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VIRAL AND VIROID DISEASES

Analysis of rice *RNA-dependent RNA polymerase 1* (*OsRDR1*) in virus-mediated RNA silencing after particle bombardment

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Abstract RNA-dependent RNA polymerases (RDRs) play a key role in various RNA silencing pathways in many organisms. Using the nucleotide sequence of *SGS2/SDE1/RDR6* in *Arabidopsis* as the search query for sequences that flank the insertions of rice retrotransposon *Tos17*, we selected rice mutant lines (*OsRDR1*). RT-PCR analysis showed that *OsRDR1* mRNA was undetectable in leaves and calli of the mutants, while it was detected in wild type. RNA silencing was induced by particle bombardment to investigate any effects of *OsRDR1* on RNA silencing with β-glucuronidase or green fluorescent protein DNA/RNA in the mutant lines. The results showed that RNA silencing was impaired in these mutant lines by inverted repeat (IR) DNA or in vitro transcribed double-stranded RNA. Further, the mutant lines were bombarded with *Brome mosaic bromovirus* (BMV, a ssRNA virus) or *Wheat dwarf geminivirus* (WDV, a ssDNA virus), each carrying the IR sequence of a reporter gene. As a result, RNA silencing was impaired by BMV. Interestingly, however, it was not impaired by WDV. Thus we propose that *OsRDR1* is required for RNA silencing mediated by *Bromovirus*, but not by *Geminivirus* in this system.

Keywords Rice *RNA-dependent RNA polymerase 1* · RNA silencing · *Brome mosaic bromovirus* · *Wheat dwarf geminivirus*

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Introduction

RNA-dependent RNA polymerases (RDRs) play a fundamental role in various RNA silencing pathways in numerous organisms (Curaba and Chen 2008; Wassenegger and Krczal 2006; Yang et al. 2008). The first *RDR* was discovered about 40 years ago in a search for enzymes that catalyze plant RNA viral replication (Astier-Manifacier and Cornuet 1971). The first plant-encoded RDR was isolated from tomato (*Lycopersicon esculentum*), and is now called *LeRDR1* (Schiebel et al. 1993). In the fungus *Neurospora crassa*, *NcRDR6* (*QDE-1*) is an essential component of quelling and can convert a single-stranded RNA (ssRNA) to a double-stranded (dsRNA) molecule in a primer-dependent/independent fashion (Makeyev and Bamford 2002). In *Arabidopsis thaliana*, six *RDR*s were identified as

AtRDR1, AtRDR2, AtRDR3a, AtRDR3b, AtRDR3c and *AtRDR6*, three of which (*RDR3a, RDR3b, RDR3c*) share the unique DFDGD amino acid motif in the catalytic domain (Voinnet 2008; Wassenegger and Krczal 2006). Recent findings indicate that *RDR1*, *RDR2* and *RDR6* act in different biological processes of RNA silencing (Dalmay et al. 2000; Mourrain et al. 2000; Xie et al. 2004). *RDR1* expression is induced by salicylic acid (SA), a defense-signaling hormone that accumulates during infection by many viruses and viroids (Dorssers et al. 1984; Khan et al. 1986; Xie et al. 2001). More recent studies with *Cucumber mosaic virus* (CMV) demonstrate that the production of small interfering RNAs (siRNAs) is strongly dependent on *RDR1* without interference by the virus-encoded 2b silencing suppressor protein called viral suppressors of RNA silencing, and *RDR1*-dependent secondary virus-derived siRNAs (viRNAs) play a key role in non-cell-autonomous antiviral silencing (Diaz-Pendon et al. 2007). It also implies that virus encoded proteins may inhibit one or more RDR pathways of siRNA biogenesis associated with silencing suppressor proteins (Donaire et al. 2008), while other reports indicate that *RDR1* mediates defense against herbivore attacks in *Nicotiana attenuata* (Pandey and Baldwin 2007). *RDR2* transcripts in *N. attenuata* are up-regulated by both UV and herbivore elicitation for protection (Pandey and Baldwin 2008), while *RDR6* is known to be involved in initiation and maintenance of post-transcriptional gene silencing (PTGS) and virus-induced gene silencing (VIGS) (Wassenegger and Krczal 2006). *RDR6* is required for VIGS induced by *Cabbage leaf curl virus* (CaLCuV) and CMV, but not by *Turnip crinkle virus* (TCV), *Tobacco rattle virus* (TRV), *Turnip mosaic virus* or *Tobacco mosaic virus* (Diaz-Pendon et al. 2007; Muangsan et al. 2004; Vaucheret 2006; Voinnet 2005).

In rice, five *RDR*s have been identified: *OsRDR1, OsRDR2, OsRDR3a, OsRDR3b* and *OsRDR6* (Wassenegger and Krczal 2006). A recent study indicates that the OsRDR6-dependent siRNA biogenesis that derives from an isocitrate lyase transcript was significantly up-regulated by the phytohormone abscisic acid (ABA), and *OsRDR6* may participate in the amplification of PTGS mediated by ABA in rice (Yang et al. 2008). The function of other *RDR* enzymes remains a key question. Here we isolated and identified several *OsRDR1* mutant lines using the nucleotide sequence of *SGS2/SDE1/RDR6* in *Arabidopsis* as the query to search for the sequences that flank the insertions of rice retrotransposon *Tos17*. We used particle bombardment to establish an RNA silencing system to investigate any effects of *OsRDR1* on RNA silencing with various silencing inducers, including inverted repeat (IR) DNA or dsRNA, in these mutant lines. We tested the role of *OsRDR1* in RNA silencing mediated by *Wheat dwarf virus* (a ssDNA geminivirus, WDV) or *Brome mosaic virus* (a ssRNA bromovirus, BMV), each carrying the IR sequence of a reporter gene. Here we used either β-glucuronidase (*GUS*) or green fluorescent protein (*GFP*) to make the data more versatile and to use the viral vectors available, pWI-GUS (Ugaki et al. 1991) and BMV-GFP. Our analyses show that *OsRDR1* is required for RNA silencing mediated by BMV, but not by WDV.

Materials and methods

Rice plants

Oryza sativa L. cv. Nipponbare and its knockout mutant lines (ND2001 and ND2059) were used. The homozygous individuals from M1 and M2 generation were screened by Southern blot analyses. Seedlings and calli of selected M2 plants were grown and used for respective experiments.

Southern blot analysis

Employing the cetyltrimethyl ammonium bromide (CTAB) method, we isolated rice genomic DNA (20 μg) and transferred it to a nylon membrane as described previously (Chen et al. 2008). Using the PCR DIG probe Synthesis Kit (Roche, Basel, Switzerland), the probe was prepared as described previously (Chen et al. 2008). A plasmid carrying the flanking sequences in each mutant was constructed as template DNA in the following protocol: a PCR product amplified from the coding region of *OsRDR1* by using forward primer (5′-AAACCCTTGCCTCCACCCAGGTG-3′) and reverse primer (5′-ATGGACTATACTCCAGCACC AA-3′), and the fragment amplified was then inserted into pUC19 (Stratagene, La Jolla, CA, USA), which had been digested with *Sma*I. The probes were labeled according to the manufacturer's instructions. DNA was then fixed to the membrane by UV irradiation, and the prehybridization and hybridization were done as described (Engler-Blum et al. 1993) for subsequent exposure to X-ray film (Boehringer Mannheim, Mannheim, Germany) for 12–18 h. Hybridizations were performed with DIG-dUTP-labeled (digoxigenin-labeled) DNA probes.

DNA sequencing and sequence analysis

DNA sequencing, sequence analysis and homologue searching were carried out as described previously (Chen et al. 2008).

Plasmids used for transient expression

The *GFP* expression plasmid p35S-GFP and the *DsRed* (*Discosoma* red fluorescent protein) expression plasmid

pUbq-DsRed driven by an enhanced 35S promoter of *Cauliflower mosaic virus* (CaMV) and an ubiquitin promoter of maize polyubiquitin, respectively, were provided by Miki and Shimamoto (2004). Plasmid *GFP* RNAi was transcribed into an RNA predicted to form a double-stranded hairpin RNA structure (Miki and Shimamoto 2004). Plasmid pIG121 contains *GUS*, driven by the CaMV 35S promoter and 35S terminator, respectively (Ohta et al. 1990). Plasmid pUbq-DsRed was used as an internal control (Miki and Shimamoto 2004). The p35S-GUS RNAi was described previously (Chen et al. 2008).

The plasmid of pWI-GUS is a plant–bacterium shuttle vector derived from WDV having *GUS* under the control of the 35S promoter and terminator (Ugaki et al. 1991). The pWI-GUS RNAi was constructed as follows (Fig. 1a): a fragment containing the *GUS* sense fragment and λ phage sequence was derived from p35S-GUS RNAi through digestion with *Eco*RI and *Sac*I, cloned to pBluescriptII KS+ (Stratagene), creating pBS-GUS + λ. A PCR product containing a 831-bp linker of λ phage and 1300 bp of the inverted-repeat *GUS* fragment was amplified with pBS-GUS + λ as a template using forward primer 5′-GCCGCATGCGAATTCGGACAGACAGTG-3′ containing the *Sph*I site (underlined) and reverse primer 5′-GCCGCATGCGGGAATGGTGATTACCGAC-3′ containing the *Sph*I site (underlined). The amplified fragment was digested with *Sph*I and inserted into the end of the *GUS* fragment of pWI-GUS, which had been digested partially with *Sph*I (5 min at 37°C), so that the plasmid pWI-GUS RNAi was constructed after the orientation was selected and verified by sequencing.

The mixture of BMV-GFP or BMV-GFP RNAi (Fig. 1b) was derived from in vitro transcription of pBTF1, pBTF2 and pBTFCP2smGFP or pBTFCP2hpGFP2, respectively (Mori et al. 1993). pBTFCP2smGFP and pBTFCP2hpGFP2 were constructed as follows (Fig. 1b): the smGFP gene was amplified by PCR using primers 5′-GGGATGCATGAGTAAAGGAGAAGAACT-3′ and 5′-GGGGAGCTCTTATTTGTATAGTTCATCCATGCC-3′ from clone smGFP from the Arabidopsis Biological Resource Center at Ohio State University. The PCR product was digested with *Nsi*I and *Sac*I and inserted between the *Nsi*I and *Sac*I site of pBTFCP2pIFN (Mori et al. 1993). The resulting plasmid was digested with *Nsi*I, blunt-ended with T4 DNA polymerase, then self-ligated, creating pBTFCP2smGFP. Then two DNA fragments, the one encoding 0.1 kbp from the C-terminus-, and the other encoding 0.4 kbp from the C-terminus of the *GFP* gene, was amplified using primer sets #1554 (5′-ATAACTA GTTTTCGAAAGATCCCAACGAA-3′)–#1553 (5′-TATG AGCTCGTCGACTAAAGCTCATCATGTTTGT-3′), and #1555 (5′-ATAACTAGTCAAGACACGT-GCTGAAGT CA-3′)–#1553, respectively. As a PCR template, pBI-erG3 (Tamai et al. 2003) was used. The #1554–#1553 and the #1555–#1553 fragments were digested by *Sac*I/*Spe*I, and *Sal*I/*Spe*I, respectively, ligated with *Sal*I/*Sac*I-digested pBTFCP2smGFP. The resulting plasmid was named pBTFCP2hpGFP2, which was engineered to carry the *GFP* 301 bp as a spacer between an IR of *GFP* 98 bp.

As a complementation study, the p35S-OsRDR1 containing a whole ORF-coding region of cDNA from *OsRDR1* (3342 bp) was constructed as follows: two RT-PCR fragments (cDNA1, 1769 bp and cDNA2, 1583 bp) were amplified with forward primer 5′-GGATCCTGCGCCA TGGGTGTCAA-3′ containing the *Bam*HI site (underlined) and reverse primer 5′-GCATGCTTTTTCTTAAGGAA AG-3′ containing the *Sph*I site (underlined) and using forward primer 5′-GCATGCTAAAGTTTCAGTCAGA-3′ containing the *Sph*I site (underlined) and reverse primer 5′-GAGCTCAAACATGCTGGCCACA-3′ containing the *Sac*I site (underlined), respectively. The cDNA1 fragment was inserted into the pdTA2 vector (Toyobo, Osaka, Japan). This resulted in pdTA2-cDNA1. The cDNA2 fragment was digested with *Sph*I and *Sac*I and inserted into pdTA2-cDNA1, which had been digested with *Sph*I and *Sac*I, creating pdTA2-cDNA1 + cDNA2, then this plasmid was digested with *Bam*HI and *Sac*I to isolate a fragment of cDNA1 + cDNA2, which was gel-purified and inserted into pBI221, which had been digested with *Bam*HI and *Sac*I (to remove *GUS*). This resulted in p35S-OsRDR1 containing a whole ORF-coding region of cDNA from *OsRDR1* (3342 bp). The construct was verified by sequencing.

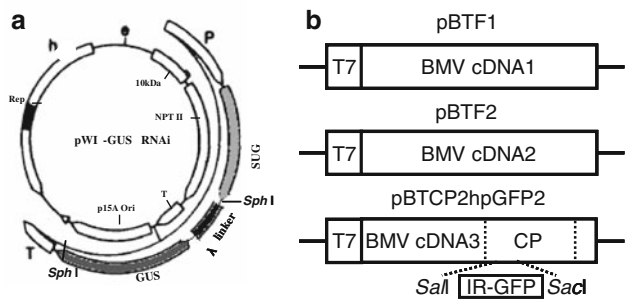

Fig. 1 Diagram of pWI-GUS RNAi (**a**), which carries IR-GUS; *e* one of five EcoO109I sites, *NPT II* neomycin phosphotransferase II gene-coding region, *p15A ori* replication origin of plasmid p15A, *T* terminator of CaMV35S transcript, *Rep* replication-associated protein gene, and BMV RNAi (**b**), which carries IR-GFP in place of the 5′-half of the CP, *T7* T7 RNA polymerase promoter

In vitro synthesis of dsRNA

In vitro synthesis of dsRNA for *GUS* was carried out as described previously (Chen et al. 2008).

Transient expression after particle bombardment

Gold particles (0.6 μm diameter, particle density 31 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) were coated with the respective mixture of plasmids, then delivered biolistically into leaf segments through a particle inflow gun (IDERA GIE-III, Tanaka, Sapporo, Japan). The samples were bombarded (2.5 kg/cm^2 force and 500 mmHg pressure) with the gold particles coated with the following amounts of plasmids or dsRNA per shot: for RNA silencing with *GUS* and WDV, (i) pIG121 (5 μg) and pBIΔGUS (control vector) (5 μg), (ii) pIG121 (5 μg) and p35S-GUS RNAi (10 μg), (iii) pIG121 (5 μg) and ds*GUS* RNA (5 μg), (iv) p35S-GUS (5 μg), p35S-GUS RNAi (10 μg) and p35S-OsRDR1 (10 μg), (v) pIG121 (5 μg) and pWI-GUS RNAi (10 μg); for RNA silencing with *GFP* gene and BMV, (i) pUbq-DsRed (5 μg), p35S-GFP (5 μg) and pBI221 (control vector) (5 μg), (ii) pUbq-DsRed (5 μg), p35S-GFP (5 μg) and *GFP* RNAi (10 μg); (iii) pUbq-DsRed (5 μg), p35S-GFP (5 μg), *GFP* RNAi (10 μg) and p35S-OsRDR1 (10 μg), (iv) pUbq-DsRed (5 μg), p35S-GFP (5 μg) and BMV-GFP RNAi (10 μg); for BMV replication, pUbq-DsRed (5 μg) and BMV-GFP (10 μg); for WDV replication, pUbq-DsRed (5 μg) and pWI-GUS RNAi (10 μg). In the case of real-time RT-PCR analysis with *GUS* silencing, *DsRed* was used for normalization as a reference gene in all treatments.

After bombardment, the expression of GFP and GUS was detected as described previously (Chen et al. 2008).

RNA isolation and analysis

Following the manufacturer's instructions (Tri reagent kit, Molecular Research Center, Cincinnati, OH, USA), total RNA was extracted from rice leaves and calli, mRNA was purified using an Oligotex^{TM}-dT30 <Super> mRNA Purification Kit (Takara, Shiga, Japan), and mRNA treated with RNase-free DNase (Stratagene) was used as a template for RT-PCR with reverse transcriptase (MonsterScript^{TM} 1st-Strand cDNA Synthesis Kit, Epicentre, Madison, WI, USA). RT-PCR was carried out using RT primer 5′-ATACGAGGCGGAATGAGAG-3′. The cDNA products of RT-PCR were then amplified by the following pairs of primers: 5′-GCTTGGATGAAACACGTAC-3′ (forward) and 5′-ATACGAGGCGGAATGAGAG-3′ (reverse). Rice ubiquitin was used as an internal standard (Miki and Shimamoto 2004).

Real-time RT-PCR experiments for detecting the expression of *GFP* and *GUS* were carried out as described previously (Chen et al. 2008), and real-time PCR experiments were done with the sets of primers specific to 10 kDa protein of WDV (5′-GGAGGCTTTTGGACCACA TCTTTT-3′ and 5′-TTCCTCGGTCCTCCTTTGCTTCTT-3′). The amount of *DsRed* transcripts was used for normalization as an internal control.

Results

Isolation of homozygous *OsRDR1* knockout mutant lines

Homozygous *OsRDR1* knockout mutant lines from the *Tos17* insertion populations available at the Rice Tos17 Insertion Mutant Database (http://tos.nias.affrc.go.jp/~miyao/pub/tos17/index.html.en) were selected based on the nucleotide sequence of *SGS2/SDE1/RDR6* in *Arabidopsis*. Two lines (ND2001 and ND2059) had the highest homology to that sequence (referred to as *AtRDR6*). In these lines, *Tos17* is inserted at the same site as *OsRDR1*, suggesting that these two were each a callus clone derived from one transformation event because these mutants were selected by tissue culture (Hirochika 2001). The phylogenetic analysis of *OsRDR1* and other *RDR*s showed that this gene is most closely related to *AtRDR1*, and thus it is referred to as *OsRDR1* (Wassenegger and Krczal 2006). The flanking sequence of each mutant line was used to design the primers and to make the specific DNA probes as shown in the materials and methods. The co-segregation analysis of *Xba*I-digested genomic DNA, isolated from each of the individual mutant lines, was carried out by Southern blot analysis; one of three ND2001 and one out of three ND2059 plants were homozygous (Fig. 2a). Furthermore, no significant differences between the wild type (WT) and the homozygous knockout mutant lines have been observed from germination through flowering (data not shown), indicating that the *OsRDR1* gene is not required for essential biological processes for morphology.

Identification of *OsRDR1* homologues in rice

To identify *OsRDR1* in rice, we searched for the *SGS2/SDE1/RDR6* sequence in the rice genome sequence database from DNA Bank at the National Institute of Agrobiological Sciences (http://www.dna.affrc.go.jp) and from the chromatin database (http://www.chromdb.org) at the University of Arizona. The flanking sequence of lines ND2001 and ND2059 each had high identity to one genomic clone from chromosome 2, AP004880, one cDNA clone from AK101440 and one transcript sequence from RDR704. Using this information, one putative cDNA of rice *RDR* was cloned by RT-PCR as described in the materials and methods. Combining these results, AK101440 might be an incomplete clone including a missing transcript in exon 4. Comparison of the putative cDNA and its corresponding genomic sequences revealed that *OsRDR1* consists of four

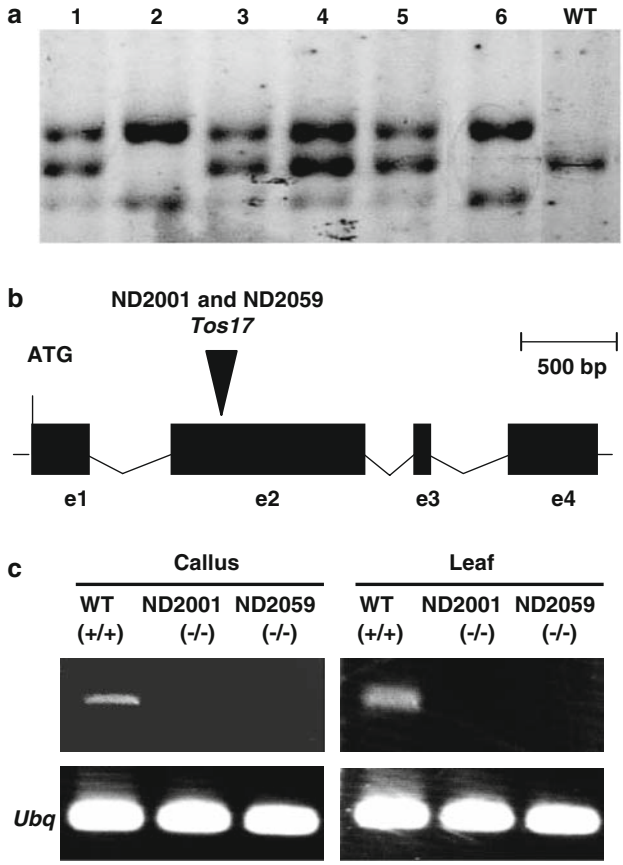

Fig. 2 **a** Southern blot analysis of *OsRDR1* mutant lines. *Lanes 1–3* and *lanes 4–6* are M1 progeny of lines ND2001 and ND2059, respectively. *Lanes 2* and *6* are homozygous in terms of the *OsRDR1* knockout. **b** Genomic structure of functional *OsRDR1* gene and *Tos17* insertion site in the *OsRDR1* knockout mutant lines. Exons (e1–e4) are shown as *black boxes*. Introns are indicated by *broken lines*. *Black triangles* indicate the *Tos17* insertion position in knockout mutant lines. **c** Comparative RT-PCR of *OsRDR1* mRNA from different tissues between wild-type and mutant lines. *OsRDR1* mRNA was amplified with 30 PCR cycles. +/+ and −/− are the wild-type and mutant line, respectively. Rice ubiquitin (*Ubq*) serves as a normalization control for RT-PCR efficiency

exons interrupted by three introns (Fig. 2b). Two mutant lines have the same *Tos17* insertions in exon 2 (Fig. 2b). A phylogenetic relationship between RDR homologues in rice and *Arabidopsis* was established. A Neighbor-Joining tree from bootstrap analysis (1000 replicates) was constructed using MEGA software version 3.1. These results indicate that *OsRDR1* is in the same clade as *AtRDR1* with a 99% consensus value of bootstrap in Fig. S1 in Electronic Supplementary Material.

The expression of *OsRDR1* in WT and mutant lines (ND2001 and ND2059) was examined with RT-PCR analysis using specific primers for *OsRDR1*. *OsRDR1* mRNA was not detected in the young leaves or calli of the mutants, while a significant level was found in the WT after running 30 PCR cycles (Fig. 2c). These results indicate that *OsRDR1* was not expressed in the mutant lines due to the insertion of *Tos17*.

Effects of *OsRDR1* disruption on RNA silencing induced by particle bombardment with IR DNA or dsRNA

As we previously reported (Chen et al. 2008), a transient RNA silencing system targeted to *GUS* was examined. The different mixtures of plasmids were used. As shown in Fig. 3a, b, substantial levels of *GUS* were expressed at 48 h post-bombardment in the histochemical GUS assay of the WT and mutant line ND2001 when bombarded with plasmid (i) pIG121. The number of *GUS* expressing cells per shot was counted for quantitative analysis in three to six independent experiments ($n = 3$–6 shots). When using mixture (ii) pIG121 and p35S-GUS RNAi, the number of *GUS*-expressing cells was reduced in the WT but not in the mutant line. When using mixture (iii) pIG121 and ds*GUS*, a dsRNA for *GUS* silencing, the number of *GUS*-expressing cells in the WT clearly decreased but not in the mutant line (Fig. 3a, b). The specific silencing of *GUS* was recovered by co-bombardment with mixture (iv) including plasmid p35S-OsRDR1 in the WT and mutant line (ND2001) (Fig. 3a, b). Moreover, the accumulation of *GUS* transcripts versus *DsRed* transcripts (as an internal control) was examined in the leaf discs from the WT and the mutant line at 48 h post-bombardment with the mixture of those plasmids by real-time RT-PCR (Fig. 3c). Similar results were obtained with three independent experiments essentially as levels of *GUS*-expressing cells (Fig. 3b). These results were similar to those from the other mutant line (ND2059) (data no shown). Thus, in the mutant lines, RNA silencing was impaired by IR-DNA and dsRNA of *GUS*.

Effects of *OsRDR1* disruption on RNA silencing mediated by plant ssDNA/ssRNA viruses

It is worthwhile examining whether the *OsRDR1* gene is required for virus-mediated RNA silencing by particle co-bombardment. There are no appropriate viral vectors that infect rice plants available; thus, we selected WDV (ssDNA) and BMV (ssRNA), both of which are reported to infect rice protoplasts (Huntley and Hall 1996; Laufs et al. 1990). Here, experiments with this co-expression system were performed to detect RNA silencing with pIG121 and pWI-GUS RNAi constructed as described in the materials and methods. Specific silencing of *GUS* was observed after co-bombardment with mixture (v) including plasmid pWI-GUS RNAi in the WT and the mutant line (ND2001) (Fig. 3a). This result indicates that *OsRDR1* is not required for WDV-mediated RNA silencing. To confirm

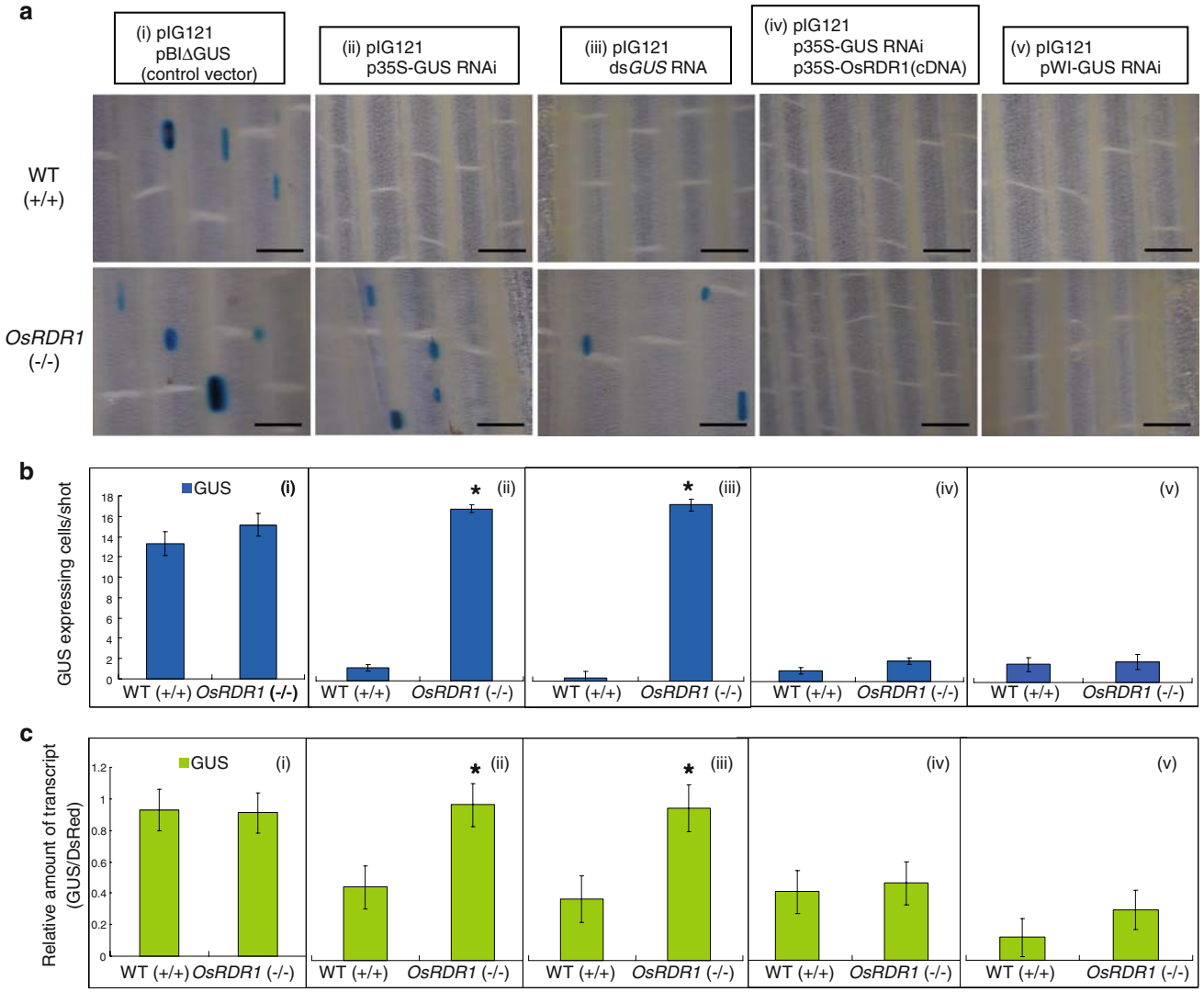

Fig. 3 RNA silencing of *GUS* in the epidermal cells of rice leaf discs of wild-type (*WT*) and mutant line after cobombardment with different mixtures of plasmids. **a** Micrographs of *GUS* expression. *Bar* 30 μm. **b** Quantitative analysis of GUS-expressing rice cells. Data are mean ± SE (n = 3–6 shots). **c** Real-time RT-PCR analysis of accumulation of *GUS* transcripts 48 h after bombardment. Data were normalized using *DsRed* transcripts as an internal control. Data are mean ± SE (n = 3). *$P < 0.05$ (Fisher's test) versus value of WT

bombardment efficiency, we also examined accumulation of *GUS* transcripts versus *DsRed* transcripts (as an internal control) in the leaf discs from the WT and the mutant line at 48 h post-bombardment with the mixture of those plasmids by real-time RT-PCR (Fig. 3c). These results indicate that the levels of *GUS* transcript in the leaf discs bombarded were accordant with the levels *GUS*-expressing cells (Fig. 3a). To check the possibility that WDV interferes with the transcription of *GUS*, an additional experiment has been done with pWI-GUS instead of pIG121. Figure S2a in ESM reveals that both the WT and mutant line (ND2001) expressed *GUS* (WDV driven) when bombarded with plasmid mixture (i) pWI-GUS and control DNA(pBIΔGUS). With mixture (iii) pWI-GUS and pWI-GUS RNAi, the number of cells that expressed *GUS* decreased markedly in the WT and in the mutant line, similar to the results shown in Fig. 3a (v). Furthermore, Fig. S2b (i) indicates that *DsRed* expression remained at a stable level as with *GUS* (WDV driven) expression. All these results showed that the WDV did not affect expression of *GUS* or *DsRed* gene.

We also examined whether BMV affected RNA silencing in the mutant line in this system. For RNA silencing induction, we used BMV-GFP RNAi, in vitro transcripts from BMV cDNA clones. These cDNA clones were the same as reported previously for RNA1 and 2 (Mori et al. 1993). For RNA3, pBTFCP2hpGFP2 was constructed to carry IR-GFP sequence in place of the 5′-half of coat protein gene (CP). When mixture (i) or (ii) in Fig. 4 was used for bombardment, respectively, again

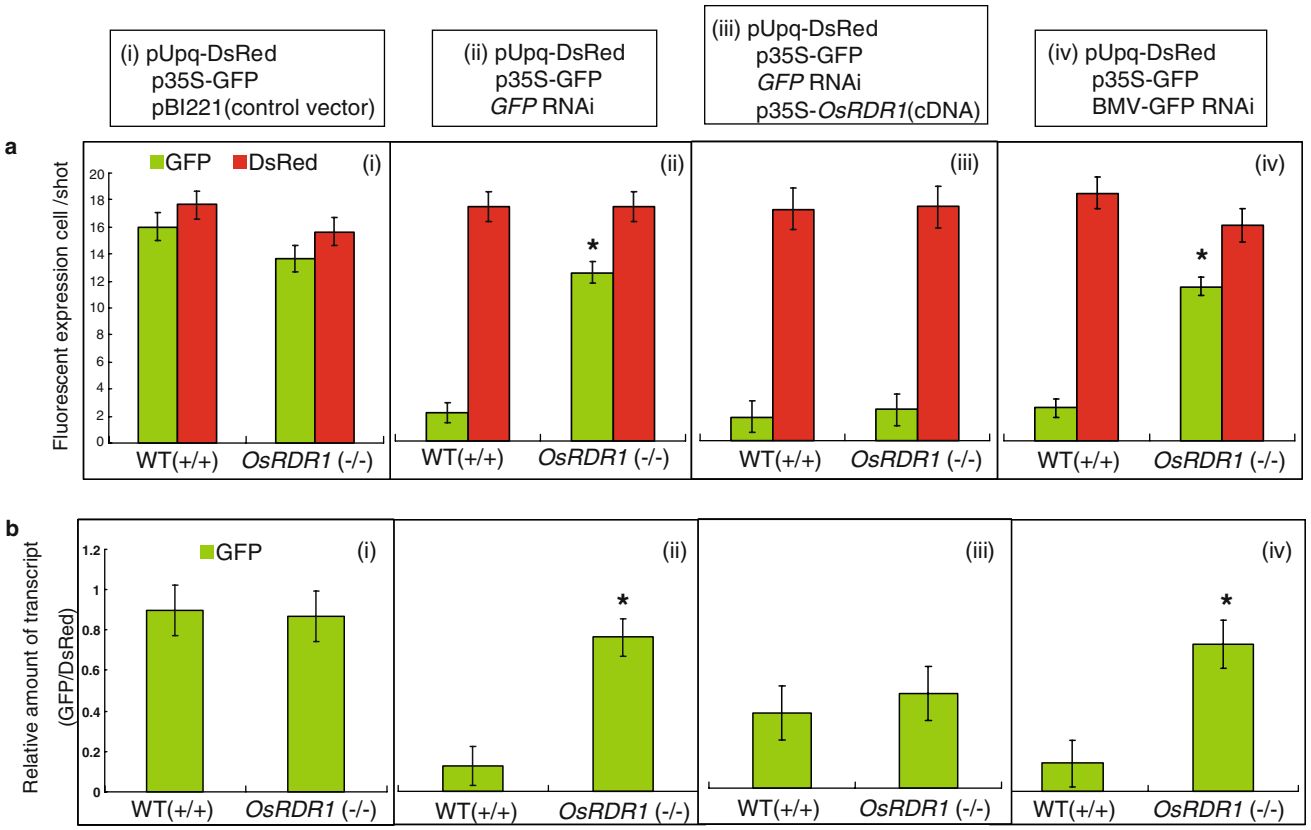

Fig. 4 Effect on RNA silencing mediated by RNA virus in rice leaf discs of wild-type (*WT*) and mutant line after cobombardment with different mixtures of plasmids. **a** Quantitative analysis of GFP-fluorescing cells per shot. Data are mean ± SE ($n = 3$). **b** Real-time RT-PCR analysis of accumulation of *GFP* transcripts in the leaf discs at 36 h after bombardment. Data were normalized using *DsRed* transcripts as an internal control. Data are mean ± SE ($n = 3$). The relative amount of transcript (*GFP/DsRed*) is shown in the *vertical line*. *$P < 0.05$ (Fisher's test) versus value of WT

the same results as in Fig. 3 were obtained, showing that RNA silencing of *GFP* was impaired in the mutant line (Fig. 4a). The number of GFP- or DsRed-fluorescing expressing cells was also counted for quantitative analysis from three independent experiments ($n = 3$ shots). With p35S-OsRDR1 in mixture (iii), which contains a whole ORF-coding region of *OsRDR1* cDNA, specific silencing of *GFP* was distinctly recovered in the mutant line, with the number of GFP-fluorescing cells decreasing as markedly in the mutant line as in the WT (Fig. 4a). When using mixture (iv), which contained BMV-GFP RNAi instead of *GFP* RNAi, there was an obvious decrease in the number of GFP-fluorescing cells in the WT but not in the mutant line, while the number of DsRed-fluorescing cells remained constant in all treatments. To verify bombardment efficiency, we also used real-time RT-PCR to compare the accumulation of *GFP* transcripts to that of *DsRed* transcripts in leaf discs from the WT and the mutant line at 36 h post-bombardment with the various mixtures (Fig. 4b). Similar results were obtained with mutant line ND2059 (data no shown). These results showed that *OsRDR1* was required for BMV-mediated RNA silencing.

To make sure that both viruses are able to replicate in bombarded rice leaf discs, we checked for BMV and WDV replication at different times post-bombardment by real-time RT-PCR and PCR, respectively (Fig. 5). The results show that BMV and WDV were able to replicate in the bombarded leaf discs of both WT and mutant rice.

Discussion

In fungi, plants and several animals, RDRs are an independent class of enzymes with an important role in RNA silencing, heterochromatin formation and natural gene regulation. Previous findings show that *RDR6*, *SDE3*, *SGS3* and *AGO1* are required for sense transgene-mediated PTGS in *Arabidopsis*, whereas these genes are not involved in IR transgene-mediated PTGS (Dalmay et al. 2000; Diaz-Pendon et al. 2007; Mourrain et al. 2000; Wassenegger and Krczal 2006). In our research, RNA silencing was induced not only by IR-DNAs but also by dsRNA in the WT. However, RNA silencing was impaired by introduction of either IR-DNA or dsRNA in the *OsRDR1*-deficient mutant

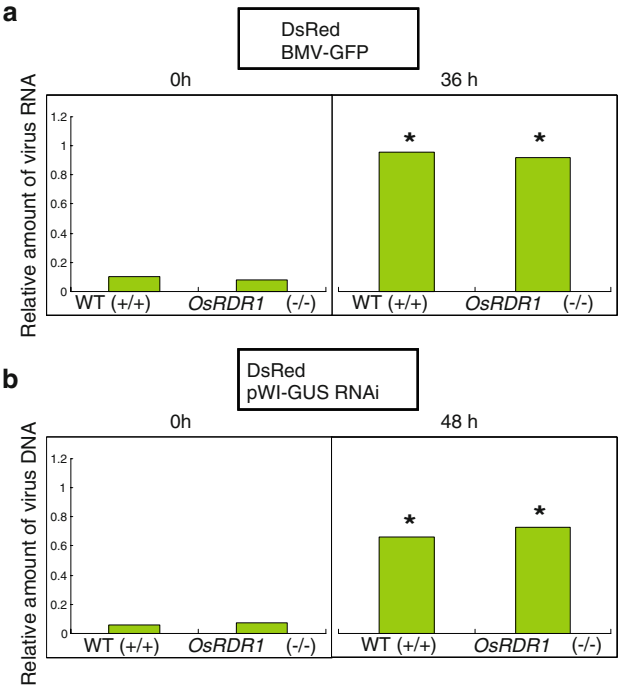

Fig. 5 Analysis of BMV (BMV-GFP) (**a**) and WDV (pWI-GUS RNAi) (**b**) replication in bombarded rice leaf discs. Total RNA (**a**) or DNA (**b**) was extracted at 36 h (BMV) or 48 h (WDV) post-bombardment and used as a template for real time RT-PCR or PCR, respectively. Data were normalized using *DsRed* as an internal control. $*P < 0.05$ (Fisher's test) versus value at 0 h

lines. These results suggest that *OsRDR1* is required at the RNA level after transcription for RNA silencing.

Previous studies show that *RDR1* is induced by SA treatment, virus and viroid infection (Dorssers et al. 1984; Khan et al. 1986; Wassenegger and Krczal 2006). The loss-of-function in the *RDR1* mutant was expressed as enhanced disease susceptibility to various viruses (Xie et al. 2001; Yu et al. 2003). In *Arabidopsis*, the accumulation of TRV RNA in the *RDR1* mutant was significantly higher than in the WT, and disease symptoms in the mutant appeared to be more severe than in the WT (Yu et al. 2003). In *N. tabacum*, a *potato virus X* strain could spread and cause a systemic infection in the *RDR1* mutant line but not in the WT (Xie et al. 2001). Most recently, the finding revealed that the biogenesis of CMV siRNAs was found to be largely dependent on *RDR1* in plants infected with CMV-Δ2b, and genes such as *SGS3*, *SDE3* and *AGO1* might also be essential for *RDR1*-dependent silencing amplification in relation to the production of second siRNAs of CMV (Diaz-Pendon et al. 2007).

In our study, the results from the BMV-GFP RNAi experiments show that *OsRDR1* might be attributed to the amplification level of silencing related to the production of second BMV siRNA (Fig. 4a, b). However, WDV-mediated RNA silencing was not impaired in the mutant lines (Fig. 3a, b), possibly because geminivirus transcription depends on host-DNA-dependent RNA polymerase (Hanley-Bowdoin et al. 2000). VIGS by geminivirus might be amplified by other host *RDR*s such as *RDR2* and *RDR6* (Muangsan et al. 2004). Geminivirus DNA was suggested to be methylated by the RNA-directed DNA methylation pathway requiring *PolIVa*, *RDR2*, *DCL3* and *AGO4* in *Arabidopsis* (Donaire et al. 2008). Thus, WDV in this study is also presumed to be affected in rice by *OsRDR2* instead of *OsRDR1*.

In this RNA-silencing system, we have no data on siRNA, a hallmark of RNA silencing. After co-bombarded with the mixture containing BMV-GFP, *GFP* RNAi and pUbqDsRed, however, BMV-GFP was clearly reduced (data not shown) like the result shown in Fig. 4, mixture (ii). This finding confirmed that RNA silencing functioned in this system as reported by English et al. (1996). Thus, the siRNA must be produced in each bombarded cell although it is difficult to check at the single cell level. The siRNA could be detected using protoplasts as shown by Qi et al. (2004).

Although rice is not a host for either BMV or WDV (http://www.agls.uidaho.edu/ebi/vdie/sppindex.htm), in some reports, each of these viruses infected and replicated in rice protoplasts (Huntley and Hall 1996; Laufs et al. 1990). Our results in Fig. 5 showed that each virus could at least replicate in the leaf discs after co-bombardment. In the BMV vector used, the 5′-half of CP was replaced by the GFP sequence, which might possibly leave the virus unable to move from cell to cell (Schmitz and Rao 1996). In the WDV vector, the region covering the CP and part of the movement protein genes was replaced by a selectable marker (NPTII) and *GUS* (Ugaki et al. 1991). Thus, its cell-to-cell movement would be impaired. In Fig. S2a (i) experiment in ESM, only a single cell expressed *GUS* (data not shown), suggesting that the virus did not spread from cell to cell. Similarly, with BMV vector, *GFP* was expressed in only one cell (data not shown). The spread *GUS/GFP* expression after co-bombardment with the wild-type virus would also be worth checking.

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