Deep Sequencing Uncovers Rice Long siRNAs and Its Involvement in Immunity Against *Rhizoctonia solani*

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ABSTRACT

Small RNA (sRNA) is a class of noncoding RNA that can silence the expression of target genes. In rice, the majority of characterized sRNAs are within the range of 21 to 24 nucleotides (nt) long, whose biogenesis and function are associated with a specific sets of components, such as Dicer-like (OsDCLs) and Argonaute proteins (OsAGOs). Rice sRNAs longer than 24 nt are occasionally reported, with biogenesis and functional mechanism uninvestigated, especially in a context of defense responses against pathogen infection. By using deep sequencing, we identified a group of rice long small interfering RNAs (lsiRNAs) that are within the range of 25 to 40 nt in length.

Our results show that some rice lsiRNAs are differentially expressed upon infection of *Rhizoctonia solani*, the causal agent of the rice sheath blight disease. Bioinformatic analysis and experimental validation indicate that some rice lsiRNAs can target defense-related genes. We further demonstrate that rice lsiRNAs are neither derived from RNA degradation nor originated as secondary small interfering RNAs (siRNAs). Moreover, lsiRNAs require *OsDCL4* for biogenesis and *OsAGO18* for function. Therefore, our study indicates that rice lsiRNAs are a unique class of endogenous sRNAs produced in rice, which may participate in response against pathogens.

Small RNA (sRNA) is a class of 21 to 24 nucleotide (nt)-long RNA molecules that can specifically target genes with reverse complementary sequences and mediate gene silencing. sRNAs regulate the expression of target genes either through transcriptional gene silencing (TGS) or posttranscriptional gene silencing (PTGS) (Baulcombe 2004). Plant sRNAs play important roles in processes such as leaf morphogenesis, floral organ identity, hormone signaling, nutrient transportation, assimilation and stress responses (Ambros and Chen 2007; Jones-Rhoades et al. 2006; Rogers and Chen 2013; Voinnet 2009). Plant sRNAs are classified into two subgroups, microRNAs (miRNAs) and short interfering RNAs (siRNAs), according to their biogenesis and precursor structures (Chen 2009). siRNAs can be further divided into heterochromatic siRNAs (hc-siRNAs), transacting siRNAs (ta-siRNAs), long siRNAs (lsiRNAs), and natural antisense transcripts-derived siRNAs (nat-siRNAs) (Chapman and Carrington 2007; Jin and Zhu 2010; Katiyar-Agarwal and Jin 2010). In plants, the functions of sRNAs depend on several conserved protein families, such as Dicer-like proteins (DCL), Argonaute (AGO) proteins, RNA-dependent RNA polymerases (RDRs), and the sRNA methyltransferase (HEN1) (Chapman and Carrington 2007).

Plant sRNAs are important players in defense response against pathogens (Duan et al. 2012; Katiyar-Agarwal and Jin 2010; Pumplin

and Voinnet 2013). miR393 is the first identified defense-related sRNA that can be induced by pathogen associated molecular pattern (PAMP) molecules such as flg22. By silencing an auxin receptor, miR393 efficiently suppresses auxin signaling and positively contributes to the PAMP triggered immunity (PTI) (Navarro et al. 2006). Interestingly, the miRNA originated from another strand of the miR393 duplex, miR393*, can be activated by the effector triggered immunity (ETI) signaling. miR393* can influence exocytosis of some pathogenesis-related protein (PRs) and contributes to plant defense against bacterial pathogens (Zhang et al. 2011). The functionally cognate miR393/miR393b* pair demonstrates that both strands of an miRNA duplex (previously considered as miRNA and its passenger miRNA without function) are biologically related and functionally coregulated. It suggests that plant immunity system can adjust PTI and ETI machineries by such a pair of miRNAs from a single transcript, more efficiently and effectively. Similar phenomenon was found on *Arabidopsis* miR825/825*, both of which are reduced by a *Bacillus cereus* strain and contribute to induced systemic resistance (ISR) (Niu et al. 2016). Several other sRNAs involved in plant defense have also been identified, including their mechanism of action through TGS or PTGS (Niu et al. 2015; Seo et al. 2013).

In crops and vegetables, endogenous plant sRNAs are also critical factors in shaping the relationship with their associated pathogens. This is demonstrated by observations made in many species such as rice, wheat, tomato, and citrus. For example, Zhai and colleagues identified several 22-nt miRNAs that target conserved domains of nucleotide-binding, leucine-rich repeats (NB-LRRs) genes in *Medicago* and potato, which trigger the production of phased, secondary siRNAs (phasiRNAs) at the cleavage site. These transacting siRNAs can target other NB-LRRs genes and affect plant immunity (Zhai et al. 2011). In wheat, diverse set of miRNAs are identified in response to powdery

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mildew infection (Xin et al. 2010). In citrus, miR399 is induced by the Huanglongbing (HLB) bacteria. The expression of citrus miR399 improves the inorganic phosphorus deficiency (one of the HLB disease symptoms) through elevating phosphorus transporter activity, which alleviates HLB symptoms (Zhao et al. 2013). When infected by *Fusarium oxysporum*, expression of two tomato miRNAs which are capable of targeting genes encoding proteins with full or partial nucleotide-binding (NB) domains are suppressed. Silencing of these target genes leads to a resistance-to-susceptibility switch phenotype in resistant cultivars (Ouyang et al. 2014). In rice, miR160 or miR398 are differentially expressed between resistant and susceptible cultivars after *Magnaporthe oryzae* challenge. Overexpression of miR160 or miR398 in a susceptible rice cultivar leads to enhance disease resistance toward *M. oryzae*, confirming that these two miRNAs are positive regulators of defense against the blast disease (Li et al. 2014). Taken together, multiple evidence supports the idea that endogenous sRNAs are critical players of plant innate immunity, and their study will be of great significance on plant disease control and management.

Although most of the functional sRNAs studied to date are 21 to 24 nt long, sRNAs with length more than 24 nt have been reported in different species sporadically. The 27- to 30-nt small scan RNAs (scnRNAs) associated with Argonaute proteins have been shown to act in the DNA elimination process in *Tetrahymena* (Hock and Meister 2008). PIWI-interacting RNAs (piRNAs), which are generally 24 to 32 nt in length, bind specifically to the PIWI subfamily of Argonaute proteins and function in germline stem-cell maintenance and self-renewal (Lin and Spradling 1997). Carone et al. (2009) reported the 35 to 42 nt sRNA (centromere repeat associated short interacting RNAs; crasiRNAs), which is produced from transcription of repeat regions and is proposed to be essential components of cellular stability and chromosome segregation in tammar wallaby (*Macropus eugenii*) (Brown and O'Neill 2010; Carone et al. 2009). In plants, a novel class of endogenous long siRNAs (lsiRNAs; 30 to 40 nt) were found to be induced by bacterial infection or specific growth conditions, such as cell suspension culture (Katiyar-Agarwal et al. 2007). For example, *AtlsiRNA1* is a cluster of heterogeneous lsiRNAs, most being 39 to 41 nt in length, is strongly induced by *Pseudomonas syringae* pv. *tomato* containing a *Rpt2* avirulent factor (*P. syringae* pv. *tomato* [*avrRpt2*]). *AtlsiRNA1* silences *AtRAP* is a negative regulator of plant defense. When *AtRAP* was knocked out, increased resistance to both virulent and avirulent strains was observed in *Arabidopsis*, indicating defense against bacterial pathogens could be enhanced through upregulation of *AtlsiRNA1* (Katiyar-Agarwal et al. 2007). Long nonprotein coding RNAs (npcRNAs) responsive to powdery mildew infection were identified in wheat. The authors found that wheat long npcRNAs showed tissue dependent expression patterns and were responsive to powdery mildew infection and heat stress (Xin et al. 2010). siRNAs independent of *DCLs* (sidRNAs; 20 to 60 nt in length) that are associated with AGO4 and capable of directing DNA methylation have also been reported. (Ye et al. 2016). In rice, a new class of miRNAs called long miRNAs (lmiRNAs) that are 24 nt long and are loaded into AGO4 clade proteins have been identified, which direct DNA methylation both in *cis* and *trans* (Wu et al. 2010). This evidence indicates that a variety of sRNAs longer than 24 nt exist in vivo and are functional. It is of great interest to investigate whether these long sRNAs are associated with any unknown biogenesis or functional characteristics.

Rice is one of the major food crops in the world, especially in Asia. Rice sheath blight, caused by *Rhizoctonia solani*, is considered as one of the major diseases that lead to significant losses of yields and quality each year (Lee and Rush 1983). Here, we used a deep sequencing approach to investigate the global expression of endogenous long siRNAs in rice against *R. solani* infection. We identified a new class of 25- to 40-nt-long rice endogenous lsiRNAs (osa-lsiRNAs) whose expression is specifically induced by *R. solani* infection. Our results indicate that *R. solani* specifically induces the expression of several osa-lsiRNAs, whose target genes include some defense-related genes. *R. solani* infection is facilitated by inhibited expression of defense-related genes through osa-lsiRNAs-mediated

gene silencing, indicating a crucial role of osa-lsiRNAs in shaping the relationship between rice and *R. solani*.

MATERIALS AND METHODS

Pathogen infection and sRNA library construction. Rice (*Oryza sativa* L. japonica. 'Nipponbare') was grown in soil in a growth room maintained at 26°C and 70% relative humidity with a 12/12-h day (200 $\mu\text{E m}^{-2} \text{s}^{-1}$)/night. The rice sheath blight disease pathogen *R. solani* AG1 IA was used in this study. The fungal strains were grown on potato dextrose agar (PDA: 200 g of potato, 20 g of dextrose, and 20 g of agar) at 28°C. For inoculation, four-leaf-stage seedlings were covered by a mycelium plug (5 mm in diameter) of actively growing *R. solani* mycelium onto the stem. The samples were collected at 0, 24, 48, and 72 h postinoculation (hpi).

RNA extraction and library construction was carried out as described previously (Katiyar-Agarwal et al. 2007; Padmanabhan et al. 2009). Briefly, total RNA was isolated from infiltrated leaves and removed large ribosomal RNA (rRNA) from total RNA by RiboMinus Plant Kit as describe (Invitrogen, A10838-08). Then the RiboMinus RNA was fractionated on 15% denaturing polyacrylamide gel. RNA molecules ranging from 20 to 45 nt were excised and ligated to 5'- and 3'-RNA adaptors using T4 RNA ligase followed by RT-PCR and gel purification as described in the Instruction from Illumina Inc. The sRNA libraries were sequenced by Illumina Inc. and UCR core facility. The 20 to 24 nt will be reported in a separate manuscript.

Long siRNAs analysis. Raw reads were processed to trim the adaptors using cutadapt. After adaptor sequence was removed, clean reads of 25 to 40 nt length were clustered into unique reads. The reads that match known rice repeats, rRNAs, tRNAs, snRNAs, and snoRNAs (allow 2 mismatches) were removed, and remaining reads were further mapped to the rice genome (version 6.1) using bowtie. The expression level of sRNAs were normalized to the all mapped reads using TPTM (transcripts per 10 million). Audic and Claverie method were used for analyzing differentially expression of sRNAs with a significant threshold ($P \leq 0.01$) as previously described. Bonferroni method was used to adjust P values for multiple comparisons. The psTarget program was used to predict the target of long sRNAs. A penalty scoring system (0 to 5) was used for sRNA target prediction, in which score 0 is the best (perfect match). The penalty score for a mismatch or gap in sRNA and target alignment is 1, or 1.5 when it is located in the seed region (2 to 13 nt); the penalty score for a G:U pair is 0.5. The R package TopGO were used for target gene GO term enrichment analysis as described before. Adrian Alexi's improved weighted scoring algorithm and Fisher's test was used to determine the significance of GO term enrichment. Significantly enriched GO terms were identified as those with a P value less than 0.05.

Northern blot analysis. RNA-blot analyses for long siRNAs from total extract were performed as described previously (Katiyar-Agarwal et al. 2007). Total RNA was extracted using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. RNA is resolved on a 17% denaturing 8 M urea-PAGE gel and then transferred and chemically crosslinked onto a Hybond N⁺ membrane (GE Healthcare Life Sciences) using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride. lsiRNAs were detected using end-labeled oligonucleotide probes and exposed to a phosphor imager screen. Expression level was quantified by using Image J as instructed.

qRT-PCR. The total RNA used for qRT-PCR was from the same set of RNA used for RNA-blot analyses. To detect the quality and quantity of RNA, a NanoDrop-1000 was accessed (NanoDrop). The RNA was then reverse transcribed into cDNA by using PrimeScript RT Reagent Kit (Takara, Japan). The qRT-PCR was performed in 15 μl of reaction mixture consisting 1.5 μl of 1 $\times$ SYBR Green (Invitrogen), 1.5 μl of PCR buffer, 0.3 μl of 10 mM dNTPs (Takara), 0.3 μl of Taq, 0.3 μl of ROX DYE2 (Vazyme, China), 1.5 μl of 2 mM

each primer and 2 µl of appropriate diluted cDNA. The conditions for real-time RT-PCR were listed as follow: 94°C for 3 min, then 40 cycles at 94°C for 30 s and 58°C for 30 s followed by 72°C for 35 s for PCR amplification. Transcript levels of each gene were measured by the Applied Biosystems 7500 (Applied Biosystems) according to the manufacturer's instructions. 18s rRNA was used as a quantitative control in the qRT-PCR analysis.

Vector construction. The enzyme digestion connection method was used to make the long siRNAs artificial constructs by using WMD3 online (<http://wmd3.weigelworld.org>), and were cloned into the pEG202 vector. The homologous recombination method was used to make target genes constructs by using ClonExpress II One Step Cloning Kit (Vazyme), and were cloned into the pEG202 vector.

Protein extraction and analysis. Tissue sample was ground in liquid nitrogen and total proteins were extracted using 2× SDS loading buffer. The samples were resolved on a 12% SDS-PAGE gel and transferred onto an Amersham Hybond-P PVDF membrane (GE Healthcare) using a Tris-Glycine transfer buffer. Membranes were blocked with 5% milk in TBS-Tween-20 0.05% (TBST) for 40 min and incubated overnight at 4°C with 1:5,000 dilutions of primary antibodies mouse anti-Flag conjugated to horseradish peroxidase (Abmart), and washed three times with TBST, then incubated overnight at 4°C with 1:1,000 dilutions of secondary antibodies (Beytime). Membranes were washed three times with TBST. Detection was performed using the ECL Plus Western Blotting Detection Reagents (GE Healthcare) and ChemiDoc Touch Imager (Bio-Rad). Image data were analyzed with Image Lab Software (Bio-Rad) and assembled in Adobe Photoshop CS6.

Transient expression analysis in *N. benthamiana*. Transient co-expression assays in *N. benthamiana* were performed by infiltrating 3-week-old *N. benthamiana* plants with Agrobacterium GV3101 (OD₆₀₀ [optical density at 600 nm] = 1.0) harboring constructs containing the long siRNAs (pEG202) with Agrobacterium GV3101 containing (OD₆₀₀ = 1.0) target genes CDS (pEG202), and miR319b (pEG202) was a control. Leaf tissue was collected at 48 hpi. Protein expression was detected by western blot.

Statistical analysis. All data obtained were subjected to two-way analysis of variance (ANOVA) and a Student's *t* test was used to determine significant differences between the treatments. The comparisons with ***P* < 0.01 and **P* < 0.05 were considered significantly different.

RESULTS

Characterization of osa-lsiRNAs by deep-sequencing analysis. To investigate the potential roles that lsiRNAs may play in rice defense against *R. solani*, osa-lsiRNAs expression profiles between rice (*japonica* cultivar Nipponbare) infected with *R. solani* (strain AG1-IA) and noninfected rice (mock) were compared and analyzed. Due to the quick responding nature of plant innate immunity to *R. solani* infection, osa-lsiRNAs libraries were constructed with stem at 24 h postinoculation (hpi; RS-24). At 48 hpi, a large number of hyphae were produced at inoculation site and the rice stalk gradually became yellow and lodging (Supplementary Fig. S1). Long siRNAs fractions ranging from 20 to 45 nt were

cloned and sequenced by Illumina HiSeq platform. A total of 59,304,915 and 45,418,644 reads were generated from the control (control-24) and treatment (RS-24) groups, respectively (Table 1). These reads were mapped to the rice genome (Rice Genome Annotation Project), which resulted in 45,229,961 and 29,210,015 rice reads (corresponding to 76.27 and 64.31% of the total reads), respectively. Reads were further searched against the Rfam database (<http://xfam.org/>) to remove known sRNAs such as ribosomal RNA (rRNA), transfer RNA (tRNA), and small nucleolar RNAs (snoRNAs). A total of 28,425,834 and 13,279,033 reads passed the aforementioned criteria and were obtained from the mock and infected group, respectively, corresponding to 2,267,454 and 1,069,396 unique osa-lsiRNAs. These identified osa-lsiRNAs were regarded as osa-lsiRNAs and were subjected to further analysis (Table 1).

Among the osa-lsiRNAs identified, lengths of 28 and 29 nt were the most abundant ones, each accounting for more than 10% of the total reads (Fig. 1A). The 27 nt and 30- to 36-nt-long siRNAs were between 5 and 10% of total reads. Collectively, osa-lsiRNAs from 27 to 36 nt accounted for more than 85% of the total reads we identified in this research, while the remaining populations represented less than 15% of the total reads (Fig. 1A). Abundance of osa-lsiRNAs longer than 37 nt dropped rapidly to less than 1%, indicating that lengths between 25 and 40 nt should cover most of the long siRNA populations.

Regardless of the varied length, four different types of nucleotide (A, U, G, and C) displayed similar frequency on the first nucleotide position (Fig. 1B), with guanine (G; 28.36%) a little bit higher presented and uracil (U) relatively less presented (20.95%) at the 5'-most position of the osa-lsiRNAs. The other two types of nucleotides, adenine (A) and cytosine (C), represented 27.59 and 23.10% of the total population, respectively. Osa-lsiRNAs were further analyzed according to their origination on the rice genome. Most of the osa-lsiRNAs (91.32%) were derived from transposable elements and repeat regions (TE and repeat); 3.86% were derived from intergenic region; 2.21% were derived from promoter region; and only 0.12% were derived from miRNA region. Osa-lsiRNAs originating from coding regions were also identified, most of which (2.24%) generated from the sense orientation, with only a small fraction from the antisense orientation (Fig. 1C).

Identification of osa-lsiRNAs responsive to *R. solani* infection. Among millions of osa-lsiRNAs identified, we focused on osa-lsiRNAs that are induced or reduced more than twofold ($|\log_2[\text{treated/mock}]| \geq 1$) (Supplementary Table S1). Bioinformatics analysis revealed that 14,415 reads fitted this criterion, implying that a big panel of osa-lsiRNAs are differentially expressed after *R. solani* infection. We hypothesized that if an osa-lsiRNA is involved in rice defense against *R. solani* infection, this specific candidate should fulfill one or more following pre-requirements: (i) candidates should not be expressed in very low level; (ii) candidates should show significantly varied expression after *R. solani* infection; (iii) candidates should target some resistance-related genes; and (iv) candidate sequence should demonstrate a relatively strict match to their target genes. To evaluate this hypothesis, we applied more stringent criteria (including the following: reads [in either treatment] ≥ 60 ; [treated/mock] ≥ 3 or [treated/mock] ≤ 0.33 ; target genes with defense-related functions; matching scores ≤ 3) that would effectively eliminate potential false positive candidates. By applying these criteria, we identified 14 differentially expressed osa-lsiRNAs, which ranged from 25 to 30 nt in length, and whose target genes are involved in a variety of functions such as disease resistance related factors, kinase, transcription factors, and signaling components (Table 2).

The differential expression pattern was further validated by using northern blotting, in which the U6 small nuclear RNA (U6) was used as a loading control. Due to low in vivo abundance, many of these candidates could not be detected even after prolonged exposure (3× longer exposure than regular). At the end, we were able

TABLE 1. Summary of osa-lsiRNAs in *Rhizoctonia solani*-infected samples

Read categories	Mock	(%)	Infected	(%)
Total reads	59,304,915	100.00	45,418,644	100.00
Rice reads (mapped to rice genome)	45,229,961	76.27	29,210,015	64.31
Aligned to Rfam (rRNA, tRNA, snRNA, and snoRNA)	16,804,127	28.34	15,930,982	35.08
Rice sRNA (total reads)	28,425,834	47.93	13,279,033	29.24
Rice sRNA (unique reads)	2,267,454	...	1,069,396	...

to validate six osa-lsiRNAs with clear northern results (Fig. 2A; Supplementary Fig. S2). We further focused on the osa-lsiRNAs whose expression signal were sufficient for Image J quantification (Fig. 2A). Osa-lsiR51031 is a 30-nt lsiRNA, whose expression was suppressed by *R. solani* infection. At as early as 24 hpi, its relative abundance (RA) was about 50% of that at 0 hpi (RA = [Exp]^{24 hpi}/Exp)^{0 hpi}), and continued to 48 and 72 hpi, respectively. After 3 days of infection, the expression level of osa-lsiR51031 was almost below detectable level (RA = 0.06) (Fig. 2A). In contrast, osa-lsiR118183, 194568, and 73750 showed a sustained increase with *R. solani* infection (Fig. 2A). Osa-lsiR118183 was rapidly induced as early as

24 hpi (RA = 3.1), and increased steadily to a medium time point (48 hpi; RA = 3.7) and a late time point (72 hpi; RA = 4.0). Osa-lsiR73750 showed a delayed but stronger induced expression tendency, which showed no increase at 24 hpi but accumulated to more than 7 folds at 48 hpi; after that, it dropped to 3.2 folds at 72 hpi. Osa-lsiR194568 was under detectable level without *R. solani* infection. After infection, it demonstrated mild increase at 24 and 48 hpi but increased quickly to more than 10 folds at 72 hpi (Fig. 2A). Taken together, our results confirmed that some osa-lsiRNAs could be differentially expressed upon *R. solani* infection. This prompted us to further investigate the biological function of these lsiRNAs.

Fig. 1. Characteristics of osa-lsiRNAs. **A**, Length distribution of osa-lsiRNAs. x-axis, osa-lsiRNAs length; y-axis, percentage (%) of osa-lsiRNAs of each length in total population; and nt: nucleotide. **B**, First nucleotide preference of osa-lsiRNAs. A, adenine; C, cytosine; G, guanine; and U, uracil. **C**, Genome distribution of osa-lsiRNAs.

TABLE 2. Differentially expressed rice lsiRNAs and their potential target genes

ID	Length (nt)	Target gene	Annotation	Score	Alignment
lsiR118183	26	Os08g06220	Transferase	0	
lsiR51031	30	Os08g15322	Cytochrome b559 subunit alpha	0	
		Os03g38930	Expressed protein	3	
lsiR194568	25	Os06g38990	Receptor-like protein kinase 2 precursor	2.5	
lsiR73750	29	Os06g39780	Expressed protein	2.5	
		Os09g14490	TIR-NBS type disease resistance protein	2.5	
lsiR4052	30	Os11g41820	RNA recognition motif containing protein	2.5	
lsiR301048	29	Os02g27620	Endonuclease/exonuclease/phosphatase	2.5	
			family domain containing protein		
lsiR267188	29	Os06g12050	mTERF family protein	2.5	
lsiR148949	30	Os06g12050	mTERF family protein	3	
		Os12g37860	Expressed protein	3	
lsiR32311	29	Os12g02540	Broad Complex BTB domain with nonphototropic hypocotyl 3 NPH3 and coiled-coil domains	0	
lsiR218517	29	Os11g47030	Receptor protein kinase	3	
		Os11g47240	Leucine-rich repeat receptor protein kinase	3	
			EXS precursor		
lsiR407572	30	Os01g64790	AP2 domain containing protein	1.5	
lsiR33332	28	Os01g26160	Exportin 1	3	
lsiR407009	29	Os01g04920	Glycosyl transferase, group 1 domain containing protein	3	
lsiR273010	29	Os01g27150	Cullin	3	

For the four osa-lsiRNAs differentially expressed in *R. solani*-infected rice plants, six target genes were identified by bioinformatics prediction. As summarized in Table 2, lsiR194568 targets a gene encoding a putative receptor-like protein kinase 2 precursor (*Os06g38990*; score = 3). Osa-lsiR118183 targets a gene encoding a putative transferase (*Os08g06220*), between which a 100% match was observed. Osa-lsiR51031 targets a gene encoding the putative cytochrome b559 subunit alpha (*Os08g15322*), between which a 100% match was observed as well. Osa-lsiR51031 also targets a gene encoding an expressed protein (*Os03g38930*). Osa-lsiR73750 targets two genes (*Os06g39780* and *Os09g14490*). *Os06g39780* encodes an expressed protein with unknown function, while

Os09g14490 encodes a TIR-NBS type disease resistance protein. To exclude the possibility that the varied expression of these lsiRNAs target genes are due to simultaneously regulation by other differentially expressed regular sRNA, we searched a 20 to 24 nt sRNAs sequencing database from rice either with or without *R. solani* infection (*unpublished data*). We did not find any potential dual-targeting events on these target genes described here. Therefore, we are confident to conclude that the observed correlation between lsiRNAs and their target genes is due to the regulatory role of lsiRNAs. We further examined the in vivo expression of each predicted target genes upon *R. solani* infection. The RNA used for qRT-PCR was from the same set RNA for RNA-blot analyses. *Os08g15322*

Fig. 2. Altered osa-lsiRNAs and targets expression on *Rhizoctonia solani* infection. **A**, Expression of selected osa-lsiRNAs on *R. solani* infection is examined by Northern blotting. Samples from plants inoculated with *R. solani* at indicated time points are collected. RNA blots are hybridized with DNA oligonucleotide probes complementary to the indicated osa-lsiRNAs. U6 is used as a loading control. Single strengths of bands are quantified using Image J, from which relative abundance (RA) levels are calculated. Size indication is at the right side of the gel. Similar results were obtained from two biological replicates. **B**, qRT-PCR analysis of osa-lsiRNAs target genes after *R. solani* infection at time points indicated. Standard deviations were calculated from three technical replicates. ** $P < 0.01$ (Student's *t* test). Similar results were obtained from three biological replicates.

showed 1.8-fold increase since 24 hpi, which continued until 72 hpi. The increased expression of *Os08g15322* is cognately inverse to the expression pattern of siR51031, further suggesting a regulatory relationship between these two parties. *Os09g14490* showed a noticeable reduction since 24 hpi and reached less than 40% of the original level at 72 hpi, which corresponds to the overall increased expression tendency of osa-lsiR73750. The expression of *Os08g06220* and *Os06g38990* also showed a reversed pattern with their cognate lsiRNAs, osa-lsiR118183 and osa-lsiR194568, respectively (Fig. 2B). Taken together, our data indicate that these genes are authentic target genes of their cognate lsiRNAs.

To validate that the predicted genes are authentic targets of these osa-lsiRNAs, both the predicted target genes and their corresponding osa-lsiRNAs were co-expressed transiently in tobacco (*N. benthamiana*). *Os08g06220* is a predicted target gene of osa-lsiR118183 with perfect reverse complementary matching sequence. When transiently expressed in tobacco, *Os08g06220* showed obviously lower expression level in a co-infiltration assay with osa-lsiR118183, than it was expressed alone, or co-infiltrated with an unrelated miRNA (OsmiR319b). Similar examinations were performed on other lsiRNAs. In conclusion, we were able to show that *Os08g06220* is a target gene of osa-lsiR118183; *Os08g15322* is a target of osa-lsiR51031; *Os06g38990* is a target of osa-lsiR194568, and *Os09g14490* is a target of osa-lsiR73750 (Fig. 3). In contrast, *Os03g38930* did not appear to be targeted by osa-lsiR51031, and *Os06g39780* is not a target of osa-lsiR73750 either (data not shown). Therefore, in the following study, we focused on these four target genes *Os08g06220*, *Os08g15322*, *Os06g38990*, and *Os09g14490*.

Osa-lsiRNAs are produced and function through canonical sRNA silencing components. Whether osa-lsiRNAs share a common biogenesis route with canonical rice sRNAs (miRNA and siRNA within 21 to 24 nt range) is not known yet. To investigate the

essential components involved in osa-lsiRNAs biogenesis, we examined osa-lsiRNA expression level in different *dcl* mutants available to us. Northern blotting results showed that expression of osa-lsiR51031 was significantly reduced in *dcl4* mutant, while hardly any noticeable change was observed in both wild type and *dcl1* and *dcl3* mutants (Fig. 4A). Likewise, although less abundant, osa-lsiR73750 and osa-lsiR118183 showed similar expression pattern as osa-lsiR51031, that is, comparable expression in wild type and *dcl1*, but measurably reduced level in *dcl4* mutant (Fig. 4A). Interestingly, osa-lsiR73750 showed slightly reduced expression in *dcl3* mutant whereas osa-lsiR118183 remained unaffected. Osa-lsiR194568 was under detectable level, which agrees with our observation that osa-lsiR194568 gradually reached to a detectable level after *R. solani* infection (Fig. 2A). These results indicate that osa-lsiRNAs require *DCL4* but not *DCL1* for maturation, and *DCL3* is likely involved. We also examined the expression level of these target genes in *dcl* mutants. In agreement, *Os08g15322*, *Os08g06220*, *Os09g14490*, and *Os06g38990* demonstrated uniformly increased expression level in the *dcl4* mutant, while only *Os06g38990* showed a dependence on *DCL1* (Fig. 4B). In summary, our results indicate that these four genes are authentic target genes of aforementioned osa-lsiRNAs, whose generation involves the *DCL4* or other dicing process.

Components involved in osa-lsiRNAs function were also investigated. We measured expression level of osa-lsiRNAs target genes in various Argonaute mutants available to us (*osago1d*, *osago2*, *osago3*, *osago11*, *osago12*, *osago14*, *osago15*, and *osago18*). The *osago* mutant information is shown in Supplementary Table S2. We also confirmed AGOs expression level in these mutants by qRT-PCR (Supplementary Fig. S3). *Os08g06220*, which is a target gene of osa-lsiR118183, showed a two- and fourfold increase in *osago2* and *osago18*, respectively (Fig. 5). *Os06g38990* (target of osa-lsiR194568) exhibited similar expression pattern, with a 15- and

Fig. 3. Validation of osa-lsiRNAs targets. Predicted target genes are transiently co-expressed with corresponding osa-lsiRNAs or an irrelevant sRNA (miR319b) and the flag-tagged target genes in tobacco by Agrobacterium-mediated infiltration. Target gene expression are detected by antibody recognizing flag tag fused to the target genes. β -actin was used as a loading control. Similar results were obtained from three biological replicates.

12-fold increase in *osago2* and *osago18*, respectively. These results indicate that *osa-lsiR118183* and *osa-lsiR194568* may regulate the expression of its target genes through *OsAGO18* and *OsAGO2*. *Os09g14490* (target of *osa-lsiR73750*) and *Os08g15322* (target of *osa-lsiR51031*) showed similar expression profiles among different *osago* mutants. In *osago18*, *Os09g14490* and *Os08g15322* were 4- and twofold increase, while *Os09g14490* showed a twofold increase, but the variation of *Os08g15322* was not significant. These results suggest that *osa-lsiR73750* and *osa-lsiR51031* function depends on *OsAGO18* but may require *OsAGO2*. Taken together, our results suggest that, similar to canonical sRNAs, *osa-lsiRNAs* function through the AGO pathway. In specific, *OsAGO18* and *OsAGO2* are associated with *osa-lsiRNAs* function, whereas involvement of other *OsAGOs* may be dispensable.

DISCUSSION

Plant sRNAs ranging from 21 to 24 nt have received enormous attention for their biological function in plant growth, development,

reproduction, and immunity. Generally, these sRNAs are divided into two categories: microRNA and siRNA, which differ both in term of biogenesis and function. Nevertheless, both canonical miRNA and siRNA are within 21 to 24 nt in length, and rely on Dicer-like and Argonaute proteins. sRNAs longer than 24 nt (lsiRNAs) have been identified in *Arabidopsis*, and implicated in plant resistance against bacterial pathogens (Katiyar-Agarwal et al. 2007). Whether these long siRNAs function in a unique way or contribute to a specific biological trait is unknown, especially in rice.

High-throughput sequencing has identified sRNAs at the whole-genome level in a context of plant–pathogen interaction (Naqvi et al. 2010; Niu et al. 2015; Seo et al. 2013). For rice endogenous sRNAs, Wu et al. (2015) characterized the sRNA profiles of rice plants infected by *Rice dwarf virus* (RDV) and *Rice stripe virus* (RSV) using high-throughput sequencing, and revealed a critical role of *OsAGO18* in resistance against RSV. Guo et al. (2012) reported seven novel RSV-responsive miRNAs identified by high-throughput sequencing. A recent study identified 23 candidate rice miRNAs that may be involved in plant immunity against *R. solani*

Fig. 4. Effects of *OsDCLs* on the expression of lsiRNAs and their targets. **A**, Expression of *osa-lsiRNAs* depends on *OsDCL4*. Accumulation of *osa-lsiRNAs* are detected with DNA oligonucleotides complementary to the indicated *osa-lsiRNAs*. U6 was used as the loading control. Similar results were obtained from two biological replicates. **B**, Expression of four *osa-lsiRNAs* target genes in *osdcl* healthy mutants is detected by real-time PCR. x-axis, different mutant plants; y-axis, relative expression of the detected target genes. Standard deviations were calculated from three technical replicates. ** $P < 0.01$ and * $P < 0.05$ (Student's *t* test). Similar results were obtained from three biological replicates.

infection (Lin et al. 2016). We hypothesized that if osa-lsiRNAs biogenesis and function follows a canonical pathway, that they might also be involved in plant defense response as well. In this study, we showed that rice endogenous lsiRNAs (25 to 40 nt) were expressed at measurable level and could suppress target gene expression, including some genes encoding defense-related proteins. We further demonstrated that the expression level of some osa-lsiRNAs were influenced by *R. solani* infection, which is facilitated by inhibited expression of defense-related genes.

lsiRNAs normally are overlooked for functional study due to their relative less prominent abundance (Jin and Zhu 2010; Voinnet 2009). Furthermore, these molecules sometimes are masked by spontaneous RNA degradation products, which makes it neglected by traditional investigation. Distribution analysis showed that osa-lsiRNAs identified in this study originated from various genome loci with sharp start and end positions (Supplementary Fig. S4). This is quite different from spontaneous RNA degradation, which shows stretched patterns along generating loci. In addition, northern detection showed that osa-lsiRNAs migrated as homogenous molecules, without trailing smears above or beneath them (Fig. 2A). These features distinguish osa-lsiRNAs from RNA degradates.

Higher plants possess many proteins with RNase III-type activity that are required for sRNA production. Dicer-like proteins (DCLs) are RNase III-type enzymes that cleave the double-stranded RNA precursors and process them into mature siRNAs. Rice encodes four types of DCLs with different functional specificities. OsDCL1 plays a critical role in miRNA processing in rice. Research showed that in rice mutant with *OsDCL1* defect, production of miRNA was severely reduced while the production of siRNA was not affected (Liu et al. 2005). *OsDCL3* is involved in production of transposable element-associated 24-nt siRNAs and control certain agricultural traits in rice (Wei et al. 2014). *OsDCL3* has two homologs, *OsDCL3a* and *OsDCL3b*, the latter of which is associated with the processing of 24-nucleotide phased sRNA (phasiRNA) (Song et al. 2012). *OsDCL4* is involved in processing of 21-nucleotide siRNAs, including transacting siRNAs (tasiRNA) and phasiRNA. By using mutants currently available to us (*osdcl1*, *osdcl3*, and *osdcl4*), we were able to show that production of osa-lsiR51031, osa-lsiR73750,

and osa-lsiR118183 requires *OsDCL4*. Osa-lsiR194568 was below detectable level and was not discussed here, as well as the *osdcl2* mutant that is unavailable to us. These results suggest *OsDCL4* plays an essential role in osa-lsiRNAs accumulation in rice. In *Arabidopsis*, generation of the 30- to 40-nt endogenous lsiRNAs requires *DCL1* and *DCL4* (Katiyar-Agarwal et al. 2007). Therefore, it seems that the involvement of *DCL4* in lsiRNA production is conserved between monocot and dicot plants.

In rice, phasiRNA is one type of siRNA whose production is dependent of *OsDCL4*. In *osdcl4* loss-of-function mutants, dramatic reduction of phasiRNA was observed, regardless if it is the 21- or 24-nt type (Song et al. 2012). Therefore, we investigated whether osa-lsiRNAs are produced as phasiRNAs. Most of the 21- and 24-nt phasiRNA clusters are initiated from the target sites of miR2118 or miR2275 (Song et al. 2012). Sequence analysis indicates that there are no target sites identified 2,000 nt up- or down-stream of the osa-lsiRNAs generating loci, indicating the generation of these osa-lsiRNAs is not triggered by cleavage of the 22-nt miRNAs such as miR2118 or miR2275. When the intermediate up- and down-stream regions were examined (−2,000 to +2,000 nt), there was no observable siRNA in a phased pattern observed either, indicating these osa-lsiRNAs are not produced as phased siRNAs. Furthermore, the accumulation of the two triggering miRNAs (miR2118 or miR2275) requires OsDCL1 activity (Song et al. 2012). None of the osa-lsiRNAs required *OsDCL1* for their biogenesis (Fig. 4A), further excluding the possibility that lsiRNAs are phasiRNAs.

Regardless of difference in biogenesis and mechanism of action, sRNAs need to be incorporate into the RNA-induced silencing complex (RISC) core by Argonaute proteins. So far about 19 rice Argonaute genes have been identified (Kapoor et al. 2008). According to phylogenetic relationships, plant Argonaute proteins can be divided into three major clades following the *Arabidopsis* AGO system (Fang and Qi 2016). The AGO1/5/10 clade includes *OsAGO1*, 5, 10, 11, 12, 13, 14, and 17. *OsAGO1* is associated with miRNA activity, defect of which causes pleiotropic developmental phenotypes (Wu et al. 2009). *OsAGO5* is associated with 21-nt phasiRNA with a preference to siRNA started with a 5' C (Komiya

Fig. 5. Effects of *OsAGOs* on the expression of lsiRNA targets. Expression of four osa-lsiRNAs target genes in *osago* healthy mutants is detected by real-time PCR. x-axis, different mutant plants; y-axis, relative expression of the detected target genes. Standard deviations were calculated from three technical replicates. ***P* < 0.01 and **P* < 0.05 (Student's *t* test). Similar results were obtained from three biological replicates.

et al. 2014). *OsAGO10* plays an important role in shoot apical meristem (SAM) maintenance, with specifically associated sRNA unidentified (Nishimura et al. 2002). In the *osago1d* mutant plants we used, expression of target genes from each osa-lsiRNAs was examined by real-time PCR. Results showed that there was no discernable difference between the wild type and mutant plants, indicating *OsAGO1d* is not involved in silencing of osa-lsiRNAs targets. We were not able to check other *AGO1* clade mutant unavailable to us. Wu and colleagues showed that rice encodes four *AGO1* homologs (*AGO1a*, *AGO1b*, *AGO1c*, and *AGO1d*). *AGO1a*, *AGO1b*, and *AGO1c* all showed a strong preference for binding sRNAs with 5' U and have slicer activity. All three *AGO1s* predominantly bound known miRNAs. Most of the miRNAs were evenly distributed in the three *AGO1* complexes (Wu et al. 2009). Taken together, we propose that *OsAGO1* is not required for osa-lsiRNAs silencing.

OsAGO2/3/7 form a unique clade, whose function is less reported. *OsAGO7* is involved in tasiRNA biogenesis, some of which are involved in the regulation of SAM development (Nagasaki et al. 2007). *AGO7* function is conserved in *Arabidopsis*, rice, and maize. Therefore, other components of the same clade might function similarly to their counterparts in *Arabidopsis*. In our study, we showed that the suppression of all the osa-lsiRNAs target genes was released in *osago2* mutant plants, indicating *OsAGO2* is required for osa-lsiRNAs silencing. In contrast, we did not observe any expression variation between *osago3* and the wild-type plants, indicating *OsAGO3* is not involved in osa-lsiRNA-mediated gene silencing. This is in agreement with previous observation that although *AGO2* and *AGO3* are close homologs from the same clade, their functions are different (Zhang et al. 2011).

Phylogenetically, *OsAGO11*, *12*, and *14* are from the same clade as *AtAGO4*. Rice encodes four *AGO4* homologs that are *OsAGO4a*, *b*, and *OsAGO15* and *16* (Kapoor et al. 2008). *OsAGO4a*, *b*, and *16* can bind 24-nt siRNAs and lmiRNAs (Wu et al. 2010). Therefore, we tested whether osa-lsiRNA function is affected by rice plants defective in *AGO4* clade members. Our results indicated that none of the target expression was affected noticeably in these mutant plants (Fig. 5), indicating osa-lsiRNAs function through an alternate pathway without employing these AGOs.

AGO18 is conserved in monocots, and has been characterized in rice and maize (Fang and Qi 2016). *OsAGO18* is involved in resistance against viral infection. Defects in *OsAGO18* leads to increased susceptibility while its overexpression is associated with enhanced viral resistance (Wu et al. 2015). It was demonstrated that *OsAGO18*'s contribution to viral defense is through preferential recruit of miR168, which liberates *OsAGO1* from miR168-mediated degradation and confer resistance function. Maize *AGO18* is associated with 24-nt phasiRNAs (Zhai et al. 2014). In *osago18* plants, all the four target genes showed measurable increase, suggesting osa-lsiRNAs regulate their target gene expression through the *OsAGO18* route.

Our results revealed that osa-lsiR51031 constitutively expressed in uninfected plants. After *R. solani* infection, the expression reduced dramatically (Fig. 2A). Osa-lsiR51031 targets a gene encoding cytochrome b559 subunit alpha (*LOC_Os08g15322*), which is verified by transient co-expression in tobacco (Fig. 3). Cytochrome b559 is part of the reaction core of Photosystem II (PSII), which participate in an electron transport pathway that helps protect PSII from photo-damage (Chu and Chiu 2016). Moreover, Cytochrome b559 reveal an important role during temperature stress by rescuing PSII from oxidative damage (Tiwari et al. 2008). The expression of *Os08g15322* is induced by *R. solani* infection, which is in accordance with the decreasing expression of osa-lsiR51031 (Fig. 2B). The biological function of Cytochrome b559 in interaction between rice and *R. solani*, and the cause and consequence associated with induced Cytochrome b559 expression upon *R. solani* infection, is unknown yet.

Osa-lsiR118183 could target *Os08g06220*, which encodes a transferase. In rice, salicylic acid (SA) treatment can induce SA glucosyl-transferase expression which is involved in plant immunity (Silverman

et al. 1995). The homolog in *Arabidopsis* (*At5g61160*) is involved in the biosynthesis of hydroxycinnamic acid amides, which play a role in defense against pathogens (Muroi et al. 2009). Our results indicate that *R. solani* infection induces lsiR118183 expression, which leads to reduced expression of its target, the transferase. Therefore, *R. solani* infection may specifically reduce plant resistance by inhibiting expression of resistance-related genes through lsiRNA-mediated gene silencing. This hypothesis is verified by osa-lsiR194568 and osa-lsiR73750, which target a receptor-like protein kinase 2 (RLPK2) and a TIR-NBS type disease resistance protein (TNRP), respectively. Many studies have reported that RLPK and TNRP are involved in plant immunity. For example, *Arabidopsis* flagellin receptor FLS2, the EF-Tu receptor EFR (Gomez-Gomez and Boller 2000; Zipfel et al. 2006), RPS4 (Zhang et al. 2004), RPP1A (Weaver et al. 2006), and the rice protein PRR XA21 (Song et al. 1995) mediate immunity during plant-microbe interaction. Not surprisingly, both RLPK2 and TNRP were reduced upon *R. solani* infection, confirming our hypothesis that *R. solani* could manipulate plant innate immunity by inhibiting expression of key defense components.

In summary, our results revealed the existence of endogenous osa-lsiRNAs in rice, some of which are induced by *R. solani* infection. It seems that osa-lsiRNA-mediated gene silencing is employed by *R. solani* to break down host defense. Whether osa-lsiRNAs could manipulate host defense genes in response to other pathogens and how osa-lsiRNAs are affected by pathogen infection need further investigation.

Results for biological replicates are included in Supplementary Figure S5.

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