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RNA-dependent RNA polymerase 6 of rice (*Oryza sativa*) plays role in host defense against negative-strand RNA virus, *Rice stripe virus*

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ABSTRACT

RNA-dependent RNA polymerases (RDRs) from fungi, plants and some invertebrate animals play fundamental roles in antiviral defense. Here, we investigated the role of *RDR6* in the defense of economically important rice plants against a negative-strand RNA virus (*Rice stripe virus*, RSV) that causes enormous crop damage. In three independent transgenic lines (OsRDR6AS line A, B and C) in which *OsRDR6* transcription levels were reduced by 70–80% through antisense silencing, the infection and disease symptoms of RSV were shown to be significantly enhanced. The hypersusceptibilities of the OsRDR6AS plants were attributed not to enhanced insect infestation but to enhanced virus infection. The rise in symptoms was associated with the increased accumulation of RSV genomic RNA in the OsRDR6AS plants. The deep sequencing data showed reduced RSV-derived siRNA accumulation in the OsRDR6AS plants compared with the wild type plants. This is the first report of the antiviral role of a RDR in a monocot crop plant in the defense against a negative-strand RNA virus and significantly expands upon the current knowledge of the antiviral roles of RDRs in the defense against different types of viral genomes in numerous groups of plants.

1. Introduction

In most eukaryotes, small RNAs of 21–30 nucleotides (nt) in length, including microRNAs (miRNAs), small-interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs), guide the RNA silencing machinery to regulate mRNA stability, translational repression or DNA methylation in a sequence-specific manner. Such small RNA-guided RNA silencing plays fundamental roles in developmental control, epigenetic modifications and antiviral defense (Baulcombe, 2005; Carthew and Sontheimer, 2009; Chapman and Carrington, 2007; Chen, 2009; Ding, 2010; Malone and Hannon, 2009; Voinnet, 2009). In plants, endogenous RNA-dependent RNA polymerases (RDRs) convert single-stranded RNA into long double-stranded (ds) RNA, which is then processed by Dicer-like (DCL) nucleases into siRNAs (Wassenegger and Krczal, 2006). Among the six RDR genes that are encoded by the *Arabidopsis thaliana* genome, the functions of RDR1, RDR2 and RDR6 have been well demonstrated. RDR1 participates in the biogenesis of viral siRNAs that are derived from several positive-strand RNA viruses (Diaz-Pendon et al., 2007; Donaire et al., 2008; Garcia-Ruiz et al., 2010; Qi et al., 2009; Wang et al., 2010). RDR2 is responsible for the biogenesis of the DCL3-dependent 24 nt repeat-associated siRNA (rasiRNA) that guides sequence-specific heterochromatic silencing (Chapman and Carrington, 2007; Li et al., 2006; Pontes et al., 2006). RDR6 is required for the biogenesis of several classes of siRNAs, including those that are derived from transgene transcripts (Dalmay et al., 2000; Mourrain et al., 2000), the 21/22 nt *trans*-acting siRNAs (ta-siRNAs) (Peragine et al., 2004; Yoshikawa et al., 2005), the 21/24 nt natural *cis* antisense transcript-associated siRNAs (nat-siRNAs) (Borsani et al., 2005) and viral siRNAs that are derived from several positive-strand RNA viruses (Donaire et al., 2008; Qi et al., 2009; Wang et al., 2010, 2011).

RDR6-deficient plants show distinct responses to specific virus infections. The RDR6-deficient *A. thaliana* and *Nicotiana benthamiana* plants show enhanced symptoms in comparison with the wild type plants when they are infected with several positive-strand RNA viruses, but this effect has not been observed using the other tested viruses (Dalmay et al., 2000; Mourrain et al., 2000; Qu et al., 2005; Schwach et al., 2005; Vaistij and Jones, 2009; Wang et al., 2011). *A. thaliana* RDR6 mutant plants have been observed

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to develop more serious symptoms than wild-type plants when they are infected with cucumber mosaic virus (CMV) but not with tobacco vein-clearing virus, turnip mosaic virus, tobacco rattle virus or tobacco mosaic virus (TMV) (Dalmay et al., 2000; Mourrain et al., 2000). *N. benthamiana* plants with down-regulated RDR6 expression show enhanced susceptibilities to turnip crinkle virus and tobacco mosaic virus in a temperature-dependent manner and increased accumulation of potato virus X and plum pox virus regardless of the temperature regimes (Qu et al., 2005; Schwach et al., 2005). In addition, the virus invasion of the shoot apical meristem (SAM) has been shown to be inhibited by RDR6 (Di Serio et al., 2010; Qu et al., 2005; Schwach et al., 2005).

These reports indicate that the broader significance of the antiviral role of RDR6 remains to be elucidated, in particular, whether RDR6 plays an antiviral role in defense against different types of viruses, especially the negative-strand RNA viruses that have not yet been investigated. Additionally, it is unknown whether RDR6 plays an antiviral role in both dicot model plants and economically important monocot crops. In this study, we investigated the role of RDR6 in rice (*Oryza sativa*) in the defense against rice stripe virus (RSV). Rice is one of the most important food crops worldwide and an emerging monocot model for many aspects of plant biology (Shimamoto and Kyozuka, 2002). *Rice stripe virus*, which belongs to the genus *Tenuivirus*, is a negative-strand RNA virus that causes severe yield losses nearly every year in rice-growing areas (Hibino, 1996; Wei et al., 2009). Thus, elucidating the role of rice RDR6 in RSV infection will significantly broaden the current knowledge of the antiviral role of this protein and further establish a monocot–virus interaction model system to address problems that are of both fundamental and practical importance.

RSV is transmitted predominantly by the small brown planthopper (*Laodelphax striatellus*) (Falk and Tsai, 1998; Hibino, 1996). It causes extremely devastating diseases in rice that frequently lead to plant death (Washio et al., 1967). The RSV genome comprises four single-stranded RNAs that encode seven proteins using ambisense coding strategies (Falk and Tsai, 1998; Ramirez and Haenni, 1994). RNA1 is negative sense and encodes a protein that is a member of the viral RNA-dependent RNA polymerase family (Toriyama et al., 1994). RNA2, RNA3 and RNA4 are all ambisense segments that contain two open reading frames (ORFs). One is located near the 5′ terminus of the viral RNA (vRNA), whereas the other is located near the 5′ terminus of the viral complementary RNA (vcRNA). The vRNA of RNA2 encodes the NS2 protein, whose function is unknown. And the vcRNA of RNA2 encodes the putative membrane glycoprotein NSvc2 (Takahashi et al., 1993). The vRNA from RNA3 encodes NS3, which functions as an RNA silencing suppressor, and the vcRNA from RNA3 encodes the nucleocapsid protein NSCP (Kakutani et al., 1991; Xiong et al., 2009). The vRNA from RNA4 encodes the disease-specific protein SP, and the vcRNA from RNA4 encodes the movement protein NSvc4 (Toriyama, 1986; Xiong et al., 2008; Zhu et al., 1992).

There are five RDR genes that have been annotated in the rice genome (Zong et al., 2009). Mutations in rice *SHOOTLESS2* (*SHL2*), which encodes the ortholog of *Arabidopsis RDR6*, cause defects in embryo development, including the complete deletion or abnormal formation of the SAM in embryos (Nagasaki et al., 2007). Plants with strong *SHL2* alleles are embryonic lethal, and those with weak alleles are able to germinate; however, most of them die within one week after germination, and the rest survive for less than two months (Itoh et al., 2000; Satoh et al., 2003). Thus, these rice mutants cannot be used for virus infection studies. To circumvent this problem, we generated transgenic rice plants in which the transcription of *OsRDR6* is down-regulated using an antisense construct to investigate the role of the OsRDR6 protein in antiviral defense.

2. Materials and methods

2.1. Plant materials

Rice (*O. sativa*) subsp. *japonica* Zhonghua11, Zhendao88 and Wuyujing3 plants were used in this study. Zhonghua11, which is one of the most widely cultivated rice varieties in China, was used as the wild type to generate the *OsRDR6* antisense transgenic plants. Zhendao88 and Wuyujing3 were used as resistant and susceptible controls, respectively, in the RSV infection experiments (Zhou et al., 2006). The rice seeds were immersed in water for 2 days to induce germination, and then the plants were grown in a greenhouse under 14 h light/10 h dark and 33 °C/28 °C (day/night) conditions.

2.2. Transformation of rice

The *Agrobacterium tumefaciens*-mediated transformations of the rice calli were performed according to the published protocol (Hiei et al., 1994).

2.3. DNA plasmids and constructs

The full-length ORF of *OsRDR6* was cloned using RNA that had been extracted from 2-week old rice seedlings (cv. Zhonghua11, *japonica*). Total RNA was isolated from rice plants using TRIZOL (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. First-strand cDNA was prepared from total RNA using Superscript II Reverse Transcriptase (Invitrogen) and the primer OsRDR6R (sequence listed in Table S1), which is specific for *OsRDR6*. The cDNA was then amplified using KOD-Plus (TOYOBO, Osaka, Japan) and the primers OsRDR6NS1 and OsRDR6R (sequences listed in Table S1). A 3.6-kb PCR product was gel purified and then ligated into pBluescript (KS) that had been digested by *EcoRV*. The *OsRDR6* fragment was then subcloned into the binary vector pWM101 (Zhang et al., 2008) that had been digested with *Xba*I and *Nru*I in the antisense expression orientation under the control of the cauliflower mosaic virus 35S promoter.

2.4. Virus infection assay

The insects (*L. striatellus*) were cultured as previously described (Washio et al., 1967). Rice plants at the two-leaf stage were exposed to viruliferous insects for 48 h. The ratio of plants to insects was 1:3. Then the insects were removed, and the plants were grown in a greenhouse under the same conditions as described above. Details of the procedures have been previously described (Shimizu et al., 2011).

2.5. Non-preference test

For each line, 30 germinated seeds were sown in three rows (10 seeds per row) in plastic trays with soil. The trays holding all of lines to be detected were placed in a large cage. The insects (*L. striatellus*) were released inside of the cage when the seedlings were at the 1.5 leaf stage at a 1:3 (plant seedling: insect) ratio. The number of insects settling on each row of plants was counted daily. The mean number of settled insects on each seedling over a period of 3 days was used as the index of non-preference for each line. Details of the procedures have been previously described (Nemoto et al., 1994). Three independent biological replicates were performed.

2.6. RNA isolation, quantitative RT-PCR and northern blot

Total RNA was isolated from rice plants using TRIZOL (Invitrogen) following the manufacturer's instructions. The RNA that was used for the quantitative RT-PCR was treated with RQ1 RNase-free

DNase (Promega, Madison, WI, USA). One microgram of total RNA was reverse transcribed by SuperScript II Reverse Transcriptase (Invitrogen) using oligo d(T) primers according to the manufacturer's instructions. The quantitative PCR reaction was performed using the SYBR Green Real-Time PCR Master Mix (TOYOBO) following the manufacturer's instructions.

The primers that were used in the quantitative PCR assay are listed in Table S1. Relative transcript levels were calculated using the $2^{-\Delta\Delta C(t)}$ method with the *OsEF1α* transcript levels serving as the internal standards. Each data set was derived from at least three repeated experiments.

To detect viral genomic RNA, 2 μg of total RNA were separated on 1.2% (w/v) formaldehyde-denaturing agarose gels and blotted onto Hybond N^+ nylon membranes (Amersham, Buckinghamshire, UK). The probes were amplified from reverse transcription products using the primers that are listed in Table S1 and labeled using α-^{32}P-dCTP with the Primer-a-Gene Labeling System (Promega). Hybridization was performed in a 50% formamide, 5× SSC, 1% SDS, 5× Denhardt's solution with 100 μg/ml sperm DNA at 42 °C. After the overnight incubation, the membranes were washed twice in 2× SSC and 0.1% SDS for 10 min at room temperature and three times in 0.2× SSC and 0.1% SDS for 30 min at 42 °C. The membranes were stripped with 0.1% SDS at 100 °C and re-probed. The details of the procedures have been previously described (Du et al., 2011).

For the siRNA analysis, 20 μg of total RNA was separated on 8 M urea/15% denaturing polyacrylamide gels and electrically transferred to Hybond N^+ nylon membranes (Amersham). Hybridization was performed in an ULTRAhyb-oligo hybridization buffer (Ambion, Austin, TX, USA) at 40 °C overnight. The blots were washed twice at 40 °C in 2× SSC and 0.5% SDS for 30 min. The probe template for *OsRDR6* was amplified by the primers OsRDR6P1661 and OsRDR6P2256 (sequences listed in Table S1) and cloned into the pGEM-T vector (Promega). The probe was labeled with α-^{32}P-UTP using the Riboprobe® *in vitro* Transcription Systems (Promega). The probe that was complementary to the U6 sequence was used as a loading control. The radioactive signals were detected using a PhosphorImager (Perkin Elmer, Shelton CT, USA).

2.7. Small RNA cloning, sequencing and bioinformatics analysis

The rice plants that showed typical disease symptoms were collected at 25 dpi (days post infection) for the small RNA analysis. Small RNA cloning for Illumina 1G sequencing was carried out as described (Wu et al., 2009). A bioinformatics analysis was also performed as described (Du et al., 2011). Small RNA sequences that were 18–28 nt in length were used for further analyses. The bioinformatics tool BOAT, which was provided by the Center of Bioinformatics (CBI), was used for mapping the small RNAs to the *O. sativa* genome sequence (TIGR Rice Annotation Release 5.0, ftp://ftp.plantbiology.msu.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/) and RSV genome sequences (ftp://ftp.ncbi.nih.gov/genomes/Viruses/Rice_stripe_virus_uid14795/) (Zhao et al., 2009). The parameters for BOAT were set as follows: 8 for seed sequence length, 5 for mask sequence length and 0 for mismatch number for the alignment. Small RNAs with perfect genomic matches were used for further analyses.

3. Results

3.1. Cloning of O. sativa RDR homolog

We first cloned the full-length cDNA of *OsRDR6* (GenBank accession number: NC008394.4) by RT-PCR using total RNA that had been isolated from 2-week-old rice seedlings as the template. The protein sequences of OsRDR6 and other RDRs from *O. sativa* were aligned with RDRs from *N. benthamiana*, *Nicotiana tabacum* and *A. thaliana* to construct a phylogenetic tree using the neighbor-joining method. It was discovered that the protein sequence of OsRDR6 is 57.0% identical and 67.5% similar to that of the AtRDR6 and 58.0% identical and 70.7% similar to that of the NbRDR6. The phylogenetic tree indicates that OsRDR6, AtRDR6 and NbRDR6 belong to the same subgroup (Fig. 1). Both OsRDR6 and AtRDR6 contain the same DLDGD motif, which is characteristic of the catalytic domains of higher plant RDRs (Wassenegger and Krczal, 2006) (Table S2).

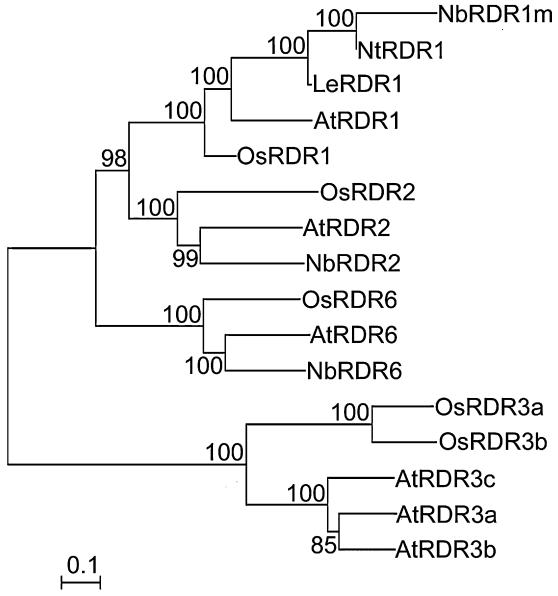

Fig. 1. Phylogenetic analysis of RDRs from *Oryza sativa*, *Arabidopsis thaliana*, *Nicotiana tabacum*, *N. benthamiana* and *Lycopersicom esculentum* (*Solanum lycopersicum*). The neighbor-joining (NJ) trees were constructed using the MEGA software (version 4; http://www.megasoftware.net/index.html) with the following parameters: Poisson correction, pairwise deletion and bootstrap (1000 replicates; random seed). The scale bar represents 0.1 substitution per site.

3.2. Down-regulated transcription of OsRDR6 in antisense rice plants

To study the function of OsRDR6, we generated transgenic rice (Zhonghua11) plants that expressed an antisense *OsRDR6* construct. In three independent lines (OsRDR6AS A, OsRDR6AS B and OsRDR6AS C), *OsRDR6* transcript levels were reduced by 70–80% (Fig. 2A). The *OsRDR1*, *OsRDR2* and *OsRDR3a* transcript levels did not significantly change. The *OsRDR3b* transcript levels increased in lines B and C but did not significantly change in line A. We also detected the siRNAs that were derived from the *OsRDR6* sequence using *OsRDR6*-specific probes. As shown in Fig. 2B, *OsRDR6*-specific siRNAs accumulated in these three transgenic lines. Furthermore, these lines did not show evident developmental phenotypes compared with the non-transgenic control (WT) plants (Fig. 3A).

3.3. Reduced transcription of OsRDR6 in rice resulted in hyper susceptibility to Rice stripe virus

To assay the impact of down-regulated *OsRDR6* transcription on viral infection, we exposed the OsRDR6AS plants (lines A–C) to RSV. The non-transgenic Zhonghua11, susceptible Wuyujing3 and resistant Zhendao88 rice varieties served as controls (Zhou et al., 2006). The plants began to show typical symptoms of infection, such as chlorotic stripes, at 12 dpi and were monitored for another 13 days (Fig. 3B). There were no significant differences in

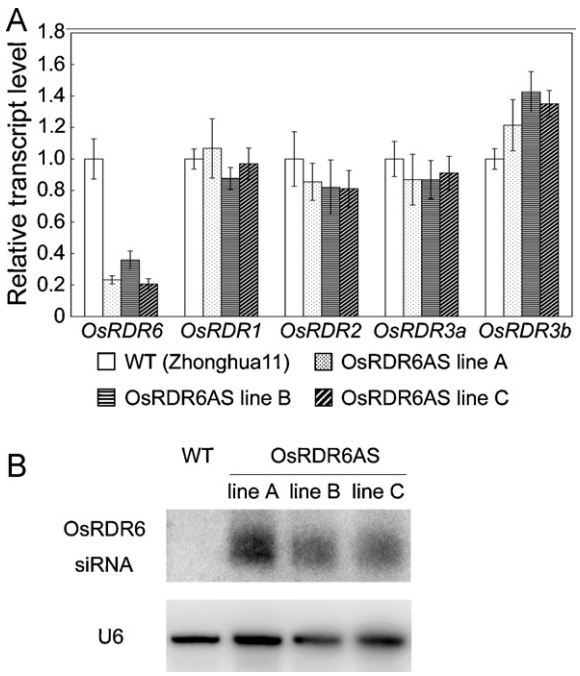

Fig. 2. Reduced transcription of *OsRDR6* in antisense transgenic rice plants. (A) Quantitative RT-PCR analysis of *OsRDR6*, *OsRDR1*, *OsRDR2*, *OsRDR3a* and *OsRDR3b* transcripts in *OsRDR6* antisense transgenic rice and non-transgenic Zhonghua11 control plants. Total RNA was extracted from 2-week-old seedlings. Relative transcript levels were calculated using the $2^{-\Delta\Delta C(t)}$ method with *OsEF1α* transcripts serving as internal standards. The steady-state level of *OsRDR6* in non-transgenic Zhonghua11 was set to 1.0, and the levels that were observed in the antisense plants were expressed as relative values. Error bars indicate standard deviations. (B) Detection of OsRDR6-specific siRNAs in OsRDR6AS transgenic lines A, B and C. Rice U6 served as a loading control.

the incidences of RSV infection among the three OsRDR6AS lines and the non-transgenic Zhonghua11, Wuyujing3 and Zhendao88 plants during early infection (14 dpi). However, the RSV incidences in the three transgenic lines began to rise compared with the non-transgenic Zhonghua11 plants at 16 dpi and continued on this trend until 24 dpi (Fig. 3B). At 24 dpi, the incidences of infection for the OsRDR6AS lines A, B and C reached 64.1, 64.0 and 65.1%, respectively; those for Zhonghua11, Wuyujing3 and Zhendao88 reached 52.7, 72.0 and 13.3%, respectively.

The infected OsRDR6AS plants exhibited more severe disease symptoms than the infected non-transgenic Zhonghua11 plants (Fig. 3C and D). In general, RSV infection in the rice plants typically led to the appearance of chlorotic stripes, mottling and necrotic streaks on the leaves, especially on those that were newly emerged. The leaves rolled up, developed increasing numbers of chlorotic stripes and turned completely yellow, eventually leading to plant death. As shown in Fig. 3A, the infected OsRDR6AS transgenic lines exhibited dwarfing symptoms. The newly emerged leaves of the OsRDR6AS transgenic rice plants turned completely yellow and rolled up (Fig. 3C). To quantitatively analyze the differences in the RSV symptoms in the different lines, we used a disease-rating index according to a previously described method with some modifications (Washio et al., 1967; Zhou et al., 2006). The rice plants that showed RSV infection symptoms were classified into four grades according to the criteria that are described in Fig. S1. The RSV disease-rating index was calculated using the following formula:

$$\text{Disease rating index} = \frac{0.25\text{A} + 0.5\text{B} + 0.75\text{C} + \text{D}}{\text{number of plants examined}}$$

where A, B, C and D represent the numbers of diseased rice plants that are classified as grades 1, 2, 3 and 4, respectively. According to the formula, a higher disease-rating index is

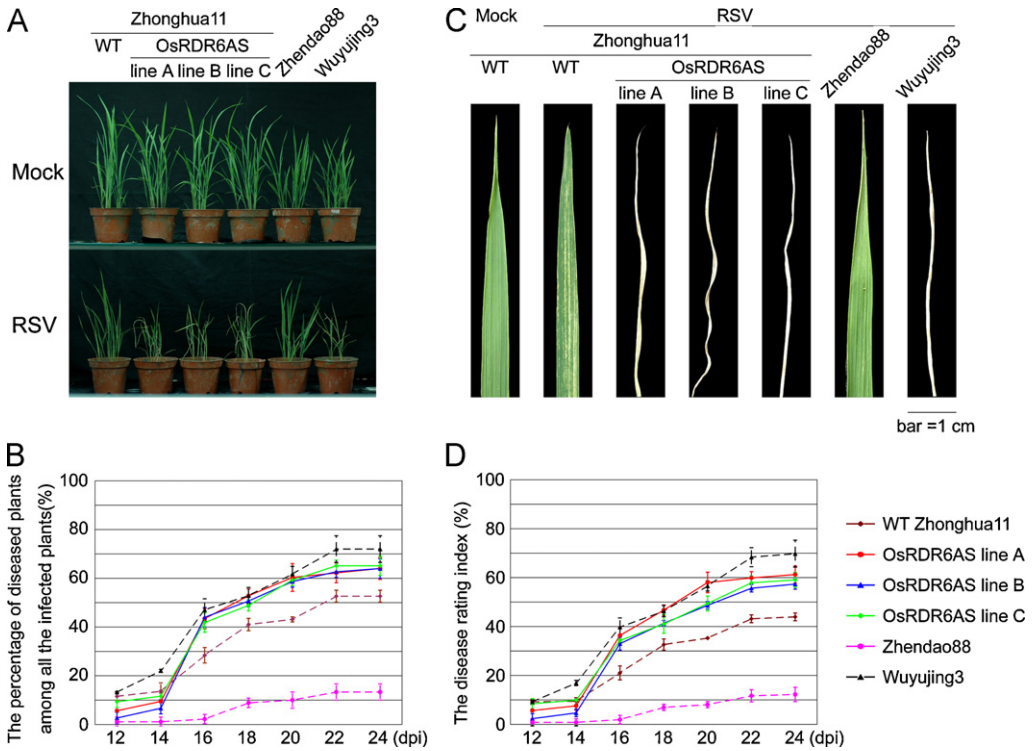

Fig. 3. Comparison of phenotypes among RSV-infected OsRDR6AS transgenic and non-transgenic Zhonghua11 plants. (A) Whole RSV-infected rice plants at 20 dpi. (B) The incidences of infection, which were determined by visual assessment of disease symptoms at 12–24 dpi from 30 individual plants for each case. Means and standard deviations were obtained from three independent experiments. (C) The first leaf from each line of RSV-infected rice plants at 20 dpi, showing distinct symptoms. (D) The disease-rating indices, which were determined by visual assessments of symptoms of viral disease at 12–24 dpi from 30 individual plants for each case. Means and standard deviations were obtained from three independent experiments.

Table 1
Non-preference test for rice varieties and transgenic lines used in this study.

Varieties	Non-preference[a]
Zhonghua11	1.56[b]
OsRDR6AS line A	1.57[b]
OsRDR6AS line B	1.57[b]
OsRDR6AS line C	1.54[b]
Zhendao88	2.02
Wuyujing3	2.94
IR36	1.27

[a] Non-preference was indicated by the number of small brown planthoppers settled on the individual plant for three days.
[b] There is no significant difference (P value > 0.05) between these data.

associated with a higher number of plants with more severe disease symptoms. As shown in Fig. 3D, at 24 dpi, the disease-rating indices reached 61.2%, 57.3% and 59.1% for the OsRDR6AS A, B and C plants. The indices reached 44.0%, 69.8% and 12.2% for the infected non-transgenic Zhonghua11, Wuyujing3 and Zhendao88 plants, respectively. These qualitative and quantitative analyses demonstrated that the down-regulated transcription of *OsRDR6* in antisense transgenic plants led to higher incidences of RSV infection and more severe disease symptoms compared with the non-transgenic Zhonghua11 plants.

Because RSV is transmitted by insects, it is possible that the differences in infection and disease symptoms between the OsRDR6AS plants and the non-transgenic Zhonghua11 control plants were attributed to differences in insect attraction and thus inoculation efficiencies. To test this possibility, we conducted a non-preference test using previously established methods (Nemoto et al., 1994). *O. sativa* subsp. *japonica* IR36 was used as the control variety, which shows resistance to RSV because of its reduced preference for the small brown planthopper (Sun et al., 2006). Our data showed no statistical differences in the non-preference indices among the transgenic and non-transgenic plants (Table 1). Thus, the hypersusceptibilities of the OsRDR6AS plants were attributed to enhanced virus infection but not to enhanced insect infestation.

3.4. Enhanced RSV infection was associated with increased accumulation of the viral RNAs

To understand the basis of the above observations, we investigated whether RSV replicated more efficiently and accumulated at higher levels in the antisense transgenic plants. We extracted total RNA from infected plants showing typical disease symptoms for a northern blot analysis to assess the accumulation of RSV genomic RNAs. As shown in Fig. 4, the levels of RSV RNA1 and RNA3 were 2.5–3-fold higher and those of RNA2 and RNA4 were 1.5–2-fold higher in OsRDR6AS lines A, B and C compared with the corresponding viral RNAs in the non-transgenic Zhonghua11 plants. We also analyzed the levels of RSV genomic RNAs in the susceptible Wuyujing3 and resistant Zhendao88. As shown in Fig. S2, the levels of all four RSV RNAs in Wuyujing3, and particularly RNA3 and RNA4, were higher than those in Zhonghua11 and Zhendao88. In fact, the levels of accumulation of the RSV genomic RNAs in Zhendao88 were the lowest among the three varieties. Thus, the accumulation level of RSV genomic RNAs was positively correlated with the severity of disease symptoms.

3.5. Reduced transcript level of OsRDR6 in rice plants depressed the abundance of RSV-derived small RNAs

To obtain a better understanding of the role that RDR6 plays in the antiviral RNA silencing pathway, we analyzed the viral siRNAs using a deep sequencing approach. A total of four libraries of two independent biological replicates of the RSV infected

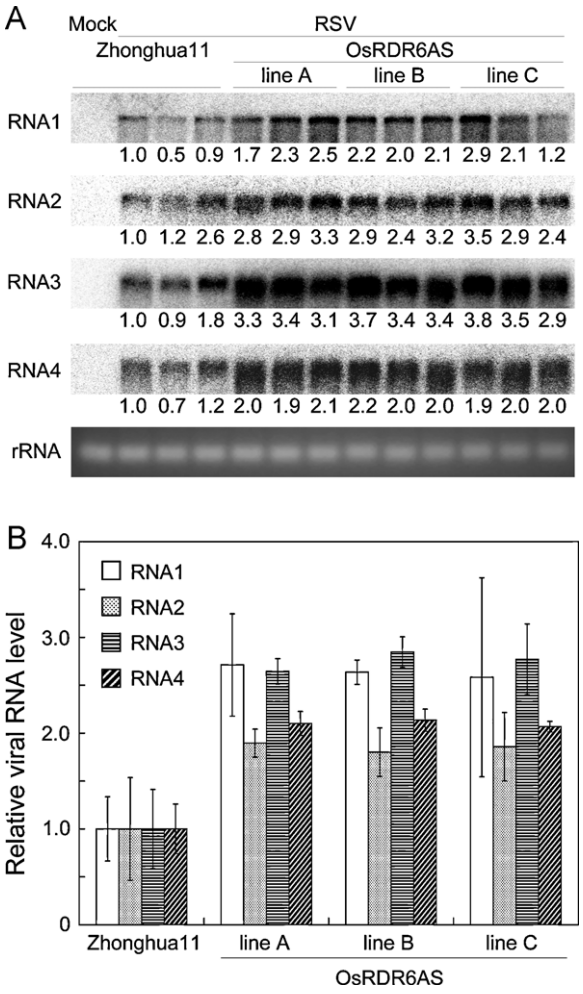

Fig. 4. Comparison of RSV genomic RNA accumulations in RSV-infected OsRDR6AS transgenic and non-transgenic Zhonghua11 plants. (A) RNA blot assays for RSV genomic RNA accumulations. Plants showing disease symptoms were harvested at 25 dpi. Three independent pools of infected plants from each line were collected with at least 10 plants in each pool. The RSV genomic RNA species are indicated on the left side. The 25S rRNA served as a loading control. The gel blot signals were quantified using ImageJ (version 1.4) and normalized using internal rRNA signals. The relative level for each sample is shown below the lane. (B) Average relative levels with standard deviations that were calculated from three biological replicates. The accumulation levels of RSV RNA1, RNA2, RNA3 and RNA4 in the non-transgenic plants were all set to 1.0.

non-transgenic Zhonghua11 and OsRDR6AS rice samples were constructed and sequenced using Illumina 1G platform. The sequences that ranged from 18 nt to 28 nt in length were isolated out for further analyses. These sequences were mapped to the rice and the RSV genomes with perfect matches. To accurately analyze the siRNAs, we normalized the total sequence reads of each library to one million. The RSV-derived siRNAs accumulated to lower levels in the infected OsRDR6AS plants than in the non-transgenic plants (Table 2). In the OsRDR6AS plants, the abundance of the RSV-derived siRNAs showed a 53% reduction. The two replicates displayed the similar results.

In the RSV-infected non-transgenic rice plants, the RSV siRNAs were predominantly 21 (38.3%), 20 (27.2%) and 22 nt (13.5%) in length (Fig. 5A, Table S3). In the OsRDR6AS plants, the abundances of all of the siRNA species were reduced. In the non-transgenic plants, most of the 21-nt siRNAs processed A (37.8%) or U (31.0%) as the 5′ terminal nucleotide (Fig. 5B, Table S4). The preference of the 5′ terminal nucleotide for

Table 2
Summary of deep sequencing results of small RNAs from RSV-infected rice small RNA libraries.

Libraries	Replicate 1		Replicate 2	
	Zhonghua11[a]	OsRDR6AS[a]	Zhonghua11[a]	OsRDR6AS[a]
Reads of total sequences[b]	5,549,056	6,842,598	5,791,793	10,764,320
Reads of total sequences mapped to the rice genome	3,487,733	3,332,295	3,629,722	5,090,024
Reads of total sequences mapped to the RSV genome	99,259	58,236	115,672	96,952
Normalized reads of sequences mapped to the rice genome[c] (Relative to that in RSV infected WT Zhonghua11 rice plants)	628,527 (1.00)	486,993 (0.77)	626,701 (1.00)	472,861 (0.75)
Normalized reads of sequences mapped to the RSV genome[c] (Relative to that in RSV infected WT Zhonghua11 rice plants)	17,885 (1.00)	8,514 (0.48)	19,978 (1.00)	9,010 (0.45)

[a] Small RNAs from the RSV-infected rice plants.
[b] Total small RNA reads within the set (18 nt ≤ length ≤ 28 nt).
[c] Reads per million reads of the total sequences of each library.

A (38.9%) or U (31.2%) did not change in the OsRDR6-silenced rice plants (Fig. 5B, Table S4).

In Fig. 5C, the distribution of siRNAs from viral-sense RNAs and complementary-sense RNAs of RSV was shown. More than half of the RSV-derived siRNAs were derived from vRNA2 and vcRNA2 (Table S5). With the exception of RNA1, vRNAs produced more siRNAs than vcRNAs. The siRNAs that were derived from each strand were reduced in the infected OsRDR6AS library,

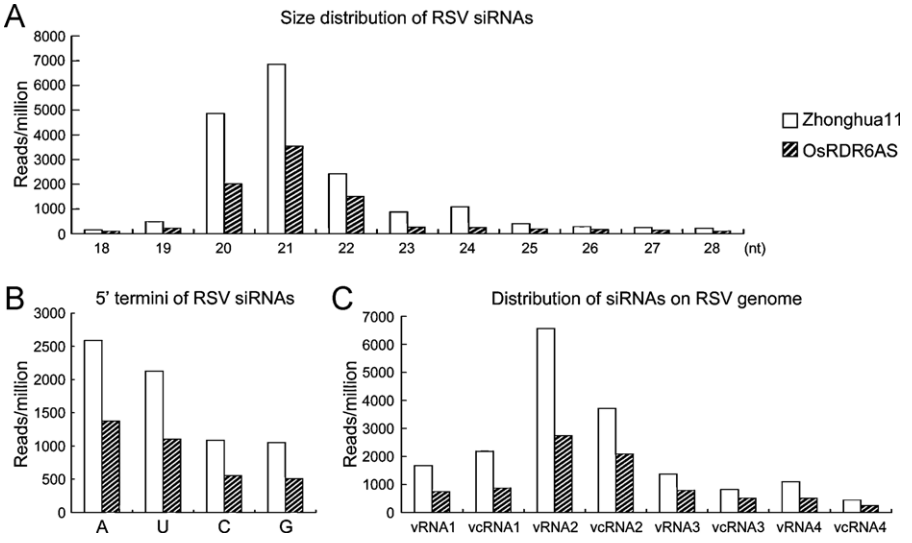

Fig. 5. Characterization of RSV-derived siRNA populations. (A) Size distribution of RSV-derived small RNA populations. (B) The relative abundance of RSV-derived 21-nt siRNAs with distinct 5′ terminal nucleotides. (C) The relative abundance of RSV-derived siRNAs that targeted each viral sense RNA and viral complementary sense RNA.

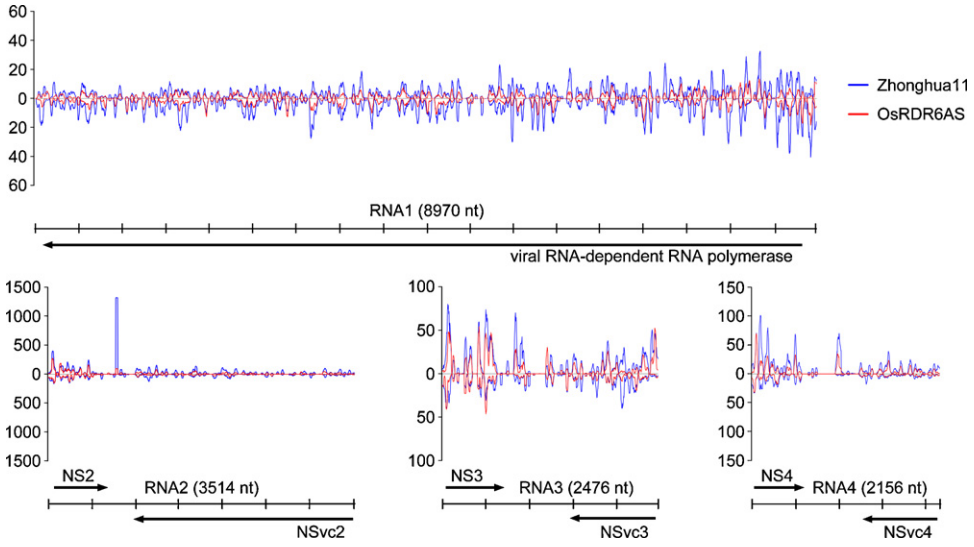

Fig. 6. Genome view of RSV-derived small RNAs that were captured from virus infected non-transgenic Zhonghua11 and OsRDR6AS transgenic rice plants. RSV-derived small RNAs per million of the total sequenced small RNAs were plotted against the viral-sense (top) or viral complementary-sense (bottom) strands of RSV genomic RNA1, 2, 3 and 4 using a single-nucleotide window. The seven known open reading frames are shown. Intervals of 500 nt are marked on the lower *x*-axes.

while the percentages of these siRNA classes showed little change (Table S5). The distribution pattern of RSV-derived siRNAs in the OsRDR6-silenced rice plants did not change significantly compared with that in the non-transgenic Zhonghua11 rice plants as indicated by the genomic view map of viral siRNAs, although the read numbers of all of the hotspots decreased (Fig. 6). It was interesting to note that the levels of accumulation of the siRNAs that targeted the 778–801 nt position of the sense RNA2 were very high in the non-transgenic Zhonghua11 infected rice plants but greatly reduced in the OsRDR6-silenced rice plants. Thus, the decreased transcript levels of *OsRDR6* in the RSV-infected rice plants inhibited the accumulation of the viral siRNAs, and OsRDR6 participated in amplifying the viral siRNAs that had been generated from hotspot regions.

4. Discussion

We generated three independent antisense transgenic rice plant lines with reduced *OsRDR6* transcript levels. The *OsRDR3b* transcript levels increased in lines B and C, but did not significantly change in line A (Fig. 2). Although differing transcript levels of *OsRDR3b* were observed, our results showed that in all three of the transgenic lines, the silencing of *OsRDR6* enhanced RSV susceptibility, promoted the accumulation of viral genomic RNAs and inhibited the accumulation of viral siRNAs (Figs. 3 and 4, and Table 2). Thus, OsRDR6 (but not OsRDR3b) played a clear role in antiviral defense in rice similar to that which has been observed in other RDR6 orthologs from *A. thaliana* and *N. benthamiana*. Further, our results indicate that the antiviral function of RDR6 may be extended to viruses with ambisense RNA genomes.

The deep sequencing data analysis of the RSV-derived siRNAs showed that a number of RSV-derived siRNA were dependent on the function of OsRDR6 (Table 2). RSV was associated with more serious disease symptoms and higher levels of accumulation of viral RNAs in the infected OsRDR6AS transgenic rice plants compared with the non-transgenic plants. These results indicate that the secondary siRNAs that were dependent on RDR6 were essential in the defense against RSV.

A prominent mechanistic question is whether an RDR utilizes a particular RNA or all RNAs of a virus in the synthesis of dsRNAs as DCL substrates. AtRDR1 and AtRDR6 exhibit specificity in the amplification of siRNAs that are derived from CMV genomic RNAs. AtRDR6 specifically amplifies the siRNA that targets the 3′ terminal region of CMV RNA3, while AtRDR1 amplifies the 5′ terminal siRNAs (Wang et al., 2010). The ratio of sense- to antisense-derived siRNAs has been shown to decrease in TMV-infected *rdr6* mutant plants compared with that in the wild type plants, indicating that AtRDR6 may affect the strand bias of the TMV-derived siRNAs in *A. thaliana* (Qi et al., 2009). Although the read numbers of the siRNAs targeting each RSV RNA strand were observed to be reduced in the OsRDR6-silenced rice plants, the genomic profiles of the RSV-derived siRNAs in the non-transgenic Zhonghua11 and OsRDR6-silenced rice plants showed little change (Table S5). These data indicate that OsRDR6 does not exhibit any preference for the amplification of viral siRNAs that target particular RSV strands and suggest that the specificity of RDR6 in the amplification of viral siRNAs differs depending on the virus and host.

The read numbers of siRNAs that targeted RNA2 at the 778–801 nt position decreased greatly in the OsRDR6-silenced rice plants compared with those in the non-transgenic Zhonghua11 rice plants. This suggests that OsRDR6 participated in the amplification of siRNAs that originated from a hotspot at the 778–801 nt position of RNA2. The 778–801 nt position of RNA2 is located at the intergenic region of the ORF NS2 and NSvc2 (Takahashi et al., 1993). We did not find a good correlation between the siRNA-generated hotspots and the potential stem-loop structures that were predicted by the RNAfold program (http://rna.tbi.univie.ac.at/) (data not shown). It is speculated that the 3′ terminal regions of the NS2 and NSvc2 transcripts overlapped in a sense-antisense pair containing the 778–801 nt region and that the siRNAs were produced from this overlapped region. It is possible that there is a mechanism here that is similar to that of the biogenesis of nat-siRNA, which is dependent upon DCL2, DCL1 and RDR6 (Borsani et al., 2005). These and other possibilities await further investigations.

RDR6 regulated the expression of viral silencing suppressor(s) and vice versa. It has been previously reported that viral silencing suppressors interfere with the functions of host RNA silencing factors (Garcia-Ruiz et al., 2010; Ren et al., 2010; Wang et al., 2011; Wu et al., 2010). In addition, it has been observed that the CMV 2b protein suppresses siRNA amplification via an RDR6-dependent pathway (Wang et al., 2010, 2011). The RSV RNA3 encodes a silencing suppressor NS3 that inhibits both local and systemic silencing through the binding of 21-nt single-stranded siRNAs, siRNA duplexes and long single-stranded RNAs (Xiong et al., 2009). Transgenic rice plants that harbor an inverted-repeat construct targeting NS3 exhibit chlorotic stripes in the early stages after inoculation with RSV but grow well at later stages of infection. RSV accumulated at lower levels in the NS3-targeted transgenic rice plants than in the wild type plants (Shimizu et al., 2011). These results suggest that NS3 is important for RSV accumulation and symptom development. In our study, the higher levels of RNA3 accumulation were accompanied by enhanced symptoms in the OsRDR6AS transgenic plants in addition to the susceptible variety Wuyujing3 (Fig. 4, Fig. S2). Thus, it is possible that OsRDR6 contributes to the repression of NS3 accumulation to enhance host defense against RSV.

In conclusion, our current results demonstrate that OsRDR6, like its orthologs in *A. thaliana* and *N. benthamiana*, plays a critical role in host antiviral defense, thereby greatly expanding upon the current knowledge of the antiviral function of RDR6 orthologs in the defense of monocots against negative-strand RNA viruses. The evolutionarily conserved function of RDR6 orthologs in the defense against different types of viral genomes make them potential targets in the use of genetic modifications to achieve broad viral resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virusres.2011.11.016.

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