
(a) Representative plots for conserved *MIRNA* loci.
(b) Representative plots for potentially mis-annotated *MIRNA* loci.

miRNA is targeting more than one gene, it is generally known to regulate the multiple members of the same gene family or at least genes related to a common biochemical pathway. In sharp contrast to this, recent findings revealed that miR168 and miR159 are targeting two different gene families that participate in entirely unrelated biochemical pathways in Arabidopsis (German *et al.*, 2008). Similarly, besides the homologous targets for conserved miRNAs in rice, we found that nine families of conserved miRNAs (miR156, miR164, miR166, miR167, miR171, miR172, miR393, miR396 and miR444) can potentially regulate 23 mRNA targets belonging to non-homologous target gene families. Thus far, computational target predictions in plants have used relatively conservative cut-offs, which typically do not allow more than 3 or 3.5 mismatches between a miRNA and its target. More importantly, no mismatches or gaps are allowed at position 10 and 11 where the slicing takes place (Schwab *et al.*, 2005). In this study, a putative retinol dehydrogenase transcript with a mismatch at position 11 was cleaved abundantly (Table 3). Gene-specific 5′-RACE assay further confirmed it as a non-conserved target for miR167 in rice (Figure 3a). These findings suggest that homology-based target predictions may have underestimated the number of targets that miRNAs are regulating in plants. However, it remains to be seen whether the slicing of these non-canonical targets directed by conserved miRNAs is biologically relevant or merely a neutral, accidental event.

Many non-conserved miRNAs may not have detectable sliced targets

Many non-conserved miRNAs are thought to be non-functional because either their predicted targets have proven impossible to validate or no targets are even predictable (Rajagopalan *et al.*, 2006; Fahlgren *et al.*, 2007). To date, 107 rice-specific miRNA families have been annotated in miRBase. Thus far, it is unknown how many of these are truly rice-specific, because their presence or absence in closely related species has not been determined. In this study, we have confirmed targets for 29 non-conserved miRNAs in rice, in addition to the four rice-specific miRNAs (miR820, miR827, miR1425 and miR1428-3p) for which targets had previously been validated (Luo *et al.*, 2006; Heisel *et al.*, 2008; Lacombe *et al.*, 2008; Lu *et al.*, 2008a; Zhu *et al.*, 2008). We have not analyzed many different tissues or developmental stages or stress conditions that could also limit the identification of targets for some of the non-conserved miRNAs. A functional miRNA is expected to regulate the expression of at least one gene at the post-transcriptional or translational level. Recently, Brodersen *et al.* (2008) and Lanet *et al.* (2009) have shown that many plant miRNAs repress the translation of their target RNAs as well as direct their slicing. Our method is incapable of finding their targets if non-conserved miRNAs tend to translationally repress targets in the absence of detectable slicing (Voinnet, 2009). Overall, the fact that targets for the most non-conserved miRNAs (about 70%) could not be identified despite using high-throughput approaches suggests that, at a minimum, conserved and non-conserved miRNAs function in consistently different manners.

Not all rice miRNAs deposited in the miRBase are bona fide miRNAs

Plants produce a vast number of endogenous siRNAs. Although they differ in origin, they closely resemble miRNAs in their structural and biochemical characteristics, and this poses a problem in distinguishing between miRNAs and siRNAs. Confident annotation of a small RNA as miRNA requires the detection of discretely produced, hairpin-derived miRNA/miRNA* duplex (Meyers *et al.*, 2008). We suggest that the sequenced tags associated with the pri-miRNA transcripts can be added to the list of ancillary criteria for annotation of a small RNA (Figures 8 and S4–S6). Most genuine miRNA precursors have typical cleavage patterns (beginning/end of miR/miR*), whereas siRNA precursors are cleaved randomly; thus, degradome patterns can distinguish miRNAs from other small RNA-producing loci.

The potential biological significance of identified miRNA targets in rice

Many of the identified rice miRNA targets belong to diverse gene families of transcription factors such as *SPL*s, *ARF*s, *MYB*s, *TCP*s, *NAC*s, *HD-ZIP*s, *NF Y* subunits, *AP2*-like factors, *SCL*s, *GRF*s and *MADS*-box factors (Table 2). Many of these transcription factors are known to regulate diverse aspects of plant growth and development. For instance, *SPL*s and *AP2*-like factors targeted by miR156 and miR172, respectively, have been shown to play an important role in phase changes (from juvenile to adult and from vegetative to the reproductive phase) in Arabidopsis (Poethig, 2009). Patterning and outgrowth of lateral organs in plants depend on the specification of adaxial/abaxial (dorsoventral) polarity in the incipient primordium, which is established through the polarized expression of *HD-ZIPIII* transcription factors that specify adaxial/upper cell fate (Emery *et al.*, 2003; Juarez *et al.*, 2004; Nogueira *et al.*, 2007). In Arabidopsis and maize (*Zea mays*), the adaxial-specific expression of *HD-ZIPIII* family members is delineated by the expression pattern of miR166 (Juarez *et al.*, 2004; Kidner and Martienssen, 2004; Nogueira *et al.*, 2007). Another important family of transcription factors worth pointing out here is the *MADS*-box gene family. Four *MADS*-box genes were validated as targets for miR444 variants in rice. Most importantly our study discovered that these four *MADS*-box factors are regulated by several miR444 variants in a combinatorial fashion (Figure 4). *MADS*-box genes are known to play a critical role in determining organ specificity during flower development in Arabidopsis (Kaufmann *et al.*, 2009). Besides, *MADS*-box factors in rice have also been hypothesized to play important roles in meristem identity, formation of the dehiscence zone, fruit ripening and embryo development as well as development of vegetative organs such as root and leaf (Arora *et al.*, 2007).

Besides their possible involvement in plant development, miRNA targets identified in this study could also play fundamental roles in biotic and abiotic stress resistance in rice. For instance, the NFYA5 transcription factor, whose transcript is a target for miR169, has been implicated in drought stress responses in Arabidopsis (Li *et al.*, 2008). Overexpression of a miR169-resistant *NFYA5* transgene significantly improves drought resistance by promoting stomatal closure under drought stress (Li *et al.*, 2008). Eight *NF* transcription factor Y subunit mRNAs were identified as targets of miR169 in rice (Table 2). Similarly, two *MYB* family members in Arabidopsis (*MYB33* and *MYB101*), which are targeted by miR159, appear to play an important role in response to ABA accumulation under drought stress, suggesting their roles in the stress response (Reyes and Chua, 2007). Two Cu/Zn-SODs targeted by miR398 in rice are well established as critical players in mitigating oxidative stress, which commonly occurs during biotic and abiotic stress (Sunkar *et al.*, 2006; Jagadeeswaran *et al.*, 2009a). Additionally, laccases (enzymes involved in cell wall metabolism), copper chaperones, plantacyanin-like proteins (involved reproduction and seed setting) and F-box proteins involved in auxin-stimulated protein degradation (TIR1-like) were

among the confirmed targets in rice (Table 2). Interestingly, several non-conserved targets including HSF (heat-shock factor)-type transcription factor, LRR kinase and retinol dehydrogenase were independently validated as genuine targets for some of the conserved miRNAs in rice. Heat-shock factor-type transcription factors (target of miR444) have been suggested to play a role in plant development and environmental stress responses (von Koskull-Doring *et al.*, 2007). The LRR kinases have been reported to play important roles in plant development, brassinosteroid and ABA signal transduction and more prominently in plant–pathogen interactions (Zhang *et al.*, 2006). Taken together, validated miRNA targets in rice are potentially involved in diverse biological processes such as plant growth and development including phase transitions, flowering, hormone signaling and biotic and abiotic stress resistance in rice.

CONCLUSIONS

Overall, degradome sequencing is an effective tool for confirming small RNA targets in plants. More specifically, this method can identify additional conserved and non-conserved targets for miRNAs, which cannot be identified with confidence by predictions alone. Application of this method also appears to be helpful in distinguishing a genuine miRNA precursor from that of a siRNA precursor. Signatures corresponding to some of the validated rice miRNA targets (Heisel *et al.*, 2008; Lu *et al.*, 2008a; Zhu *et al.*, 2008) could not be found in our reads, which could be due to the possibility that either miRNAs or targets are not expressed in the tissue analyzed. The identification of miRNA targets is restricted to 3-week-old rice seedlings in this study. We have not analyzed many different tissues and developmental stages or other conditions (biotic, abiotic and nutrient-deprived conditions), which potentially limits the identification of additional targets both for conserved and non-conserved miRNAs in rice. Thus, the potential to identify additional miRNA targets by extending this type of analysis to diverse tissues, developmental stages and stress conditions is fairly high. In summary, the current study has confirmed a large set of targets (165 genes) that are subjected to small RNA-guided degradation and sets a stage to explore potential roles of conserved and rice-specific miRNAs.

Note

While our manuscript was in revision, Wu *et al.* (2009) reported the identification of 66 sliced miRNA targets in rice using degradome sequencing. However, our degradome sequencing depth is at least three fold greater and revealed a greater number of miRNA targets in rice. More importantly, we have uncovered that *CCS1* as a conserved target for miR398 and we provide independent confirmation of several unexpected targets for well-established miRNA families in rice.

EXPERIMENTAL PROCEDURES

Degradome library construction

Construction of a small cDNA library using the sliced ends of polyadenylated transcripts from 3-week-old rice seedlings was according to Addo-Quaye *et al.* (2008) and German *et al.* (2008). In brief, polyadenylated transcripts possessing 5′ monophosphates were ligated to an RNA adapter consisting of a *Mme*I recognition site in its 3′ end. After ligation, first-strand cDNA was generated using oligo d(T) and the PCR product was amplified using five PCR cycles. The PCR product was purified and digested with *Mme*I. The digested PCR product was then ligated to a double-stranded DNA oligo with degenerate nucleotides at the 5′ or 3′ end. The ligation product was further gel purified and amplified using 10 PCR cycles. The final PCR product was purified and sequenced using SBS (Illumina, http://www.illumina.com/) sequencing technology.

Computational analysis

Initial processing and analysis of reads in our sequencing libraries

The sequences of CDS, 5′ UTRs, 3′ UTRs, introns, intergenic regions, 1 kb upstream regions, chloroplasts, mitochondria and chromosomes of rice were downloaded from the TIGR Rice Genome Annotation database (version 5, http://rice.plantbiology.msu.edu/). The sequences of rRNAs, tRNAs, snoRNAs and snRNAs were retrieved from the Rfam database (http://www.sanger.ac.uk/Software/Rfam/). The sequences of pre-miRNAs were obtained from miRBase (v13.0, http://microrna.sanger.ac.uk/sequences/). The sequences of *TAS3* genes were downloaded from the NCBI EST database and obtained from Lu *et al.* (2008a). The repeat elements were downloaded from the TIGR Rice Genome Annotation database and RepBase (http://www.girinst.org/server/RepBase/).

The 39.4 million sequences (reads) of 20 nt in length were aligned to the sequences of CDS, 5′ UTRs, 3′ UTRs, introns, intergenic regions, repeat elements, ncRNAs (rRNAs, tRNAs, snoRNAs and snRNAs), 1 kbp upstream sequences, chloroplasts, mitochondria, pre-miRNAs, *TAS3* transcripts and chromosomes using NCBI BLASTN. The numbers of valid reads that were mapped to different categories of sequences are summarized in Table 1. Raw data are available at NCBI-GEO accession no. GSE17398.

Identification of sliced miRNA targets

The *CleaveLand* pipeline (Addo-Quaye *et al.*, 2009a) was used to find sliced miRNA targets using the TIGR transcripts (version 5) and all *Oryza* miRBase 13.0 mature miRNA sequences as input. The initial *CleaveLand* output, including all matches for alignment scores up to 7, was first condensed to remove redundant cleavage sites, which often resulted from multiple sequence variants from the same miRNA family. Candidate targets were further filtered to eliminate instances with alignment scores exceeding 4.5, 3.5 and 2.5 for category one, two and three targets, respectively. Ten randomized shuffles were analyzed for each miRNA query. If any of these shuffles found a hit with an alignment score at or below the initial thresholds, the final alignment score cut-off for that query was lowered to 0.5 below the score of the random hit.

Small RNA blot analysis

Total RNA was extracted from seedlings or rosette leaves with TRIZOL reagent (Invitrogen, http://www.invitrogen.com/). The miRNA expression analysis was determined using total RNA

isolated from rice seedlings as reported previously (Sunkar *et al.*, 2005).

Quantitative real-time PCR

Total RNA was extracted from seedlings or rosette leaves with TRIzol reagent (Invitrogen). Real-time PCR was carried out using the same RNA samples that were used for northern analysis. Total RNA (2 μg) was treated with DNase I and reverse transcribed using oligo-dT primer, reverse transcriptase and deoxynucleotide triphosphates. Real-time PCR analysis was carried out using Maxima® SYBR Green qPCR Master Mix (Fermentas, http://www.fermentas.com/) in a 7500 Real-Time PCR System using 100 ng cDNA and 7.5 pmol of each gene-specific primer. The analysis was performed using two independent cDNA preparations and triplicate PCR reactions. The relative expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method with actin as the reference gene.

Co-expression of miR398 and CCS1 in *N. benthamiana* leaves

The constructs harboring *MIR398a* or *CCS1* under the control of super promoter (pBIB) were transformed into *A. tumefaciens* strain GV3101 and these cells were infiltrated into *N. benthamiana* leaves as described by English *et al.* (1997). For co-expression analysis, equal volumes of *Agrobacterium* culture containing *Ath-MIR398a* and Arabidopsis *CCS1* were mixed before infiltration into leaves of *N. benthamiana*.

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SUPPORTING INFORMATION

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

Figure S1. t-plots for category I targets.

Figure S2. t-plots for category II targets.

Figure S3. t-plots for category III targets.

Figure S4. Plots of degradome tags on rice-specific *MIRNA* precursors.

Figure S5. Plots of degradome tags on likely mis-annotated *MIRNA* precursors.

Figure S6. Plots of degradome tags on possibly mis-annotated *MIRNA* precursors.

Table S1. Targets of non-conserved microRNAs (miRNAs) identified by rice degradome sequencing.

Table S2. Observed frequencies and patterns of degradome tags on *MIRNA* pri-transcripts.

Table S3. Primer sequences used for 5′-rapid amplification of cDNA ends (5′-RACE) to validate microRNA (miRNA) targets.

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