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RESEARCH ARTICLE

Degradome sequencing reveals endogenous small RNA targets in rice (*Oryza sativa* L. ssp. *indica*)

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Abstract MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) regulate gene expression in eukaryotes. Plant miRNAs modulate their targets mainly via messenger RNA (mRNA) cleavage. Small RNA (sRNA) targets have been extensively investigated in *Arabidopsis* using computational prediction, experimental validation, and degradome sequencing. However, small RNA targets are largely unknown in rice (*Oryza sativa*). Here, we report global identification of small RNA targets using high throughput degradome sequencing in the rice *indica* cultivar *93-11* (*Oryza sativa* L. *ssp*. *indica*). One hundred and seventy-seven transcripts targeted by a total of 87 unique miRNAs were identified. Of targets for the conserved miRNAs between *Arabidopsis* and rice, transcription factors comprise around 70% (58 in 82), indicating that these miRNAs act as masters of gene regulatory nodes in rice. In contrast, non-conserved miRNAs targeted diverse genes which provide more complex regulatory networks. In addition, 5 *AUXIN RESPONSE FACTORs* (*ARFs*) cleaved by the *TAS3* derived ta-siRNAs were also detected. A total of 40 sRNA targets were further validated via RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM 5′-RACE). Our degradome results present a detailed sRNA-target interaction atlas, which provides a guide for the study of the roles of sRNAs and their targets in rice.

Keywords Degradome, miRNA, ta-siRNA, small RNA targets, rice

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1 Introduction

MicroRNAs (miRNAs) and small interfering RNAs (siRNAs) are the two major types of 21-24-nucleotide (nt), endogenous non-coding RNAs in higher plants (Llave et al., 2002b). MiRNAs are originally transcribed from single RNA molecules and form primary miRNAs (pri-miRNAs), which contain imperfect local stem-loop structures (Bartel, 2004). In higher plants, the Dicer or Dicer-like (DCL) protein 1 (DCL1) subsequently processes the pri-miRNAs and pre-miRNA precursors, forming the miRNA:miRNA* duplex. The mature miRNAs are then incorporated into ARGONAUTE (AGO) protein-containing RNA-induced silencing complex (RISC), where they direct gene silencing (Song et al., 2004; Mi et al., 2008). In animals, miRNAs regulate their targets via translational repression, whereas many plant miRNAs modulate their targets via messenger RNA (mRNA) cleavage at sites with nearly perfect complementarity to the miRNA sequence (Llave et al., 2002a; Bartel, 2009). Recently, small RNA mediated translational repression was also recognized as a widespread mode of action in higher plants (Brodersen et al., 2008).

In *Arabidopsis*, miRNAs have been shown to play important roles in various biological processes, including developmental regulation, hormone response, and stress adaptation (Jones-Rhoades et al., 2006). Increasing evidence demonstrates the critical roles of miRNAs in developmental regulation mainly through identification of miRNA mutations or overexpression of miRNA-resistant targets (Jones-Rhoades et al., 2006). For example, miR160 directs the cleavage of *AUXIN RESPONSE FACTOR 10* (*ARF10*), *ARF16*, and *ARF17*. Plants expressing a

miR160-resistant version of *ARF17* have dramatic developmental defects and miR160-overexpression plants show disorganized root tips and fewer starch granules (Mallory et al., 2005; Wang et al., 2005). Moreover, miR162 and miR168 target *DCL1* and *AGO1*, respectively, suggesting that miRNAs auto-regulate their own biogenesis and function (Xie et al., 2003; Vaucheret et al., 2004). In addition to targeting important mRNAs, miRNAs can also act on non-coding RNAs resulting in the biogenesis of plant specific trans-acting siRNAs (ta-siRNAs) in higher plants (Peragine et al., 2004; Vazquez et al., 2004).

Unlike *Arabidopsis*, much less is known about the important roles of miRNAs in rice development. In rice, loss-of-function of *Os DCL1* (*OsDCL1IR*) results in reduction of miRNA accumulation and *OsDCL1IR* transgenic plants display developmental arrest or pleiotropic developmental defects, indicating that miRNAs also play a key role in rice development (Liu et al., 2005). Overexpression of OsmiR156b and OsmiR156h genes result in severe dwarfism, strongly reduced panicle size and delayed heading date, indicating that miRNAs play critical roles in agricultural traits (Xie et al., 2006). In addition, *TAS3* transcripts, which are conserved between rice and *Arabidopsis*, are processed into ta-siRNAs by Os DCL4, a homolog of *Arabidopsis* DCL4 (Liu et al., 2007a). The *osdcl4-1* null mutant shows a more severely defective phenotype than the corresponding mutation in *Arabidopsis*, suggesting Os DCL4 plays a broader role in rice (Liu et al., 2007a).

So far, hundreds of small RNAs have been isolated by direct cloning and by deep sequencing in higher plants (Griffiths-Jones et al., 2008). Elucidating the function of these tiny molecules requires efficient approaches to identify their targets. Originally, plant miRNA targets have been studied via computational prediction, which is based on either the perfect or nearly perfect sequence complementarity between a miRNA and the target mRNA or sequence conservation among different species (Rhoades et al., 2002). However, targets prediction is very challenging, especially when a high level of mismatches exists in miRNA:target pairing (Jones-Rhoades and Bartel, 2004). Recently, a new method called degradome sequencing, which combines high throughput deep sequencing with bioinformatics tools, has been successfully established to screen for miRNA targets in *Arabidopsis* (Addo-Quaye et al., 2008; German et al., 2008; Gregory et al., 2008). Using degradome sequencing, over 100 of the previously validated and predicted targets of miRNAs and ta-siRNAs were experimentally identified (Addo-Quaye et al., 2008; German et al., 2008), indicating that it is an efficient strategy to identify small RNA targets in a large scale in plants.

Rice is one of the most important crops for human food resources in the world. The rice genome has been sequenced both in the cultivar *93-11* (*Oryza sativa* L. *ssp*. *indica*) and *Nipponbare* (*Oryza sativa* L. *ssp*. *japonica*) (Goff et al., 2002; Yu et al., 2002). Small RNAs have also been extensively discovered in rice (Reinhart et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Liu et al., 2005; Sunkar et al., 2005; Luo et al., 2006; Lacombe et al., 2008; Lu et al., 2008; Sunkar et al., 2008; Zhu et al., 2008; Johnson et al., 2009; Xue et al., 2009). However, rice small RNA targets were mainly predicted via computational approaches, and only a small proportion of targets have been validated experimentally (Sunkar and Zhu, 2004; Sunkar et al., 2005; Yang et al., 2006; Liu et al., 2007a; Lacombe et al., 2008; Lu et al., 2008; Sunkar et al., 2008; Zhu et al., 2008; Liu et al., 2009). Especially, validated targets of the conserved small RNAs largely remain elusive. To comprehensively investigate small RNA targets and provide basic information for further understanding the miRNA-mediated posttranscriptional regulation network in rice, a degradome library derived from young panicles of a typical *indica* rice cultivar, *93-11*, was constructed and used for sequencing by synthesis (SBS) sequencing. Here, we show a total of 182 distinct transcripts sliced by small RNAs and then captured as part of the degradome. One hundred and seventy-four genes targeted by 87 unique miRNAs were detected in our rice degradome library. Compared with *Arabidopsis*, approximately 70% of the identified targets for conserved miRNAs (existing both in *Arabidopsis* and rice) were members of transcription factors within a gene family, whereas non-conserved miRNAs (rice-specific) tended to target diverse genes. Moreover, 25 of the conserved and 7 of the non-conserved miRNA targets were further verified by RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM 5′-RACE). In addition, 3 *TAS3* transcripts cleaved by osa-miR390 and 5 *ARF* genes by ta-siRNAs were also identified and experimentally verified. These data showed that each small RNA family serves as a master node in gene regulation networks via down-regulation of their targets in rice.

2 Materials and methods

2.1 Plant materials

Rice (*Oryza sativa*) ssp. *indica* (*93-11*) plants were grown in the field. Young inflorescences (4-6 cm) were collected and frozen in liquid nitrogen immediately after harvest.

2.2 RNA extraction and degradome library construction

Young inflorescences were ground into a fine powder in liquid nitrogen and processed using Trizol reagent (Invitrogen). Approximately 150 μg of total RNA was then selected for polyadenylated RNA using the Oligotex mRNA mini kit (Qiagen). The degradome library was

constructed as previously described (German et al., 2008; German et al., 2009). Briefly, using T4 RNA ligase (NEB), a 5′ RNA adapter (cx4669, 5′-GUUCAGAGUUCUACAGUCCGAC-3′, from TaKaRa) was added to the cleavage products which possess a free 5′-phosphate on their 3′ termini. Then the ligated products were purified by Oligotex mRNA mini kit (Qiagen) again and reverse transcribed by a primer (cx4670, 5′-CGAGCACAGAATTAATACGACT$_{(18)}$V-3′, from Invitrogen) via M-MLV reverse transcriptase (Invitrogen). The generated cDNA library was amplified for 6 cycles (94°C for 30 s, 60°C for 20 s, and 72°C for 3 min) with a pair of primers (forward, cx4671: 5′-GTTCAGAGTTCTACAGTCCGAC-3′ and reverse, cx4672: 5′-CGAGCACAGAATTAATACGAC-3′, from Invitrogen) using Phusion Taq (NEB). The PCR products were digested with an enzyme *Mme* I (NEB). Next, a double-strand DNA adapter (top, cx4908: 5′-TCGTATGCCGTCTTCTGCTTG-3′, bottom, cx4674: 5′-CAAGCAGAAGACGGCATACGANN-3′, from Invitrogen) was incorporated into the digested products using T4 DNA ligase (NEB) (Addo-Quaye et al., 2008) and gel purified for PCR amplification (94°C for 30 s, 60°C for 20 s, and 72°C for 20 s) with primers (forward, cx4675: 5′-AATGATACGGCGACCACCGACAGGTTCA-GAGTTCTACAGTCCGAC-3′, reverse, cx4676: 5′-CAAGCAGAAGACGGCATACGA-3′, from Invitrogen) until a distinct band appeared. PCR products were gel purified and subjected to SBS sequencing by the Solexa analyzer.

2.3 MiRNAs and extract ta-siRNA sequences from *TAS3* sequences

All miRNA sequences were downloaded from Sanger miRBase 13.0 (Griffiths-Jones et al., 2008). *TAS3a*, *TAS3b*, and *TASc* were three known *TAS3* genes in *Nipponbare* (Lu et al., 2008). We used all *TAS3* transcript sequences and extracted 21 nt phased ta-siRNAs from the miR390 cleavage site as a query to search for ta-siRNA target sites.

2.4 Bioinformatics analysis

The *93-11* genome sequences and transcript sequences were downloaded from TIGR6.1 (Ouyang et al., 2007). The Short Oligonucleotide Analysis Package (SOAP) (Li et al., 2008) was applied to detect the alignment of all signatures to *93-11* genome sequences and transcript sequences. The CleaveLand (Addo-Quaye et al., 2009) was used to detect potentially cleaved targets based on degradome sequences.

The 20 and 21 nt distinct reads were subjected to the CleaveLand pipeline for small RNA targets identification as previously described (Addo-Quaye et al., 2009). Briefly, the 20 and 21 nt distinct reads were first normalized to give "reads per million" (RPM). Subsequently, the degradome reads were mapped to the rice annotated cDNA (TIGR6.1) and the cDNA hit number of each degradome read was recorded. The RPM abundance was then divided by each degradome reads hit number to give "normalized reads per million" (NRPM). For these cDNA matched reads, a 26 nt long mRNA tag is generated by extracting 13 nt of sequence upstream and downstream of the 5′ end of the matching degradome sequences. All resulting reads were aligned to each registered miRNA (miRBase 13.0) by the Needle program in the EMBOSS package (Rice et al., 2000). Alignments are then scored according to a previously described method (Allen et al., 2005). All alignments with scores not exceeding 7 and having the 5′ end of the degradome sequence coincident with the 10th nucleotide of complementarity to the small RNA were retained. To clearly represent identified targets in degradome library, all hits were categorized based on the abundance of the resulting mRNA tag relative to the overall profile of degradome reads matching the target (Addo-Quaye et al., 2008; Addo-Quaye et al., 2009). The resulting output was selected with the optimized score thresholds as previously reported with category I set as 4.5, category II as 4 and category III as 3 (Addo-Quaye et al., 2008). Gene Ontology analysis was performed according to the previous report (Maere et al., 2005).

The degradome sequencing data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE19050.

2.5 RLM 5′-RACE

For mapping the cleavage site within the microRNA target, a modified RNA ligase-mediated 5′ rapid amplification of cDNA ends (RLM 5′-RACE) was done. Total RNA was isolated from the inflorescence tissue (4-6 cm) of *93-11* (*Oryza sativa*. *indica*) using Trizol agent (Invitrogen). Poly (A)$^+$ RNAs were purified using Oligotex mRNA mini kit (Qiagen). A 5′ RNA adaptor (cx1558: 5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3') was ligated to approximately 100 ng of mRNA using T4 RNA ligase. The ligated mRNAs were then reverse transcribed using oligo(dT)$_{15}$ primer (cx2251: 5'-TTTTTTTTTTTTTTT-3') via M-MLV reverse transcriptase (Invitrogen). Two rounds of 5′ RACE reactions were performed with two nested primers (outer, cx1544: 5'-GCTGATGGCGATGAATGAACACTG-3'; inner, cx1545: 5'-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3', from Invitrogen) and two gene-specific primers (Supplemental Table 1) designed from about 200 to 600 nucleotides from the 3′ end of the predicted target site. PCR products were gel purified, cloned (pGEM-T Easy, Promega) and sequenced. A minimum of four clones were sequenced for each PCR product.

3 Results

3.1 Degradome library construction and data summary

In higher plants, most miRNAs regulate their targets via cleavage, which normally occurs at the tenth nucleotide of the complementary region between the miRNA and the mRNA (Bartel, 2004). The 3′ cleavage fragments, which result from cleavage mediated by miRNAs, contain both a free 5′ monophosphate and a 3′ polyA tail. Thus, these cleavage products can be recovered by RNA ligase-mediated ligation, whereas full length cDNAs with a 5′ cap

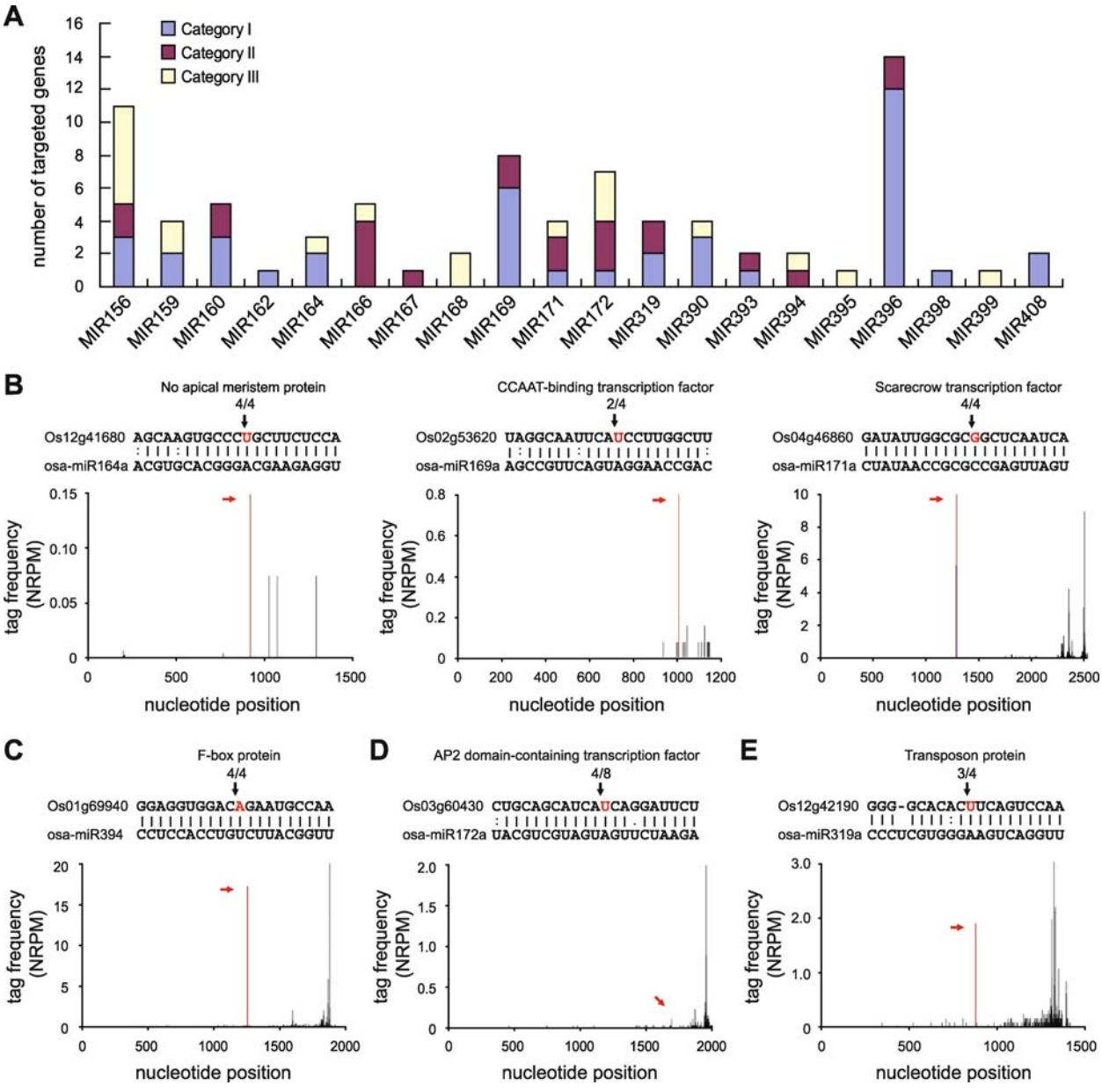

Fig. 1 Summary of conserved miRNA targets and validation of targets in different categories. (A) Summary of 82 transcripts targeted by 20 conserved miRNA families identified in the *93-11* degradome library. (B) Alignment of the miRNA with a portion of its target sequence (top) and distribution of 5′ end of the degradome tags within the full-length target mRNA sequence (bottom) corresponding to Os12g41680, Os02g53620, and Os04g46860, three category I targets. The solid lines indicate matched RNA base pairs. One dot shows G-U mismatch whereas two dots represent other types of mismatch. The black arrow indicates a site verified by RLM 5'-RACE with the frequency of cloned RACE products shown above the alignment. The cleavage site is shown as a red letter. Tags aligned with the ninth through eleventh nucleotides of the osa-miR164a, osa-miR169a and osa-miR171a complementary sites are combined and shown as a red line indicated by a red arrow. (C) As in (B) for Os01g69940, a category II target for osa-miR394. (D) As in (B) for Os03g60430, a category III target for osa-miR172a. (E) As in (B) for Os12g42190, a novel target for osa-miR319a.

Table 1 Data summary of

Table 2 Conserved miRNA targets in *93-11* (*Oryza sativa* L. *ssp*. *indica*)

miRNA family [a]	target gene family	TIGR accession [b]	cleavage site [c]	abundance [d]	category	target site location	conserved in *Arabidopsis*
osa-miR156	SBP-box gene family	Os01g69830	1163	0.244	I	ORF	Y
		Os02g04680	1975	1.665	II	ORF	Y
		Os04g46580	828	0.081	III	ORF	Y
		Os06g45310	864	0.081	III	ORF	Y
		Os06g49010	1696	0.284	II	ORF	Y
		Os07g32170	862	0.487	III	3' UTR	Y
		Os08g39890	1002	1.218	III	ORF	Y
		Os08g41940	1064	0.162	I	ORF	Y
		Os09g31438	819	0.081	III	ORF	Y
		Os09g32944	1044	0.162	I	ORF	Y
		Os11g30370	1101	0.081	III	ORF	Y
osa-miR159	MYB family transcription factor	Os01g59660 # [2]	1271	2.822	I	ORF	Y
	expressed protein	Os01g11430	1123	0.568	I	ORF	N
	expressed protein	Os09g36650	710	0.081	III	ORF	N
	hypothetical protein	Os05g42240	637	0.081	III	ORF	N
osa-miR160	Auxin response factor	Os02g41800 #	1495	5.969	II	ORF	Y
		Os04g43910 #	1355	1.543	I	ORF	Y
		Os04g59430 #	1345	0.244	I	ORF	Y
		Os06g47150 #	2053	7.455	I	ORF	Y
		Os10g33940 #	1646	5.969	II	ORF	Y
osa-miR162	DICER-like protein	Os03g02970 #	2988	1.2999	I	ORF	Y
osa-miR164	no apical meristem protein	Os06g23650	805	6.903	I	ORF	Y
		Os12g41680 #	922	0.162	I	ORF	Y
	phytosulfokines precursor	Os12g05260	213	0.568	III	ORF	N
osa-miR166	HD-Zip transcription factor	Os03g01890 #	935	1.462	II	ORF	Y
		Os03g43930 [3]	1100	2.274	II	ORF	Y
		Os10g33960	966	0.406	II	ORF	Y
		Os12g41860 #	888	2.274	II	ORF	Y
	DUF581 domain containing protein	Os01g08520	589	0.081	III	ORF	Y
osa-miR167	Auxin response factor	Os04g57610 # [3]	2669	0.934	II	ORF	Y
osa-miR168	ARGONAUTE protein	Os02g45070 #	720	0.162	III	ORF	Y
		Os04g47870 #	678	0.487	III	ORF	Y
osa-miR169	nuclear transcription factor Y subunit	Os02g53620 # [3]	999	0.812	I	3′ UTR	Y
		Os03g07880	855	1.570	I	3′ UTR	Y
		Os03g29760	1295	1.570	I	3′ UTR	Y
		Os03g44540	1729	7.877	II	3′ UTR	Y
		Os03g48970	1247	0.670	I	3′ UTR	Y
		Os07g06470	1436	0.406	I	3′ UTR	Y
		Os07g41720	1180	4.466	I	3′ UTR	Y
		Os12g42400 [3]	1267	3.167	II	3′ UTR	Y
osa-miR171	Scarecrow transcription factor family protein	Os02g44360	1362	3.086	II	ORF	Y
		Os02g44370	1537	2.274	II	ORF	Y
		Os04g46860 #	1337	8.527	I	ORF	Y
		Os10g40390	179	0.162	III	ORF	Y

(*Continued*)

miRNA family [a]	target gene family	TIGR accession [b]	cleavage site [c]	abundance [d]	category	target site location	conserved in *Arabidopsis*
osa-miR172	AP2 domain containing protein	Os03g60430 [#]	1766	0.122	III	ORF	Y
		Os04g55560	1634	0.135	II	ORF	Y
		Os05g03040 [3]	1987	0.162	II	ORF	Y
		Os06g43220	1491	0.081	III	ORF	Y
		Os07g13170	1415	0.244	III	ORF	Y
	Auxin response factor	Os04g36054	2946	0.244	II	3′ UTR	N
	helix-loop-helix DNA-binding domain containing protein	Os08g39630	1879	0.731	I	3′ UTR	N
osa-miR319	TCP family transcription factor	Os01g55100 [#]	1313	1.218	I	ORF	Y
		Os03g57190	1192	0.974	II	ORF	Y
		Os07g05720 [#]	1340	5.360	I	ORF	Y
	transposon protein	Os12g42190 [#]	857	2.030	II	ORF	N
osa-miR390	TAS3	TAS3a [#]	357	3.248	I	ORF	Y
		TAS3b [#]	443	1.908	I	ORF	Y
		TAS3c [#]	311	1.908	I	ORF	Y
	leucine-rich repeat receptor protein kinase EXS precursor	Os02g10100 [1 #]	2181	0.081	III	ORF	N
osa-miR393	TIR1-like protein	Os04g32460	2235	2.233	II	ORF	Y
		Os05g05800 [#]	1708	11.125	I	ORF	Y
osa-miR394	F-box domain containing protein [3]	Os01g69940 [#]	1251	13.561	II	ORF	Y
	RNA polymerase sigma factor	Os05g51150	1646	0.041	III	ORF	N
osa-miR395	cytochrome b5-like heme/steroid binding domain containing protein	Os10g35870	663	0.162	III	ORF	N
osa-miR396	growth-regulating factor	Os02g45570	640	6.090	I	ORF	Y
		Os02g47280	678	4.101	I	ORF	Y
		Os02g53690	581	0.568	I	ORF	Y
		Os03g47140	958	17.134	I	ORF	Y
		Os03g51970	430	10.151	I	ORF	Y
		Os04g48510	869	0.406	I	ORF	Y
		Os04g51190	546	2.220	I	ORF	Y
		Os06g02560	616	7.011	I	ORF	Y
		Os06g10310	434	0.325	I	ORF	Y
		Os11g35030	880	3.248	I	ORF	Y
		Os12g29980	785	0.934	II	ORF	Y
	transcription factor X1	Os01g44230	2106	0.650	II	ORF	Y
	50S ribosomal protein L20	Os05g45220	629	0.325	I	3′ UTR	N
	expressed protein	Os12g05000	817	0.247	I	ORF	N
osa-miR398	copper/zinc superoxide dismutase	Os07g46990 [#]	135	10.557	I	5′ UTR	Y
osa-miR399	ubiquitin conjugating enzyme protein	Os05g48390	915	0.081	III	5′ UTR	Y
osa-miR408	plastocyanin-like domain containing protein	Os03g50140 [#]	263	1.056	I	ORF	Y
		Os08g37670 [#]	668	5.603	I	3′ UTR	Y

[a] MiRNA data from miRBase 13.0. [b] Gene annotations from TIGR 6.1. [c, d] Calculation based on the method in Addo-Quaye et al., (2008). [#] MiRNA targets validated by gene-specific 5′ RLM-RACE in this paper. [1] MiRNA targets experimentally validated by Sunker et al., (2005). [2] MiRNA targets experimentally validated by Luo et al., (2006). [3] MiRNA targets experimentally validated by Liu et al., (2009).

structure or other RNAs lacking the 5′ monophosphate group are not compatible for ligation (Llave et al., 2002a). A degradome library, which captures the cleaved mRNAs, was constructed from young panicles of *93-11*, which is the type genome sequenced for *indica* rice. The degradome library was then submitted for parallel analysis of RNA end (PARE) sequencing by Solexa Analyzer (Addo-Quaye et al., 2008; German et al., 2008). In total, we obtained more than 13 million raw reads with the majority (> 98%) of length 20 and 21 nt from our library (Table 1). Totally 6630999 distinct reads (20 and 21 nt) were obtained and 4901253 (73.9%) signatures (refer as mapped reads) were perfectly mapped to the *93-11* genome. Alignment results showed that 87.2% (4276053 out of 4901253) signatures have a single hit in the *93-11* genome. Due to lack of publicly available cDNA information in *93-11* genome, we used *Nipponbare* cDNA information provided by TIGR Rice Genome Annotation Project. Nearly 4 million of these signatures were mapped to rice annotated cDNAs, known as cDNA matched reads (TIGR 6.1), which represented 74.9% (42562) of the annotated rice genes (Table 1). These data show that our degradome library recovered most of the degraded mRNAs which contained the sequence profile of small RNA-mediated cleavage and allowed us to conduct further analysis.

3.2 Systematic identification of miRNA targets in rice

Using previously described methods (Addo-Quaye et al., 2009), 20 and 21 nt distinct reads were subjected to the CleaveLand pipeline for small RNA target identification (details can be seen in **Materials and methods**). As previously reported (Addo-Quaye et al., 2008), the identified targets are grouped into three categories based on the relative abundance of the signatures at the target sites. For example, one mRNA may be hit by multiple tags, and if the most abundant tags represent the margin of miRNA-directed cleavage end, then it would be classified as category I. Except for category I targets, those with the frequency of tags diagnostic of miRNA-directed cleavage being in the top one-third would be regarded as category II. The rest of the miRNA-aligned reads, which formed minor peaks, were grouped into category III targets. The resulting output was further selected with the previous described parameter in category I, II and III, respectively (for details see **Materials and methods**) (Addo-Quaye et al., 2008).

3.3 Conserved miRNA targets

MicroRNAs have been extensively reported in rice and *Arabidopsis* (Bartel, 2009). Currently, more than 370 miRNAs have been reported in rice (miRBase 13.0). Twenty-one miRNA families are evolutionarily conserved between *Arabidopsis* and rice, whereas the rest are non-conserved (Jones-Rhoades et al., 2006; Lu et al., 2008). In our degradome data set, except for targets of miR397 family, 20 of the 21 conserved miRNA families targeting 79 protein coding genes and 3 conserved *TAS3* non-coding transcripts were detected (Table 2).

Targets distributing among three categories revealed that 40 targets (about 50%) of conserved miRNA families belonged to category I, whereas the category II and category III targets accounted for about 27% (22) and 24% (20), respectively (Fig. 1A). For osa-miR396, 12 out 14 targets belonged to category I, strongly suggesting that these targets were efficiently cleaved and represented high abundance tags in the degradome library.

It was noticed that a conserved miRNA family could target various numbers of genes ranging from 1 up to 14 with an around of 4 genes. In addition, the conserved miRNAs tended to target multiple genes belonging to a gene family (Table 2). Among those conserved miRNA families, osa-miR156 and osa-miR396 confer the highest number of targets with 11 and 14 unique transcripts, respectively (Table 2), indicating that osa-miR156 and osa-miR396 families might be center nodes in gene regulation networks. In contrast, fewer targets were identified for osa-miR162, osa-miR167, osa-miR395, osa-miR398 and osa-miR399, indicating that these miRNAs might act on a specialized pathway. Compared with *Arabidopsis*, the conserved miRNA targets are largely conserved, which indicates the miRNA-target relationship was evolutionally conserved between monocot and dicot.

Previous studies indicate that miRNAs predominantly target transcription factors both in *Arabidopsis* and *Populus* (Jones-Rhoades et al., 2006). In our data set, about 70% (58 out of 82) targets of genes cleaved by the conserved miRNA families were transcription factors. Moreover, in the category I group a higher proportion (80%, 32 out of 40) of the targets were transcription factors. Many of these transcription factors have been shown to act as master modulators that regulate growth and development in *Arabidopsis* (Jones-Rhoades et al., 2006), suggesting that the conserved miRNAs might act as key nodes in gene expression networks by regulating transcription factors.

3.4 Validation of conserved miRNA targets

In rice, conserved miRNA targets were previously

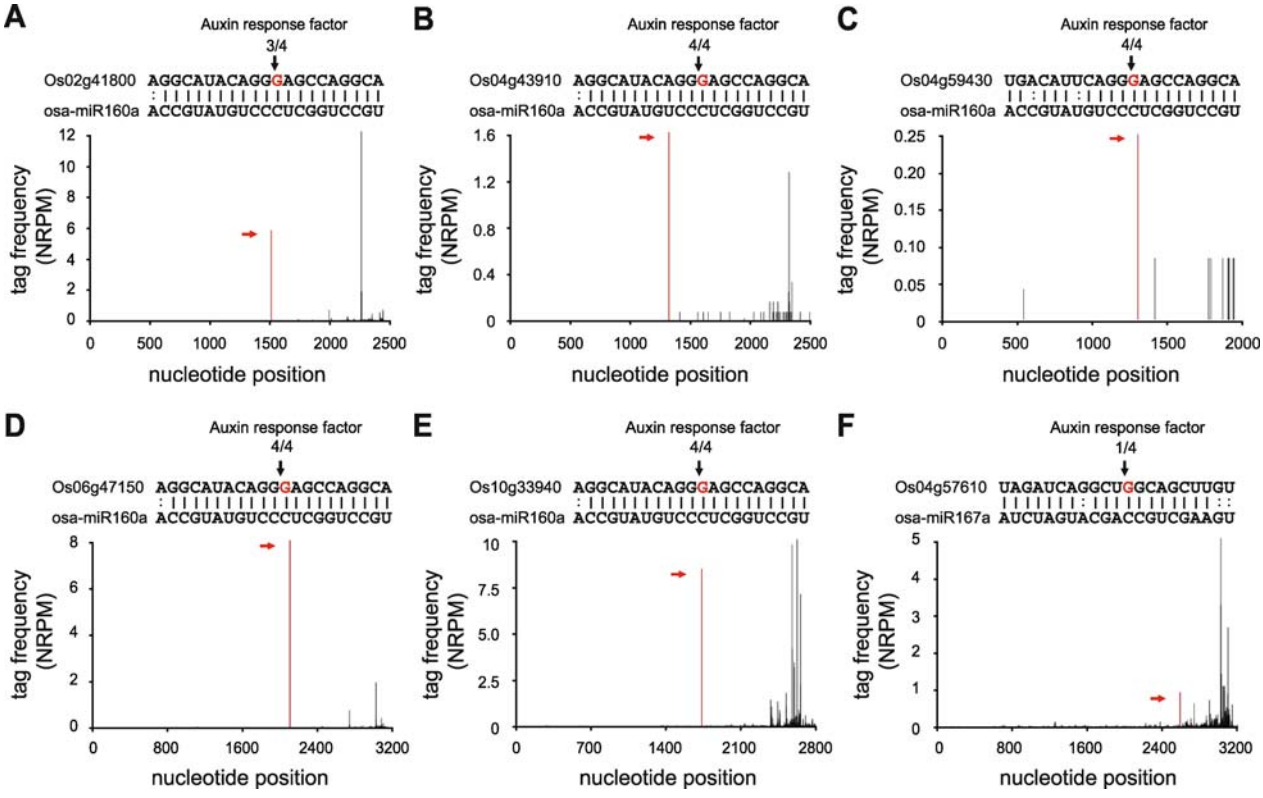

Fig. 2 Validation of *AUXIN RESPONSE FACTORs* regulated by osa-miR160 and osa-miR167. (A) Alignment of the miRNA with a portion of its target sequence (top) and distribution of 5′ end of the degradome tags within the full-length target mRNA sequence (bottom) corresponding to Os02g41800. The solid lines indicate matched RNA base pairs. Two dots represent other types of mismatch. The black arrow indicates a site verified by RLM 5′-RACE with the frequency of cloned RACE products shown above the alignment. The cleavage site is shown as a red letter. Tags aligned with the ninth through eleventh nucleotides of the osa-miR160a complementary sites were combined and shown as a red line indicated by a red arrow. (B) As in (A) for Os04g43910, the target for osa-miR160a. (C) As in (A) for Os04g59430, the target for osa-miR160a. (D) As in (A) for Os06g47150, the target for osa-miR160a. (E) As in (A) for Os10g33940, the target for osa-miR160a. (F) As in (A) for Os04g57610, the target for osa-miR167a.

investigated mainly via bioinformatics prediction (Reinhart et al., 2002; Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Archak and Nagaraju, 2007). Only a few conserved miRNA targets have been experimentally validated (Sunkar et al., 2005; Luo et al., 2006; Xie et al., 2006; Liu et al., 2007a; Liu et al., 2009). We showed here that many targets were captured by the degradome analysis, which provided experimental evidence to support previous predictions. To further confirm the degradome data, RLM 5′-RACE experiments were applied and several types of targets of conserved miRNAs were selected for validation.

First, some conserved miRNAs targets in category I, such as Os12g41680 (osa-miR164a), Os02g53620 (osa-miR169a), and Os04g46860 (osa-miR171a), respectively, were amplified and found to be cleaved at the same position as found in the degradome library (Fig. 1B). In addition, some targets such as Os01g69940 (osa-miR394) in category II and Os03g60430 (osa-miR172a) in category III, respectively, were also successfully validated by RLM 5′-RACE experiments (Fig. 1C and 1D). In addition to the conserved targets between *Arabidopsis* and rice, some non-conserved targets were also identified (Table 2). For instance, in addition to targeting *TEOSINTE BRANCHED/CYCLOIDEA/PCF* (*TCP*) transcription factors, osa-miR319a was found to be involved in the transposon protein regulation which was further confirmed by RLM 5′-RACE (Fig. 1E). This suggested that the conserved miRNAs in rice might regulate diverse genes except for the evolutionarily conserved targets.

Auxin is a key phytohormone in higher plant development. In *Arabidopsis*, regulation of *ARF10*, *ARF16*, and *ARF17* by miR160 appears to be vital in many aspects of shoot and root development (Mallory et al., 2005; Wang et al., 2005; Liu et al., 2007b), whereas *ARF6* and *ARF8*, targeted by miR167, act redundantly to regulate ovule and anther development (Wu et al., 2006). The miR160 family and the *ARF* genes are highly conserved in rice and *Arabidopsis* (Jones-Rhoades et al., 2006; Wang et al., 2007). Degradome analysis revealed five osa-miR160

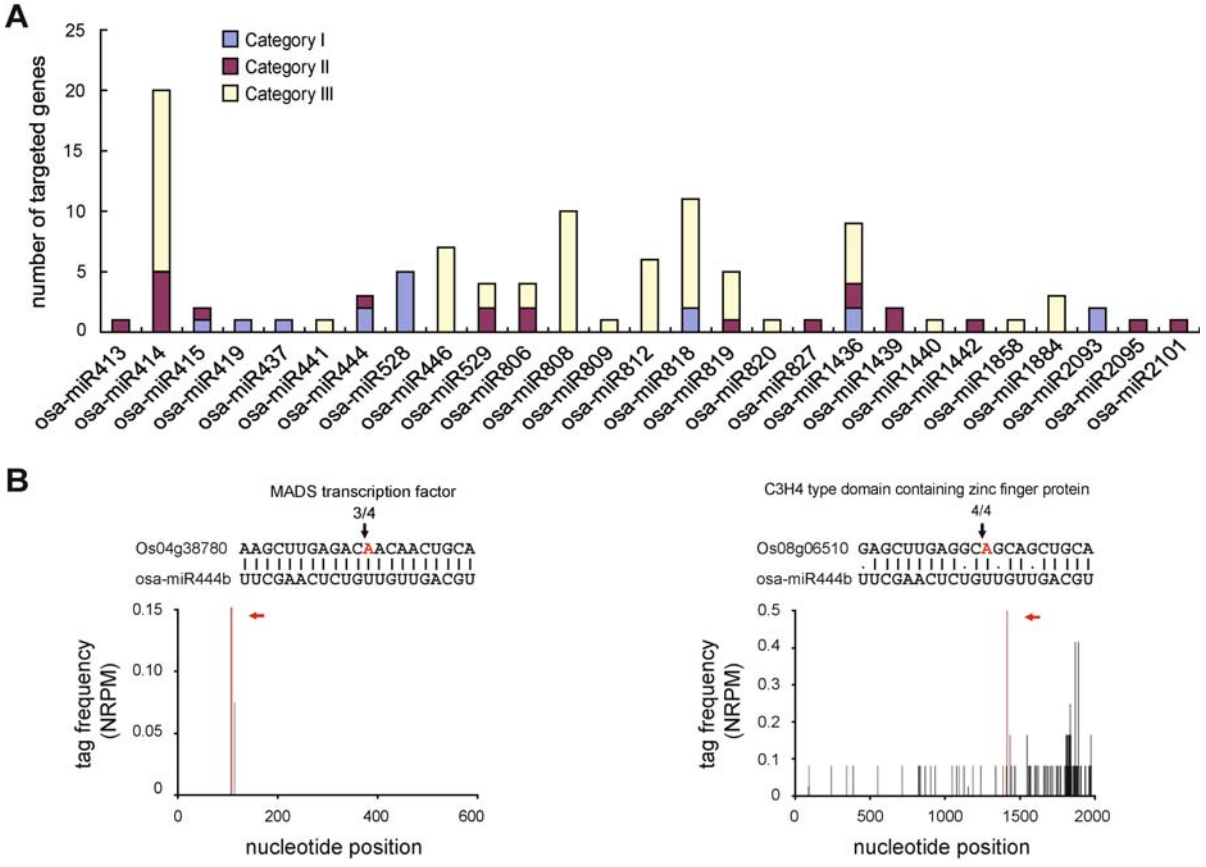

Fig. 3 Summary of the non-conserved miRNA targets and validation of targets for osa-miR444. (A) Summary of 105 transcripts targeted by 27 non-conserved miRNAs in *93-11* degradome library. (B) Alignment of the miRNA with a portion of its target sequence (top) and distribution of 5′ end of the degradome tags within the full-length target mRNA sequence (bottom) corresponding to a MADS transcription factor (Os04g38780) and a zinc finger protein (Os08g06510). The solid lines indicate matched RNA base pairs. One dot shows G-U mismatch. The black arrow indicates a site verified by RLM 5′-RACE with the frequency of cloned RACE products shown above the alignment. The cleavage site is shown as a red letter. Tags aligned with the ninth through eleventh nucleotides complementary sites are combined and shown as a red line indicated by a red arrow.

directed cleavages of *ARF* genes including Os02g41800, Os04g43910, Os04g59430, Os06g47150, and Os10g33940, respectively (Table 2). In addition, in rice, *OsARF8* (Os04g57610) which was previously shown to be cleaved by osa-miR167 in cultured cells in respond to exogenous auxin levels (Yang et al., 2006), was also captured in this degradome analysis. All of the six *ARF* genes examined above were further validated by RLM 5′-RACE showing cleavage precisely as expected (Fig. 2A-F). These results suggested that osa-miR160 and osa-miR167 could respond in auxin signaling for shoot, root cap formation and flower development via down-regulation of *ARFs* in rice.

3.5 The non-conserved osa-mirRNAs regulate multiple targets in rice

Compared with miRNAs in *Arabidopsis*, in rice 27 non-conserved miRNA families targeting 105 distinct genes were also identified (Table 3). Category III targets comprised 64% (68 out of 105) the non-conserved miRNAs, which might be caused by the low-abundance of the non-conserved miRNA-aligned reads. Sixteen targets of category I (about 15%) were identified as being regulated by osa-miR415, osa-miR419, osa-miR437, oas-miR444, osa-miR528, osa-miR818, osa-miR1436 and osa-miR2093, respectively (Fig. 3A). In contrast with conserved miRNA families, in which the sliced positions were most likely located in the open reading frame (ORF), 11 out of 16 category I targets occurred in either the 5′ or 3′ untranslated regions (UTR) (Tables 2 and 3). A similar situation was also observed in all targets mediated by non-conserved miRNA families (Table 3). Given that most miRNAs in animals regulate genes at the 3′ UTR through translational repression, in rice translational repression might also be a common

Table 3 Non-conserved miRNAs and their targets in *93-11* (*Oryza sativa* L. *ssp. indica*)

miRNA family [a]	TIGR accession [b]	targeted genes [c]	cleavage site [d]	abundance [e]	category [f]	target site location
osa-miR413	Os03g63380	expressed protein	811	0.162	II	3′ UTR
osa-miR414	Os01g03410	ubiquitin	623	0.009	III	ORF
	Os01g08560	DnaK family protein	2206	0.162	III	ORF
	Os01g16670	BSD domain-containing protein	1250	0.081	III	ORF
	Os01g48180	DDT domain containing protein	4724	0.027	III	ORF
	Os01g70330	prefoldin subunit family protein	701	0.016	III	ORF
	Os03g58590	expressed protein	1325	0.016	III	ORF
	Os03g59760	RING finger protein 126	564	0.010	III	ORF
	Os03g62620	late embryogenesis abundant protein	1094	0.183	II	ORF
	Os04g13880	retrotransposon protein	950	0.009	III	ORF
	Os04g37940	expressed protein	545	0.009	III	ORF
	Os05g51490	SIT4 phosphatase-associated protein domain containing protein	2021	0.041	III	ORF
	Os05g51830	ZOS5-12, C2H2 zinc finger protein	660	0.650	III	ORF
	Os06g33520	DEAD/DEAH box helicase	452	0.081	III	ORF
	Os06g50880	WD domain, G-beta repeat domain containing protein	1454	0.081	III	ORF
	Os07g40450	expressed protein	814	0.041	III	ORF
	Os08g09270	pentatricopeptide	1848	0.122	II	ORF
	Os08g33370	14-3-3 protein	821	0.325	II	ORF
	Os09g38400	prostatic sepermine-binding protein precursor	260	1.543	II	ORF
	Os10g16520	retrotransposon protein	695	0.009	III	ORF
	Os11g14220	tubulin/FtsZ domain containing protein	1450	11.125	II	ORF
osa-miR415	Os01g73160	40S ribosomal protein S10	795	0.406	I	3′ UTR
	Os04g52050	TNP1	3146	0.162	II	3′ UTR
osa-miR419	Os05g06280	ATKINESIN-13A/KINESIN-13A	2364	0.108	I	ORF
osa-miR437	Os02g18080	NB-ARC domain containing protein	4068	1.299	I	3′ UTR
osa-miR441	Os09g09820	retrotransposon protein	796	0.050	III	5′ UTR
osa-miR444	Os04g38780 [# 1]	a MADS transcription factor	108	0.162	I	ORF
	Os08g06510 [#]	zinc finger, C3H4 type domain containing protein	1382	0.487	I	ORF
	Os03g32499	expressed protein	220	0.487	II	ORF
osa-miR446	Os02g29140	ankyrin	1566	0.020	III	3′ UTR
	Os03g52010	lecithin cholesterol acyltransferase	1813	0.010	III	3′ UTR
	Os04g38450	gamma-glutamyltranspeptidase 1 precursor	2150	0.020	III	3′ UTR
	Os06g19990	GPI-anchored protein	699	0.020	III	3′ UTR
	Os08g44850	C2 domain containing protein	1846	0.010	III	3′ UTR
	Os09g09820	retrotransposon protein	793	0.010	III	5′ UTR
	Os10g26720	exonuclease	1409	0.020	III	3′ UTR
osa-miR528	Os06g06050 [#]	F-box domain and LRR containing protein	2659	0.650	I	3′ UTR
	Os0611310	plastocyanin-like domain containing protein	695	0.162	I	3′ UTR
	Os06g37150 [#]	L-ascorbate oxidase precursor	2011	0.487	I	3′ UTR
	Os07g38290 [#]	plastocyanin-like domain containing protein	538	24.443	I	ORF
	Os08g04310 [#]	plastocyanin-like domain containing protein	258	1.462	I	ORF
osa-miR529	Os01g69830	OsSPL2	1159	0.244	III	ORF
	Os08g39890 [#]	OsSPL14	998	2.842	II	ORF
	Os09g31438	OsSPL17	815	0.893	II	ORF
	Os09g32944	OsSPL18	1040	0.081	III	ORF

(*Continued*)

miRNA family [a]	TIGR accession [b]	targeted genes [c]	cleavage site [d]	abundance [e]	category [f]	target site location
osa-miR806	Os02g34950	ATP binding protein	1211	0.771	II	3′ UTR
	Os02g43370	transposon protein	2269	0.771	II	3′ UTR
	Os03g09230	LTPL69	869	0.081	III	3′ UTR
	Os12g40560	KH domain containing protein	2005	0.040	III	3′ UTR
osa-miR808	Os02g44990	OsFBDUF13, F-box and DUF domain containing protein	1815	0.018	III	3′ UTR
	Os02g45650	peptidase	1743	0.081	III	3′ UTR
	Os03g50070	DUF1295 domain containing protein	1201	0.018	III	3′ UTR
	Os04g02640	3-ketoacyl-CoA synthase 6	1643	0.016	III	3′ UTR
	Os04g32610	expressed protein	281	0.016	III	5′ UTR
	Os06g47850	zinc finger family protein	103	0.018	III	5′ UTR
	Os08g06500	PPR repeat domain containing protein	4574	0.018	III	3′ UTR
	Os08g19114	expressed protein	2056	0.018	III	5′ UTR
	Os08g36840	glycoprotein 3-alpha-L-fucosyltrans-ferase A	1783	0.406	III	3′ UTR
	Os08g40440	dihydroflavonol-4-reductase	1321	0.018	III	3′ UTR
osa-miR809	Os04g58070	aspartic proteinase nepenthesin precursor	1534	0.081	III	3′ UTR
osa-miR812	Os02g23823	helix-loop-helix DNA binding domain containing protein	400	0.014	III	ORF
	Os03g12620	glycosyl hydrolases family 17	1620	0.014	III	3′ UTR
	Os03g22050	CAMK	2926	0.081	III	3′ UTR
	Os06g38210	expressed protein	2519	0.081	III	5′ UTR
	Os06g50146	calcium-dependent protein kinase CPK1 adapter protein 2	1251	0.081	III	3′ UTR
	Os11g09260	expressed protein	910	0.027	III	5′ UTR
osa-miR818	Os01g63880	expressed protein	2170	0.050	III	3′ UTR
	Os02g10210	expressed protein	834	0.081	III	3′ UTR
	Os03g48010	exostosin	2445	0.050	III	3′ UTR
	Os03g49126	expressed protein	2042	0.081	III	3′ UTR
	Os04g39160	RNA-dependent RNA polymerase	3905	0.050	III	3′ UTR
	Os04g57154	expressed protein	2692	0.162	I	3′ UTR
	Os06g11500	MCM9	2497	0.081	III	3′ UTR
	Os06g39330	UDP-glucoronosyl and UDP-glucosyl trans-ferase domain containing protein	1646	0.050	III	3′ UTR
	Os08g29760	membrane protein	1917	0.081	III	3′ UTR
	Os08g41080	expressed protein	698	0.162	I	3′ UTR
	Os09g36320	tyrosine protein kinase domain containing protein	1601	0.036	III	3′ UTR
osa-miR819	Os03g31180	diacyglycerol kinase 1	1914	0.027	III	3′ UTR
	Os08g38620	expressed protein	971	0.081	III	3′ UTR
	Os09g30140	expressed protein	844	0.054	III	3′ UTR
	Os10g39970	harpin-induced protein 1 domain containing protein	968	0.244	II	3′ UTR
	Os12g02520	endo beta mannanase	1275	0.027	III	3′ UTR
osa-miR820	Os03g02010 [1]	DNA Methyltransferase protein	315	0.041	III	ORF
osa-miR827	Os04g48390 [2]	uncharacterized membrane protein	345	0.325	II	5′ UTR

(*Continued*)

miRNA family [a]	TIGR accession [b]	targeted genes [c]	cleavage site [d]	abundance [e]	category [f]	target site location
osa-miR1436	Os01g63880	expressed protein	2162	0.014	III	3′ UTR
	Os02g13210	transposable element protein	322	0.122	II	ORF
	Os02g43560	OsWRKY34	2736	0.244	II	3′ UTR
	Os03g15350	Sel1 repeat domain containing protein	2452	0.014	III	3′ UTR
	Os05g50570	OsSCP29	1507	1.299	I	3′ UTR
	Os06g11500	MCM9	2489	0.014	III	3′ UTR
	Os07g03110	OsFBX213	1477	0.379	I	3′ UTR
	Os07g40450	expressed protein	95	0.014	III	5′ UTR
	Os09g36320	tyrosine protein kinase domain containing protein	1593	0.014	III	3′ UTR
osa-miR1439	Os06g50146	calcium-dependent protein kinase CPK1 adapter protein 2	1205	0.325	II	3′ UTR
	Os07g17250	disease resistance RPP13-like protein 1	3639	0.325	II	3′ UTR
osa-miR1440	Os03g05200	DENN domain containing protein	1120	0.041	III	ORF
osa-miR1442	Os02g58670	bZIP transcription factor domain containing protein	958	0.122	II	3′ UTR
osa-miR1858	Os03g56060	CSLC9	105	0.005	III	5′ UTR
	Os01g59720	expressed protein	877	0.041	III	3′ UTR
osa-miR1884	Os03g19380	calvin cycle protein CP12	869	0.041	III	3′ UTR
	Os02g22610	transposon protein	1005	0.047	III	3′ UTR
osa-miR2093	Os01g72650	RNA recognition motif containing protein	1843	1.056	I	ORF
	Os11g06370	expressed protein	1395	0.247	I	3′ UTR
osa-miR2095	Os04g32340	RNA-binding motif protein	3294	0.406	II	3′ UTR
osa-miR2101	Os04g42090	CPuORF7	1903	0.406	II	3′ UTR

[a] miRNA data from miRBase 13.0. [b, c] Gene annotations from TIGR 6.1. [d, e, f] Calculation based on the method in Addo-Quaye et al., (2008). [#] miRNA targets validated by gene-specific RLM-5'RACE in this paper. [1] MiRNA targets experimentally validated by Lu et al., (2008). [2] MiRNA targets experimentally validated by Lacombe et al., (2008).

mechanism employed by non-conserved miRNAs. In addition, different from the conserved miRNAs, the non-conserved miRNAs tended to regulate several diverse genes via mRNA cleavage. For example, osa-miR808 and osa-miR1436 regulated 10 and 9 different targets, respectively (Table 3). In our degradome analysis, several previously validated non-conserved miRNA targets, such as a DNA methlytransferase encoding gene (Os03g02010) targeting by osa-miR820 and an SPX coding gene (Os04g48390) by osa-miR827, were also identified (Lacombe et al., 2008; Lu et al., 2008).

Both osa-miR444 and its target sites are conserved in monocots (Sunkar et al., 2005; Sunkar and Jagadeeswaran, 2008). Osa-miR444 targeting a MADS box transcription factor gene (Os04g38780) was detected in the degradome as previously reported (Sunkar et al., 2005; Lu et al., 2008). Another novel transcription factor (Os08g06510) targeted by osa-miR444 was identified in our degradome library (Table 3), RLM 5′-RACE further confirmed these two transcription factors are targets of osa-miR444 (Fig. 3B).

Interestingly, 10 transcripts were targeted by two osa-miRNAs in our degradome library. For instances, Os08g39890 was targeted by osa-miR156 at the 1002 nt position and it could also be cleaved by osa-miR529 at the 998 nt position, 4 nt before the osa-miR156 target site. Besides, osa-miR1436 could target the Os07g07450 transcript at 98 nt, and osa-miR414 would cleave the same transcript 719 nt downstream of the osa-miR1436 target site. Another 8 transcripts were also cleaved by two different osa-miRNAs (Supplemental Table 2). This phenomenon shows the complex nature of miRNA regulation in higher plants.

3.6 Validation of non-conserved osa-miRNA528 targets in rice

Osa-miR528 expresses at a relatively high level in several tissues of rice and its unique precursor shows a nearly perfect folding structure (Liu et al., 2005). Therefore, the osa-miR528 precursor has been successfully selected as a general model for expression of artificial miRNAs (amiRNA) in rice (Warthmann et al., 2008). In our data set, osa-miR528 appeared to target five protein encoding transcripts which belonged to three different gene families (Table 3). Three plastocyanin-like domain containing

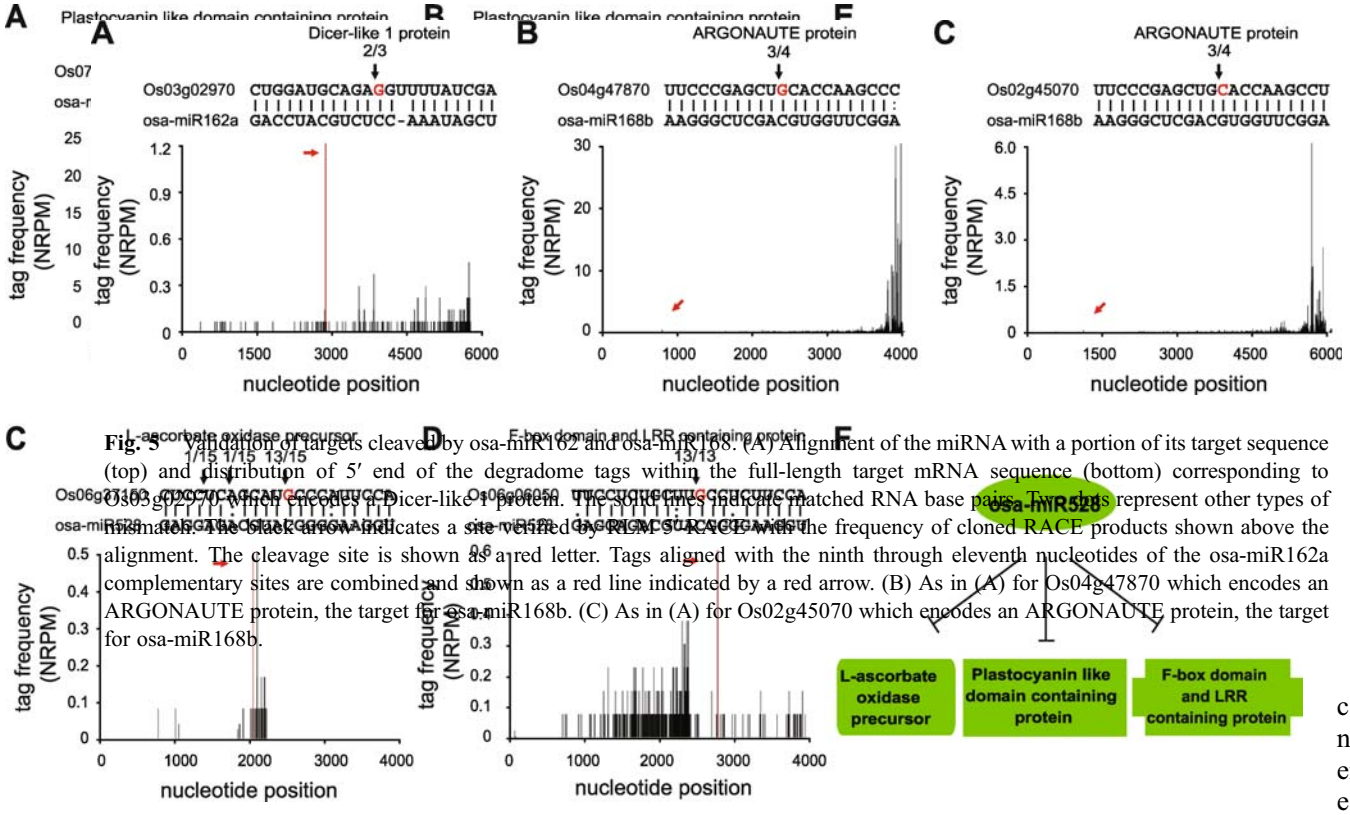

Fig. 3 Validation of targets cleaved by osa-miR162 and osa-miR168. (A) Alignment of the miRNA with a portion of its target sequence (top) and distribution of 5′ end of the degradome tags within the full-length target mRNA sequence (bottom) corresponding to Os03g02970 which encodes a Dicer-like 1 protein. The solid lines indicate matched RNA base pairs. Two dots represent other types of mismatch. The black arrow indicates a site verified by RLM 5′-RACE with the frequency of cloned RACE products shown above the alignment. The cleavage site is shown as a red letter. Tags aligned with the ninth through eleventh nucleotides of the osa-miR162a complementary sites are combined and shown as a red line indicated by a red arrow. (B) As in (A) for Os04g47870 which encodes an ARGONAUTE protein, the target for osa-miR168b. (C) As in (A) for Os02g45070 which encodes an ARGONAUTE protein, the target for osa-miR168b.

Fig. 4 Validation of trargets cleaved by osa-miR528, alignment of the target sites and action model of osa-miR528. (A) Alignment of the miRNA with a portion of its target sequence (top) and distribution of 5′ end of the degradome tags within the full-length target mRNA sequence (bottom) corresponding to Os07g38290 which encodes a plastocyanin like domain containing protein. The solid lines indicate matched RNA base pairs. One dot shows G-U mismatch whereas two dots represent other types of mismatch. The black arrow indicates a site verified by RLM 5′-RACE with the frequency of cloned RACE products shown above the alignment. The cleavage site is shown as a red letter. Tags aligned with the ninth through eleventh nucleotides of the osa-miR528 complementary sites are combined and shown as a red line indicated by a red arrow. (B) As in (A) for Os08g04310 which encodes a plastocyanin like domain containing protein, the target for osa-miR528. (C) As in (A) for Os06g37150 which encodes an L-ascorbate oxidase, the target for osa-miR528. (D) As in (A) for Os06g06050 which encodes an F-box domain and LRR containing protein, the target for osa-miR528. (E) Alignment of osa-miR528 target sites of different genes. The asterisks represent consensus sequence of osa-miR528 target sites of different genes. (F) Model of osa-miR528-targets node in rice. Distinct genes are linked to osa-miR528. Vertical lines represent negative regulation.

genes (Os07g38290, Os06g11310, and Os08g04310) were targeted by osa-miR528, with the target site of Os07g38290 located in the open reading frame (ORF) whereas the sites in the other two transcripts are located in the 3′ UTR. Further more, oas-miR528 also targets mRNAs encoding an L-ascorbate oxidase (Os06g37150) and an F-box and LRR containing protein (Os06g06050), respectively. The RLM 5′-RACE mapped the cleavage sites of these targets to the predicted sites, showing these genes were *bona fide* targets of osa-miR528 (Fig. 4A-D). Except for the target sites of osa-miR528, these three classes of genes belong to different gene families (Fig. 4E and data not shown). The diversity of osa-miR528 targets suggested that osa-miR528 might play crucial roles in various processes, including physiological processes, stress responses, and ubiquitination. Alternatively, three different types of genes regulated by osa-miR528 might converge into a common pathway in rice (Fig. 4F).

3.7 Autoregulation of miRNA biogenesis

Dicer or Dicer-like proteins serve as key components in miRNA and siRNA biogenesis (Bartel, 2004). The *dcl1* loss-of-function mutants impaired miRNA accumulation resulting in pleiotropic developmental defects both in *Arabidopsis* and rice (Park et al., 2002; Liu et al., 2005). Computational approaches predict that miR162 and osa-miR162 target *DCL1* or *Os DCL1* in *Arabidopsis* and rice respectively (Jones-Rhoades and Bartel, 2004). The degradome sequencing data showed that osa-miR162 directed the cleavage of the Os03g02970 (*Os DCL1*), the *DCL1* homolog in rice (Table 2) which is further confirmed by the RLM 5'-RACE experiment (Fig. 5A). Regulation of

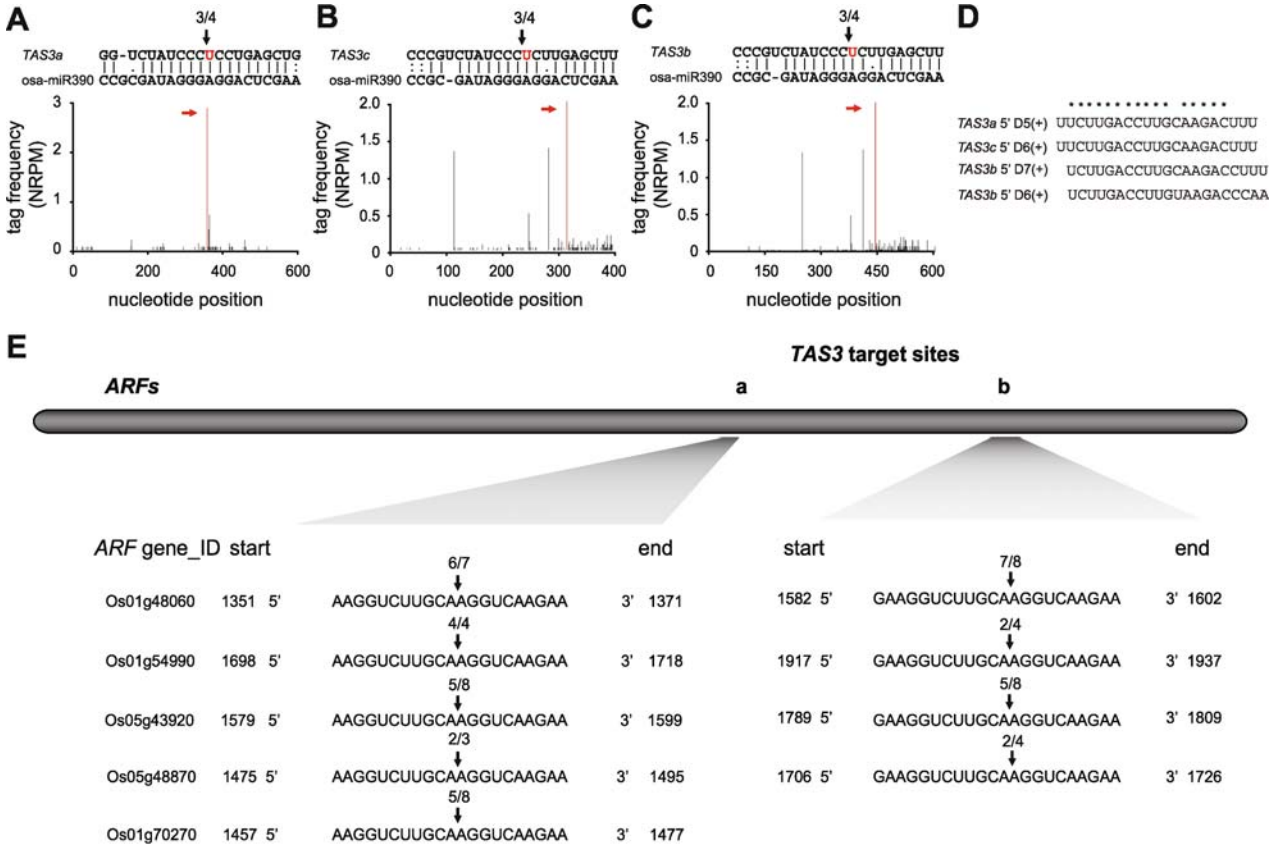

Fig. 6 Validation of *TAS3* transcripts and *AUXIN RESPONSE FACTORs* cleaved by osa-miR390 and ta-siRNAs. (A) Alignment of the miRNA with a portion of its target sequence (top) and distribution of 5′ end of the degradome tags within the full-length target mRNA sequence (bottom) corresponding to *TAS3a*. The solid lines indicate matched RNA base pairs. One dot shows G-U mismatch whereas two dots represent other types of mismatch. The black arrow indicates a site verified by RLM 5′-RACE with the frequency of cloned RACE products shown above the alignment. The cleavage site is shown as a red letter. Tags aligned with the ninth through eleventh nucleotides of the osa-miR390 complementary sites are combined and shown as a red line indicated by a red arrow. (B) As in (A) for *TAS3c*, the target for osa-miR390. (C) As in (A) for *TAS3b*, the target for osa-miR390. (D) Alignment of distinct ta-siRNAs from different *TAS3* transcripts. The asterisks indicate consensus sequences. (E) The diagram represents *ARFs* targeted by ta-siRNAs. Two *TAS3* target sites, a and b, are shown on the top. Five different *ARF* genes and ta-siRNAs are shown in their corresponding positions on the *ARF* gene schematic. The black arrow indicates a site verified by RLM 5′-RACE with the frequency of cloned RACE products shown at each position.

osa-miR162 on *Os DCL1* suggested miRNA input and output might be regulated in a feedback loop.

AGO is another core component of RISC and regarded as the integral player in all known small RNA-directed regulatory pathways (Hock and Meister, 2008; Vaucheret, 2008). *Arabidopsis* genome contains ten AGO coding genes and the rice genome has 18 putative AGO proteins (Vaucheret, 2008). Studies show that *AGO1* has extensive complementarity to miR168 and regulation of miR168 on AGO1 mRNA is needed for proper plant development (Vaucheret et al., 2004). Interestingly, two members of AGO gene family (Os02g45070 and Os04g47870, also known as *Os AGO711* and *Os AGO708*, respectively), which belong to the AGO1 group, were targeted by osa-miR168 (Table 2) in rice. RLM 5′-RACE experiments revealed that Os02g45070 and Os04g47870 mRNA fragments were cleaved precisely at the complementary sites of osa-miR168 (Fig. 5B and 5C). Multiple AGO proteins regulated by osa-miR168 in rice suggested that these AGO proteins acquired fine-tuning in small RNAs metabolism.

3.8 Identification and validation of ta-siRNA targets in rice

Like miRNAs, trans-acting siRNAs repress gene expression through mRNA degradation. Three *TAS3* loci have been identified in *Nipponbare* (Liu et al., 2007a; Lu et al., 2008; Zhu et al., 2008). As in *Arabidopsis*, osa-miR390 directed the cleavage of the *TAS3* transcripts (Table 2, Fig. 6A-C). In *93-11*, these *TAS3* loci were obtained by

computational prediction, namely *TAS3a*, *TAS3b*, and *TAS3c*. The *TAS3* derived ta-siRNAs including TAS3a D5(+), TAS3c D6(+), TAS3b D6(+), and TAS3b D7(+) (Fig. 6D) were submitted to the CleaveLand pipeline. Five *ARF* genes were shown to be cleaved by ta-siRNAs. Four *ARF* genes (Os01g48060, Os01g54990, Os05g43920, and Os05g48870) had two, whereas Os01g70270 had one ta-siRNAs target site (Fig. 6E). Previously studies show that in the *osdcl4-1* mutant, ta-siRNAs are greatly reduced resulting in up-regulation of *ARF* genes (Os01g48060, Os01g54990, Os05g43920, and Os05g48870) (Liu et al., 2007a). Moreover, three *ARF* genes (Os01g48060, Os05g43920, and Os01g70270) are cleaved by ta-siRNAs (Zhu et al., 2008). We further investigated the cleavage of the five *ARF* genes. Gene specific RLM 5′-RACE showed that all these target sites were cut, as shown by the degradome sequencing (Fig. 6E). Consistent with the up-regulation of *ARFs* and dramatic defects of leaves and spikelet organs in *osdcl4-1* mutants (Liu et al., 2007a), ta-siRNAs play important roles in rice development.

4 Discussion

Small RNAs play fundamental roles in gene regulation in animals and plants (Bartel, 2004). A huge number of miRNAs and siRNAs have been identified by cloning and deep sequencing in higher plants. Using degradome sequencing, we present a detailed sRNA:target interaction atlas in rice (*Oryza sativa* L. *ssp*. *indica*). In total, 182 transcripts targeted by 91 small RNAs including 87 miNRAs and 4 ta-siRNAs are identified. Of 82 targets for the 20 conserved miRNA families, around 70% of them are transcription factors. Besides, 105 genes targeted by the non-conserved miRNAs are also detected (Table 3). Moreover, 10 transcripts are shown to be targeted by two distinct miRNAs. In addition, 5 *ARF* genes cleaved by ta-siRNAs are also included. Eventually, 40 targets are validated employing RLM 5′-RACE. These results indicate that degradome sequencing serves as an efficient strategy to identified small RNA targets in plants.

Degradome sequencing, which combines high throughput deep sequencing with an effective computational approach, can globally identify sRNA-mediated slicing targets in higher plants. Using this strategy, over 100 small RNA targets have been successfully identified in *Arabidopsis* (Addo-Quaye et al., 2008; German et al., 2008; Gregory et al., 2008). We generated a degradome library from young panicles of *93-11*, an *indica* variety, and totally identified more than 180 transcripts cleaved by miRNAs and ta-siRNAs. Our results show that degradome technique can be applied as a powerful tool to detect targets of miRNAs and ta-siRNAs in rice. Ideally, all transcripts which possess a free 5′ monophosphate group and a 3′ polyA tail can be captured in the degradome library. Therefore, this method can be used to identify targets of not only miRNAs/ta-siRNAs, but also novel types of small RNAs. Although degradome sequencing is a powerful technique, many *bona fide* miRNA targets could also be excluded due to the natural mode of sRNA:target interaction. For example, translational repression of miRNAs exists widely in higher plants and targets regulated by such a mechanism would not be obtained by degradome sequencing (Brodersen et al., 2008). In addition, miRNAs are differentially expressed in various tissues (Xie et al., 2005) and in different biotic and abiotic stress conditions (such as miR395 induced by low SO_4^{2-} concentration) (Kawashima et al., 2009). Therefore, degradome libraries from different tissues, developmental stages and growth conditions might be required to comprehensively cover small RNA targets.

DCL and AGO proteins are central components in small RNA biogenesis in higher plants. Nearly all small RNAs are products of DCL protein and effectors of AGO protein. Interestingly, these key players are, in turn, regulated by miRNAs. MiR162 and miR168 regulate *DCL1* and *AGO1* in a feedback cycle, respectively (Xie et al., 2003; Vaucheret et al., 2004). In rice, *Os DCL1*, *Os AGO708*, and *Os AGO711* are also regulated by osa-miR162 and osa-miR168, respectively, via mRNA cleavage (Table 2, Fig. 5). In moss, *Pp DCL1a* and *Pp AGOs* are also regulated by ppt-miR1047 and ppt-miR904 (Axtell et al., 2007), respectively, suggesting that this mechanism might be conserved through plant kingdom.

Auxin acts as a central player in plant development (Rubio-Somoza et al., 2009). As the transducer of auxin signaling, ARFs play crucial roles in plant development, including shoot, root, leaves, and flower formation. In *Arabidopsis*, miR160 and miR167 are involved in auxin signaling via regulation of *ARF* genes (Jones-Rhoades et al., 2006). In rice, we discovered five and one *ARF* encoding genes which are regulated by osa-miR160 and osa-miR167, respectively (Table 2, Fig. 2). In addition, ta-siRNAs, a class of plant specific siRNA, are involved in *ARF* gene regulation through mRNA cleavage. Together, osa-miR160, osa-miR167, and ta-siRNAs would orchestrally regulate auxin response in rice development (Supplemental Fig. 1). As shown in *Arabidopsis*, the biosynthesis is indirectly controlled by miR319 through regulation of *TCP* transcripts, whereas the reception and output of auxin is directly regulated posttranscriptionally by miR393 on TIR-like protein, and miR164-CUC, respectively (Rubio-Somoza et al., 2009). In rice, *TIR-like* and *TCP* genes osa-miR164-CUC are cleaved by osa-miR393, osa-miR319, and osa-miR164, respectively, indicating that the conserved regulatory

mechanism between dicot *Arabidopsis* and monocot rice for auxin biogenesis and signaling transduction (Table 2) (Rubio-Somoza et al., 2009).

In *Arabidopsis*, miRNA targets are mainly involved in major transition between each stage of development and transcription factors account for more than half of these targets (Jones-Rhoades et al., 2006). Of 179 identified targets in our rice degradome library (except for 3 *TAS3* transcripts), 105 transcripts have Gene Ontology (GO) annotations (Maere et al., 2005) and they are obviously enriched in transcription factor and transcription regulatory activity (Supplemental Fig. 2A). Further, GO analysis for biological function indicates that these genes are mainly involved in developmental and metabolic processes (Supplemental Fig. 2B). This is consistent with roles of miRNA targets in *Arabidopsis*, and suggested that miRNAs are master nodes of gene regulatory networks.

Using degradome sequencing, we present a detailed sRNA:target interaction atlas in rice. The identified and verified small RNA targets will provide useful information for understanding the interplay between small RNAs and their targets and further revealing the important roles of small RNAs in rice.

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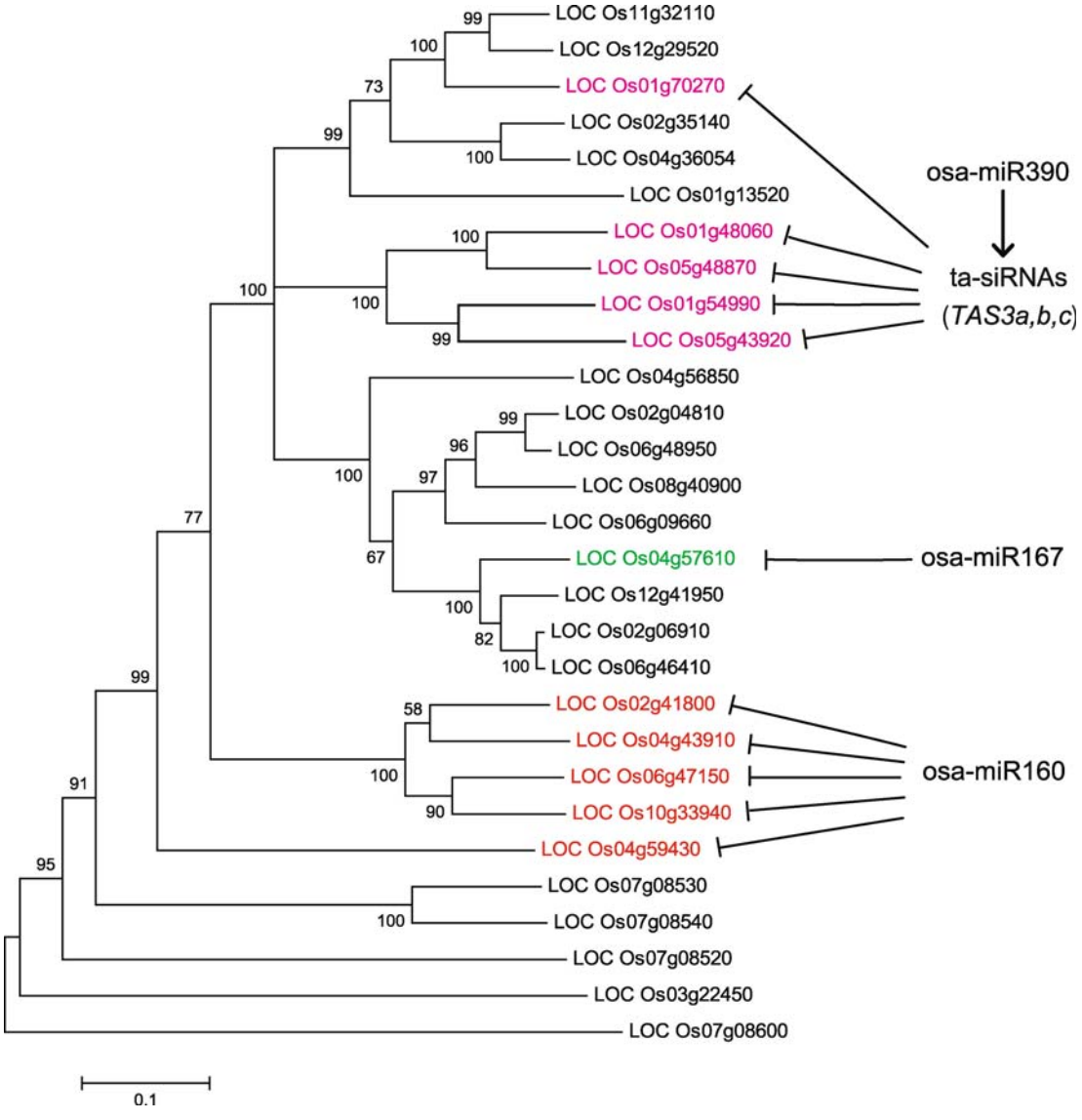
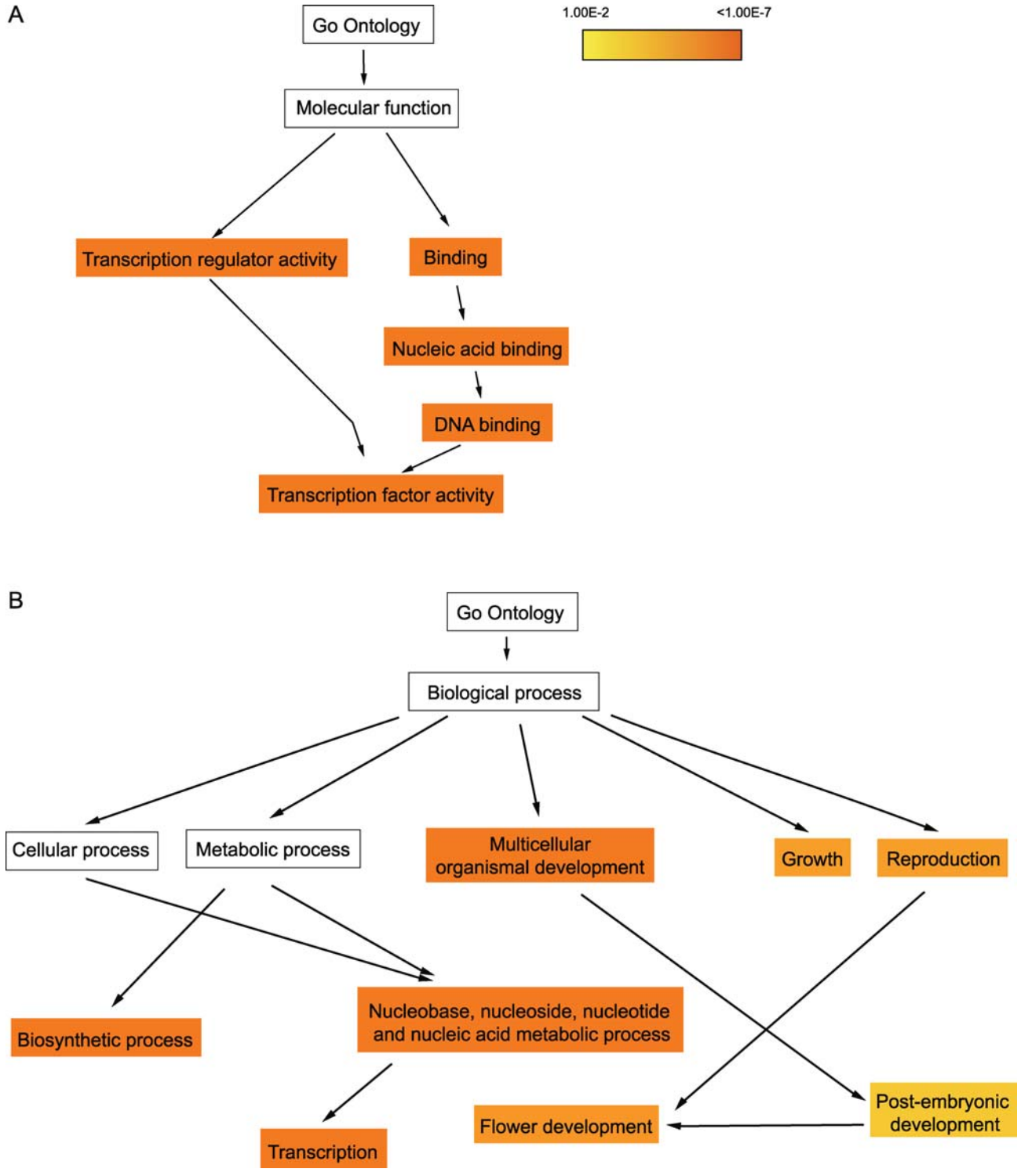


Supplemental Fig.1 Rice AUXIN RESPONSE FACTOR genes orchestrally regulated by distinct small RNAs. Genes in pink are targets of osa-miR390 ta-siRNAs; gene in green is the target of osa-miR167, and the genes in red are targets of osa-miR160, respectinely.



Supplemental Fig.2 Gene Ontology (GO) analysis of the small RNA targets in rice. (A) GO analysis function. The p-value varies from 1.00E-2 (bright yellow) to 1.00E-7 (dark brown). (B) GO analysis for biological function.

Supplemental Table 1 Primers for RLM-5′RACE and targets validation of sRNA

miRNA	target gene	primer ID [a]	primer sequences	predicted length/bp	target/osa-miRNA pairs [b]	RLM-5'RACE
osa-miR159a	LOC_Os01g11430	cx6373 (O)	TTGCAGTGAGCAAAGTCAGA	293	CGGAGCCCCCUUCAAACCAAA	3/4
		cx6374 (I)	AAGTCAGATGTCCAGTCTGA		GUCUCGAGGGAAGUUAGGUUU	
osa-miR159a	LOC_Os01g59660	cx6195 (O)	TCAGGATGAGGTGAAGTGTC	460	UGGAGCUCCCUUCACUCCAAG	
		cx6196 (I)	CTTGCTCTCCAGATCCCATT		GUCUCGAGGGAAGUUAGGUUU	4/4
osa-miR160a	LOC_Os02g41800	cx6536 (O)	AACCACTGGAGCCTCTCCGA	322		
		cx6537 (I)	TCGGTGTTCCAGTTGAGAGA			
osa-miR160a	LOC_Os04g43910	cx6538 (O)	TTGATCTGCTCCTCGGTTAGA	266		
		cx6539 (I)	AATTGCCTGTCCAAAGAGGA			
osa-miR160a	LOC_Os04g59430	cx6540 (O)	AGGCTTGTGTCAGAAGCTTCT	301		
		cx6541 (I)	TCATCATCTACACCCTCTGGA			
osa-miR160a	LOC_Os10g33940	cx6542 (O)	GCATTGCCATTAGGTGAGCTA	284		
		cx6543 (I)	TGAAGTTATCTGCTGCTCAGT			
osa-miR160a	LOC_Os06g47150	cx6197 (O)	CCAACAACAGAGAGATCAAG	499		
		cx6198 (I)	ACATCCTCCGACTGCATGAA			
osa-miR162b	LOC_Os03g02970	cx6213 (O)	AATGTCCTCTATCACGAGCA	473		
		cx6214 (I)	CAGATGCTTTCACAATGTCA			
osa-miR164c	LOC_Os12g41680	cx6191 (O)	TCCCTAGAAGTGATTCATCC	283		
		cx6192 (I)	AAGAAGAGCTGACAGCAGCA			
osa-miR166a	LOC_Os12g41860	cx6672 (O)	CCTCACATGGTCGAATCAGAT	299	GGUUGGGAUGAAGCCUGGUCCGG	
		cx6673 (I)	CAGATCACAAGACTCCCATCT		CC–CCUUACUUCGGACCAGGCU	4/4
osa-miR166a	LOC_Os03g01890	cx6237 (O)	ACACTCCATGCCTCAAGATC	431	GGG–AUGAAGCCUGGUCCGG	
		cx6238 (I)	ACTATGTGCACAATTGATCC		CCCCUUACUUCGGACCAGGCU	4/4
osa-miR167a	LOC_Os04g57610	cx6532 (O)	CGATACTGATTCCTAAGTGGA	308		
		cx6533 (I)	TAGGTAACAGAGAGTTCTGGA			
osa-miR168b	LOC_Os02g45070	cx6391 (O)	TAGACACATCATACTGGTGA	251		
		cx6392 (I)	TGGTTGGCTTTCACGATGCA			
osa-miR168b	LOC_Os04g47870	cx6393 (O)	CAGCGTTACTAACTCAAACA	300		
		cx6394 (I)	TACGTCGTATTGGTGAAGGT			
osa-miR169a	LOC_Os02g53620	cx6410 (O)	ACAGGGACAACTTCATCACA	125		
		cx6411 (I)	AACTTCATCACACCAATACA			
osa-miR171b	LOC_Os04g46860	cx6203 (O)	AACCTCATCAGCTGTAGGAG	450		
		cx6204 (I)	GAATACCAAGATCAGCAGCA			
osa-miR172a	LOC_Os03g60430	cx6599 (O)	TCAATGAGCTGTGGGTGTCA	225		
		cx6600 (I)	CTAGTACTACCTCACTGCCT			

(*Continued*)

miRNA	target gene	primer ID [a]	primer sequences	predicted length/bp	target/osa-miRNA pairs [b]	RLM-5'RACE
osa-miR319	LOC_Os07g05720	cx6676 (O)	CTTCCATCCAAGAAGAGACGT	242	GGG-GGACCCUUCAGUCCAA	
		cx6677 (I)	CTGCTGATCTGATCAGTGCT		CCCUCGUGGGAAGUCAGGUU	2/4
osa-miR319	LOC_Os01g55100	cx6678 (O)	AAGCACACACCTCCTGCAT	218	GGGAAC-CCCUUCAGUCCAG	
		cx6679 (I)	CCTGATCACCTCACTTCCGT		CCCUCGUGGGAAGUCAGGUU	4/4
osa-miR319a	LOC_Os12g42190	cx6355 (O)	GCTGACATCATCTTCAGTTC	270		
		cx6356 (I)	CTTTGACTTGTGGCACAACA			
osa-miR390	TAS3a	cx6442 (O)	TGCACTCTGACTGCAGAAATA	172		
		cx6443 (I)	ACATTATATCACGCAAGCAA			
osa-miR390	TAS3b	cx6444 (O)	GAGAGAGAGATCAATCACCA	151		
		cx6445 (I)	AAGGAACTACTGACGAGTGA			
osa-miR390	TAS3c	cx6448 (O)	GACATATCACAAGCACAAGGT	241		
		cx6449 (I)	GCTTACTAGCATGGCATATCT			
osa-miR393	LOC_Os05g05800	cx6205 (O)	TAGAGTCCATGCATTTCCAT	213	GA-CAAUGCGAUCCCUUUGGA	
		cx6206 (I)	GTCCTGAAAAAGGCTGTGAA		CUAGUUACGCUAGGGAAACCU	2/2
osa-miR394	LOC_Os01g69940	cx6416 (O)	CAATGATCCGGAATGTGTGA	220		
		cx6417 (I)	ATGGACCAAATGCTGTGCCA			
osa-miR398a	LOC_Os07g46990	cx6381 (O)	CCAGTGGTCTTGCTAAGCTC	521	CAGGGGUCGCCUGAGAACACA	
		cx6382 (I)	CATCAGGATCAGCATGGACA		UUCCCCACUGGACUCUUGUGU	3/4
osa-miR408	LOC_Os03g50140	cx6383 (O)	GTGCAGACGAAGAAGTTGT	238	GCUCGGGGAAGAGGCAGUGCA	
		cx6384 (I)	CGTACCCGTCTTCGTCGA		CG-GUCCCUUCUCCGUCACGUC	1/4
osa-miR408	LOC_Os08g37670	cx6385 (O)	GAGTGATATAGAGTGTCCGA	149	GCCAGGAUAGAGGCAGUGCA	
		cx6386 (I)	GTACTAAGTACACGATGGA		CGGUCCCUUCUCCGUCACGU	2/3
osa-miR444b.2	LOC_Os04g38780	cx6395 (O)	GAGACTTCCCTTTCGATTCA	257		
		cx63696 (I)	TCAGTTCGTGGATTTCATCA			
osa-miR444b.2	LOC_Os08g06510	cx6397 (O)	GCTTGCTCCACAGATGATGA	240		
		cx6398 (I)	CAGCACCATTAGCATCAGGA			
osa-miR528	LOC_Os07g38290	cx6211 (O)	GAGATGAAACCCACAATTCC	390		
		cx6212 (I)	GTCCAAAACATGAGGTGACA			
osa-miR528	LOC_Os08g04310	cx6424 (O)	ATACAAGAGCACGAGGACTA	293		
		cx6425 (I)	GAGATGTTGTTGGCGTTGGA			
osa-miR528	LOC_Os06g06050	cx2144 (O)	CATCTGAATTTCCAACACATATCT	332		
		cx2100 (I)	TGTGTATGTGCTTGTGTGACGTGG			
osa-miR528	LOC_Os06g37150	cx2146 (O)	ACATTGCAACAATATTACACAATCAT	166		
		cx2147 (I)	CACAATCATCTTAATTAGCAAACATG			

(*Continued*)

miRNA	target gene	primer ID [a]	primer sequences	predicted length/bp	target/osa-miRNA pairs [b]	RLM-5'RACE
osa-miR529	LOC_Os08g39890	cx6666 (O)	CCTGCAGAGCAAGCTCAAGCT	219	GAGCUGUGCUCUCUCUCUUCU	
		cx6667 (I)	AGACTTCATGTGGTAGCTGGT		UUCGACAUGAGAGAGAGAAGA	3/4
ta-siRNA	LOC_Os01g48060-cleavage sit 1	cx4577 (O)	GAGATTCCCCAAAGCCTGAGC	191		
		cx4578 (I)	AGCAATGGTAGGAAAACCCAG			
	LOC_Os01g48060-cleavage sit 2	cx4579 (O)	TACACTCATCTAGCACTTTGC	268		
		cx4580 (I)	AAGATGAGGCCAGAGTCGTAA			
ta-siRNA	LOC_Os01g54990-cleavage site 1	cx4581 (O)	AATCCAATAGATTCACCGAAGC	182		
		cx5482 (I)	TTACAGATTGGTGGGTATAGC			
	LOC_Os01g54990-cleavage site 2	cx4583 (O)	CCATTACCAGGCTTAGTAGCA	310		
		cx4584 (I)	CTGGTCCCAGCTAGTTTCACT			
ta-siRNA	LOC_Os05g43920-cleavage site 1	cx4585 (O)	AATCCAAGAGACTCACTGAAG	171		
		cx4586 (I)	ATGGGCTGATAAGTAAACTCT			
	LOC_Os05g43920-cleavage site 2	cx4587 (O)	AACTGGTTGCTCGTGATTTCG	303		
		cx4588 (I)	GTTTCTGAGAGCATCTCCTG			
ta-siRNA	LOC_Os05g48870-cleavage site 1	cx4589 (O)	GTCTGTGAGACTCCCCAAAG	194		
		cx4590 (I)	CTGAGCAATGGTAGGAAAATC			
	LOC_Os05g48870-cleavage site 2	cx4591 (O)	TCAACTGTAACCTGTCCATCT	358		
		cx4592 (I)	TTCTCCAGAAGTAGGATAAGG			
ta-siRNA	LOC_Os01g70270	cx6456 (O)	GATCCTGAAACTGGTTCTTGA	245		
		cx6457 (I)	GAGCCTCATCAAAATTCTGCA			

[a]"O" represents the gene specific outer primer. "I" represents the gene specific inner primer. [b]The target/osa-sRNA pairs. The cleavage site directed by sRNA was shown in red capital letter.

Supplemental Table 2 Eight transcripts cleaved by two different osa-miRNAs

transcirpt	MiRNA family	target site
Os01g69830	osa-miR156a-l	1163
	osa-miR529b	1159
Os08g39890	osa-miR156a-l	1002
	osa-miR529b	998
Os09g31438	osa-miR156a-l	819
	osa-miR529b	815
Os09g32944	osa-miR156a-l	1044
	osa-miR529b	1040
Os07g40450	osa-miR414	814
	osa-miR1436	95
Os09g09820	osa-miR441a-c	796
	osa-miR446	793
Os06g50146	osa-miR812a-e	1251
	osa-miR1439	1205
Os01g63880	osa-miR818a-e	2170
	osa-miR1436	2162
Os06g11500	osa-miR818a-e	2497
	osa-miR1436	2489
Os09g36320	osa-miR818a-e	1601
	osa-miR1436	1593