Journal of Biotechnology 157 (2012) 421–427

Contents lists available at SciVerse ScienceDirect

Journal of Biotechnology

journal homepage: www.elsevier.com/locate/jbiotec

Hairpin RNA derived from the gene for Pns9, a viroplasm matrix protein of *Rice gall dwarf virus*, confers strong resistance to virus infection in transgenic rice plants

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ARTICLE INFO

Article history:
Received 17 June 2011
Received in revised form 7 December 2011
Accepted 20 December 2011
Available online 27 December 2011

Key words:
Phytoreovirus
RNA interference
Transgenic rice
Viral resistance

ABSTRACT

The nonstructural Pns9 protein of *Rice gall dwarf virus* (RGDV) accumulates in viroplasm inclusions, which are structures that appear to play an important role in viral morphogenesis and are commonly found in host cells infected by viruses in the family *Reoviridae*. An RNA interference construct was designed to target the gene for Pns9 of RGDV, namely Trigger_G9. The resultant transgenic plants accumulated short interfering RNAs specific for the construct. All progenies from self-fertilized transgenic plants had strong and heritable resistance to RGDV infection and did not allow the propagation of RGDV. By contrast, our transgenic plants remained susceptible to *Rice dwarf virus*, another phytoreovirus. There were no significant changes in the morphology of our transgenic plants compared with non-inoculated wild-type rice plants, suggesting that genes critical for the growth of rice plants were unaffected. Our results demonstrate that the resistance to RGDV of our transgenic rice plants is not due to resistance to the vector insects but to specific inhibition of RGDV replication and that the designed trigger sequence is functioning normally. Thus, our strategy to target a gene for viroplasm matrix protein should be applicable to plant viruses that belong to the family *Reoviridae*.

1. Introduction

Rice gall dwarf virus (RGDV), which belongs to the genus *Phytoreovirus* in the family *Reoviridae*, infects rice and the grass weed *Alopecurus aequalis* (Xie et al., 1983) and seriously damages rice production in Thailand (Omura et al., 1980), Malaysia (Ong and Omura, 1982), and Fujian and Guandong provinces in China (Xie et al., 1994). Infection of rice plants by RGDV leads to severe stunting with small galls along the leaf veins on the abaxial surface of leaves and the outer surface of sheaths, considerably decreasing grain yield. RGDV is transmitted to rice plants in a persistent manner by leafhoppers (*Nephotettix* spp.). The virus replicates in the insect vector (Omura et al., 1980). In addition to the relatively long-distance migration of the virus in such viruliferous insects, RGDV is transmitted transovarially to progeny via their eggs (Hibino, 1996). Thus, eradication of the disease is very difficult once widespread infection has occurred (Hibino, 1996).

Genetic resistance is one of the most effective means of protecting crop plants from viral infection. However, there are no reports, to the best of our knowledge, of naturally occurring genes that endow resistance to RGDV infection. Although the high cost of insecticide application is a major burden to growers, insecticides against the vector insect are generally the most common control measure in affected regions. Additionally, the vector insects are likely to become resistant to certain insecticides after continuous, widespread use of these chemicals (Brogdon and McAllister, 1998). Therefore, we are developing genetic engineering strategies as potential solutions to control RGDV disease.

RNA silencing or RNA interference (RNAi), an evolutionarily conserved process that is active in a wide variety of eukaryotic organisms, is a form of gene suppression that occurs at transcriptional and post-transcriptional levels (Baulcombe, 2005; Voinnet, 2005). Double-stranded RNA (dsRNA) is diced into short/small interfering RNAs (siRNAs) of 21- to 24-nucleotide (nt) (Bernstein et al., 2001; Hamilton et al., 2002). These siRNAs are then incorporated into the RNA-induced silencing complex to guide the degradation or translational repression in a sequence-specific manner (Hammond et al., 2000; Liu et al., 2004). As hairpin constructs (Miki et al., 2005; Waterhouse et al., 2001), dsRNA can be expressed in plants relatively easily, enabling us to apply this technology to a wide range of species to silence the expression of both specific endogenous genes and genes of invading pathogens (Mansoor et al., 2006). RNAi has been an important tool to render plants resistant to plant virus infection (Collinge et al., 2010; Mansoor et al., 2006;

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doi:10.1016/j.jbiotec.2011.12.015

Prins et al., 2008). There are several reports of the induction of viral resistance in plants by targeting the genes of certain plant viruses (Bonfim et al., 2007; Di Nicola-Negri et al., 2005; Fahim et al., 2010; Kalantidis et al., 2002; Patil et al., 2011; Reyes et al., 2011; Ribeiro et al., 2007; Shimizu et al., 2009, 2011a,b; Wang et al., 2000). However, the levels of achieved resistance varied from immunity to a delay in the appearance of symptoms or the absence of resistance (Mansoor et al., 2006).

RGDV is an icosahedral double-shelled particle of approximately 65–70 nm in diameter, with two concentric layers of proteins that enclose core. The viral genome consists of 12 segments of dsRNA (Hibi et al., 1984), designated S1 through S12 in order of their migration during SDS-PAGE, that encode six structural and six nonstructural proteins (Ichimi et al., 2002; Koganezawa et al., 1990; Maruyama et al., 1997; Moriyasu et al., 2000, 2007; Noda et al., 1991; Omura et al., 1985; Takahashi et al., 1994). In our previous cytopathological studies, we characterized the role of these 12 proteins encoded by the genome segments of *Rice dwarf virus* (RDV), a member of the same genus in the *Reoviridae*. We have shown that viral structural proteins assemble inside and at the periphery of the viroplasm, which is the putative site of viral replication and composed of three nonstructural proteins, namely, Pns6, Pns11 and Pns12 (Wei et al., 2006). We have demonstrated that Pns12 can initiate the formation of viroplasm-like inclusions in the absence of other viral proteins and that it is the first detectable viral protein in infected insect-vector cells (Wei et al., 2006). Furthermore, we have shown that transgenic plants that harbored RNAi constructs comprising 500 nt that correspond to the 5′ termini of the coding region of the S12 genome segment, which encodes Pns12, conferred strong resistance to the virus (Shimizu et al., 2009). These observations suggested to us that the gene for Pns9 of RGDV, which is the functional orthologue of RDV Pns12 through homology searches between both viral genomic RNAs (Moriyasu et al., 2007) and cytopathological studies (Akita et al., 2011; Wei et al., 2009), might be a critical target for suppression of the proliferation of RGDV in infected rice plants.

In the present study, we analyzed the effects of target sequences that correspond to 500 nt of the S9 genome segment of RGDV, which encodes Pns9, using transgenic rice plants that expressed an inverted-repeat (IR) construct. We demonstrated that targeting the gene for the viroplasm matrix protein is effective in conferring resistance to RGDV in transgenic rice plants.

2. Materials and methods

2.1. Construction of plasmids

For generating an RNAi-inducing construct, a 5′ terminal 500-bp fragment of the gene for Pns9 of RGDV, namely Trigger_G9, was amplified from cDNA clone using the following oligonucleotides: Trigger_G9F, 5′-CACCATGTTTACATCTTCTGCAGCC-3′; and Trigger_G9R, 5′-TTGTGAATATCATAATTTAGCATTAC-3′. The Trigger_G9F primer contained CACC at the 5′ end for directional TOPO® cloning (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA). The product was subcloned into a Gateway entry vector, the pENTR™/D-TOPO® (Invitrogen). The final RNAi vector was produced by an LR clonase®-catalyzed reaction (Invitrogen) between this entry vector and a Gateway destination vector, pANDA (Miki et al., 2005; Miki and Shimamoto, 2004) for transformation of rice calli, according to the manufacturer's instructions. In these reactions, the PCR-derived fragments were inserted into two regions flanked by two recombination sites (attB1 and attB2) in opposite directions, and the *β-glucuronidase* (*gus*) linker sequence was flanked by the two inverted repeats. The resulting plasmid was introduced into *Agrobacterium tumefaciens* EHA101.

2.2. Transformation of rice calli

Rice calli (cv. Nipponbare) were subjected to *Agrobacterium*-mediated transformation according to a previously published method (Hiei et al., 1994). Transformants were selected in a medium containing hygromycin B (50 mg/L). Regenerated plants (T_0 generation) from hygromycin-resistant calli were eventually transferred to commercial soil (Bonsol; Sumitomo Chemical, Tokyo, Japan) in pots and grown to maturity in a greenhouse at 25 ± 3 °C under natural sunlight.

2.3. Screening by PCR

For multiplex PCR screening of transgenic lines, genomic DNA was extracted from all plants (T_0, T_1 and T_2 generations) used in this study according to the published protocols (Edwards et al., 1991). To amplify the transgene-specific *gus* linker and an endogeneous rice gene for actin, we used the following oligonucleotides: GUS-F, 5′-CATGAAGATGCGGACTTACG-3′; GUS-R, 5′-ATCCACGCCGTATTCGG-3′; ACT-F, 5′-TCCATCTTGGCATCTCTCAG-3′; and ACT-R, 5′-GTACCCGCATCAGGCATCTG-3′.

2.4. Isolation and detection of small RNAs

Total RNA was isolated from leaves of rice plants used in this study with TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Northern blot analysis was performed as described previously (Shimizu et al., 2011b). For detection of siRNAs, Digoxigenin (DIG)-labeled sense riboprobe corresponding to Trigger_G9 was prepared by in vitro transcription, with a DIG RNA Labeling Kit (Roche Diagnostics GmbH., Mannheim, Germany).

2.5. Assessment of resistance to RGDV

Transgenic plants (T_1 or T_2 generation) were inoculated with RGDV using a method similar to that described by Shimizu et al. (2007). Wild-type rice plants (cv. Nipponbare) were used as controls. In brief, 10-day-old rice seedlings were exposed to approximately 10 viruliferous leafhoppers per plant in an inoculation chamber (70 cm wide by 70 cm deep by 75 cm high) for 2 days. After the inoculation period, the leafhoppers were killed with insecticide (Kadan-A; Fumakilla, Tokyo, Japan) according to the manufacturer's instructions and plants were transferred to an insect-free greenhouse at 25 ± 3 °C under natural sunlight for development and evaluation of symptoms. The appearance of symptoms on developing leaves was monitored daily until harvest.

2.6. Detection of RGDV by ELISA

Infection by RGDV and its concentration in plants were evaluated by double antibody-sandwich (DAS) enzyme-linked immunosorbent assays (ELISA) using an antiserum against RGDV, described by Takahashi et al. (1991). To evaluate RGDV infection, we harvested pieces (about 100 mg) of leaf sheath plus stem tissue from RGDV-inoculated rice plants. These samples were flash-frozen in liquid nitrogen and then ground in the Multi-beads shocker® (Yasui Kikai, Osaka, Japan). The ground samples were suspended with 10 volumes of phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 2.7 mM KCl, 1.5 mM KH_2PO_4, pH 7.4). Each sap extract was diluted 20-fold with PBS and subjected to DAS-ELISA as described by Satoh et al. (2010).

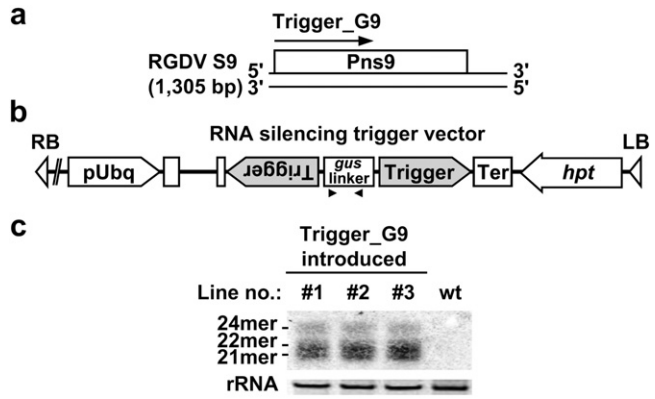

Fig. 1. RNA silencing of the *Rice gall dwarf virus* (RGDV) S9-derived transgene. (a) Genetic map of S9 of the RGDV genome. The line represents dsRNA genome, the box denotes Pns9-protein-coding region, the arrow shows the origin of fragment for the RNAi-trigger, Trigger_G9, which was amplified and cloned into the vector for plant transformation. (b) Schematic diagram of the construct for Pns9-specific RNAi. Trigger_G9, derived from the S9 genome was cloned in two orientations in pANDA to trigger RNAi. Transcripts of the RNAi-trigger region were designed to be expressed constitutively under the control of promoter pUbq of the maize gene for ubiquitin. Downstream of pUbq, the two unlabeled boxes represent exons and the thin line separating the exons marks an intron of the *Ubq* gene. Small arrowheads indicate position of primers used to detect a transgene. (c) Detection of siRNAs in three independent lines of transgenic T_0 plants that harbor Trigger_G9. Numeric designations of transgenic lines are shown above the panels. Approximately 3 μg of total RNA was probed with digoxigenin-labeled transgene-specific RNA. The lower panel shows the detection of 5.8S rRNA, used as a control to confirm loading of equal amounts of RNA in each lane.

3. Results

3.1. Accumulation of transgene-specific siRNAs in the T_0 generation

For generating an RNAi-inducing construct, an internal 500-nt region of the S9 genome segment of RGDV, namely Trigger_G9 (Fig. 1a), was amplified and cloned into the vector pANDA that would transcribe hairpin RNA (hpRNA). As shown in Fig. 1b, the Trigger_G9 fragments were inserted into two sites in opposite directions, separated by the *gus* linker sequence and were driven by the promoter of the maize (*Zea mays*) gene for ubiquitin (*Ubq*) (Christensen et al., 1992). We introduced this RNAi construct into rice calli derived from seeds of an RGDV-susceptible rice cultivar (cv. Nipponbare). We generated 16 transgenic plants from independent transformed calli by selection for hygromycin resistance and confirmed the presence of the transgene in the genome DNA by PCR analysis (data not shown).

To monitor the extent of RNA silencing in transgenic plants, we examined the induction of RNAi by analyzing total RNA from individual Trigger_G9 transgenic T_0 rice plants by Northern blotting, using Trigger_G9-specific digoxigenin (DIG)-labeled RNA probes. As shown in Fig. 1c, we selected three lines (#1–3) that accumulated siRNAs specific to the 5′-proximal region of the gene for Pns9, which migrated as RNA molecules of 21- to 24-nt, among our transgenic T_0 plants. Then, we self-fertilized these transgenic T_0 plants to generate T_1 progeny for further analysis.

3.2. High-level resistance to RGDV in transgenic rice plants that harbored Pns9-specific sequence

To evaluate the response to infection by RGDV, we exposed transgenic T_1 plants to viruliferous (RGDV-carrying) leafhoppers, using wild-type plants as controls. After the insects were killed with insecticide, we monitored plants daily for symptoms. The progeny from each line of Trigger_G9 transgenic rice plants were significantly resistant to RGDV infection (Table 1 and Fig. 2). Furthermore, all the plants that had no symptoms 2 weeks post-inoculation (wpi), when all infected plants normally exhibit symptoms, were symptoms-free until the harvest time, as was the case, for example, for line #1 among the Trigger_G9 plants (Fig. 3). The extent of the resistance to RGDV was similar in each line. By contrast, newly developed leaves of the wild-type plants had typical stunting symptoms by 2 wpi. Not all the wild-type control plants were infected (Table 1), and some escaped infection as observed previously (Shimizu et al., 2009, 2011a, 2011b). As shown in Fig. 4a, all the transgenic T_1 plants of line #1 of the Trigger_G9 that were resistant to RGDV infection had inherited a transgene, whereas eight T_1 plants of this line that had symptoms of RGDV infection had not inherited a transgene. Among all 97 plants in 3 lines analyzed, all the transgenic Trigger_G9 T_1 plants that were infected with RGDV within 2 wpi did not harbor a transgene (data not shown), as was the case for line #1 of the Trigger_G9 T_1 plants described above.

Table 1
RGDV resistance of T_1 generation of transgenic rice plants that harbored Trigger_G9 for RNAi and of control plants.

Transgene	Parent line		Reactions of test plants	
	T_0	n[a]	S[b]	R[c]
Trigger_G9	#1	32	8[e]	24
	#2	31	6[e]	25
	#3	34	7[e]	27
Nipponbare (susceptible cv.[d])		66	60	6[f]

[a] Number of rice plants examined.
[b] Typical symptoms were observed 2 weeks after inoculation.
[c] Plants remained symptomless for the 8-week of observation period.
[d] Cultivars used as controls.
[e] None of the plants inherited the transgene when detected by PCR.
[f] Plant escaped from infection by RGDV.

To confirm the resistance to RGDV infection in inoculated transgenic rice plants, extracts of leaf sheath plus stem from each of all plants 50 days post inoculation were examined for the presence of RGDV by DAS-ELISA with polyclonal antibodies raised against RGDV particle. We were unable to detect RGDV in all the transgenic T_1 plants of line #1 of Trigger_G9 that accumulated

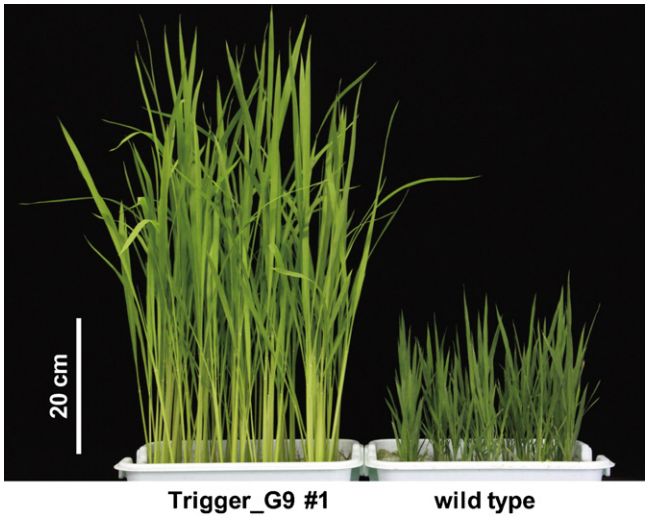

Fig. 2. Highly enhanced resistance to *Rice gall dwarf virus* (RGDV) of self-fertilized transgenic rice plants (T_1) due to Pns9-specific RNAi. Phenotypes of RGDV-inoculated transgenic T_1 rice plants, 50 days post inoculation. Plants in containers from left to right are: resistant progeny of line #1 of Trigger_G9 plants, showing healthy growth after RGDV inoculation; RGDV-susceptible nontransgenic wild-type rice plants (cv. Nipponbare), showing typical stunting after RGDV infection. Bar, 20 cm.

Fig. 3. Appearance of transgenic T_1 rice plants that harbored Trigger_G9 at harvest time. Plants in pots from left to right are: a mock-inoculated wild-type rice plant, showing normal growth (left); an RGDV-resistant progeny line, showing healthy growth and fertile after RGDV inoculation (center); an RGDV-susceptible nontransgenic wild-type rice plant, showing typical stunting after RGDV infection (right). RGDV, inoculated with RGDV by viruliferous leafhoppers; and Mock, "inoculated" with virus-free leafhoppers. Bar = 30 cm.

transgene-specific siRNAs (Fig. 4b and c). By contrast, in eight T_1 plants of this line that had not inherited a transgene and did not accumulate these siRNAs, RGDV was detected at levels as high as those in the control, wild-type plants (Fig. 4). Among all 97 plants in 3 lines analyzed, we were unable to detect RGDV in all the transgenic Trigger_G9 T_1 plants that had inherited a transgene (data not shown), as was the case for #1 of Trigger_G9 T_1 plants, described above.

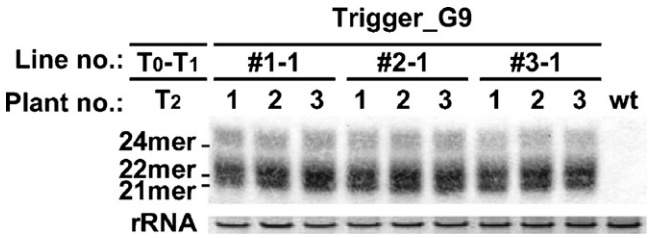

Fig. 5. Accumulation of siRNAs in three independent plants from three lines of transgenic T_2 plants that harbored Trigger_G9. Numeric designations of transgenic plants are shown above the panels. For others, refer to the legend for Fig. 1c.

3.3. Genetic stability of the transgenic RGDV resistance

To monitor the maintenance of RNAi in the T_2 generation, we examined the accumulation of transgene-specific siRNAs by analyzing total RNA from Trigger_G9 transgenic T_2 rice plants. As shown in Fig. 5, siRNAs specific to Trigger_G9, which migrated as RNA molecules of 21- to 24-nt, accumulated in transgenic T_2 plants. Then, to assess the stability of the resistance phenotype, we exposed seedlings from the T_2 generation of Trigger_G9 transgenic rice plants and wild-type rice plants to RGDV-carrying leafhoppers. The inoculated Trigger_G9 transgenic T_2 rice plants were significantly resistant to RGDV infection (Table 2). Among all 148 plants in 9 lines analyzed, all 125 transgenic Trigger_G9 T_2 plants that exhibited strong resistance to RGDV infection had inherited a transgene (data not shown). By contrast, all 23 plants that were infected with RGDV had not inherited a transgene (data not shown). Thus, our observations revealed that the resistance to RGDV of plants with Trigger_G9 RNAi constructs was stably inherited.

3.4. No resistance to another phytoreovirus in transgenic rice plants that expressed RGDV Pns9-specific siRNAs

To evaluate the response to infection by other phytoreoviruses, progeny T_1 plants from each transgenic line generated with

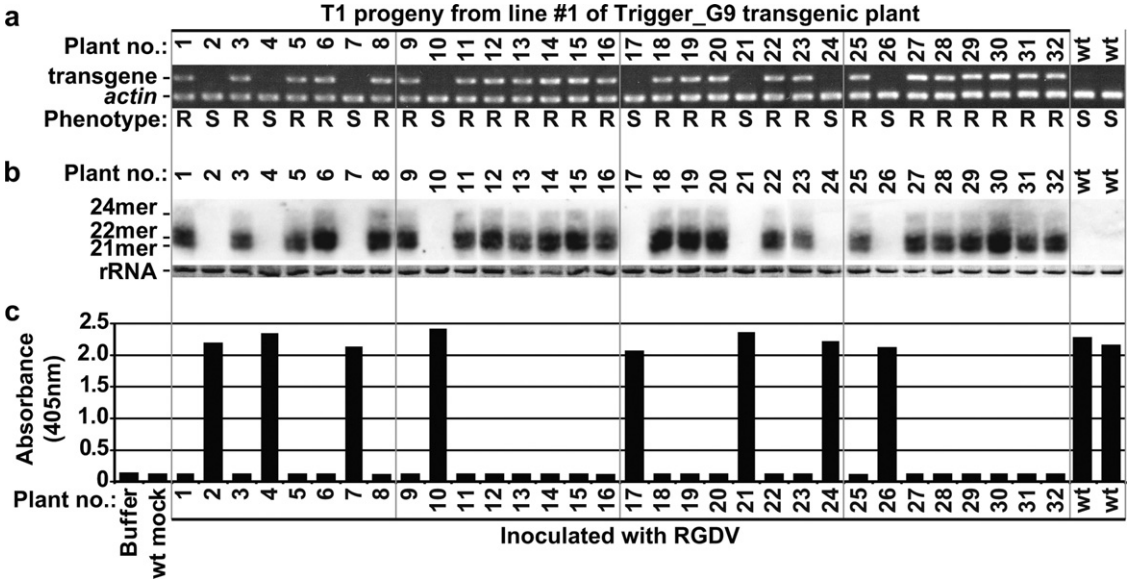

Fig. 4. Correlation between inheritance of a transgene and RGDV accumulation in a population of segregating progeny from line #1 of Trigger_G9 transgenic rice plant. (a) PCR screening for the inheritance of a transgene. Genomic DNA from transgenic and wild-type (wt) plant was analyzed by multiplex PCR with transgene-specific *β-glucuronidase* (*gus*) linker primers and with primers specific for an endogeneous rice gene for actin, as a control. Numbers above lanes represent individual plants. Response to infection of each plant is indicated below the panel by "R" or "S". R, no symptoms of disease (i.e. resistance) 50 days post inoculation (dpi); and S, typical symptoms were apparent 14 dpi. (b) Accumulation of siRNAs in transgenic and wild-type (wt) rice plants. Numbers above lanes represent individual plants. Approximately 1 μg of total RNA was probed with digoxigenin-labeled transgene-specific RNA. For others, refer to the legend for Fig. 1c. (c) Detection by ELISA of RGDV particle in transgenic and wild-type (wt) rice plants 50 dpi. Values refer to absorbance at 405 nm, measured after hydrolysis of substrate for 1 h. wt mock, non-transgenic wild-type exposed to virus-free leafhoppers.

Table 2
RGDV resistance of T_2 generation of transgenic rice plants that harbored Trigger_G9 for RNAi and of control plants.

Transgene	Parent line		Reactions of test plants	
	T_0–T_1	n[a]	S[b]	R[c]
Trigger_G9	#1–1	17	0	17
	#1–2	15	3[e]	12
	#1–3	17	4[e]	13
	#2–1	16	0	16
	#2–2	16	4[e]	12
	#2–3	17	5[e]	12
	#3–1	16	0	16
	#3–2	17	3[e]	14
	#3–3	17	4[e]	13
Nipponbare (susceptible cv.[d])		51	46	5[f]

[a] Number of rice plants examined.
[b] Typical symptoms were observed 2 weeks after inoculation.
[c] Plants remained symptomless for the 8-week of observation period.
[d] Cultivars used as controls.
[e] None of the plants inherited the transgene when detected by PCR.
[f] Plant escaped from infection by RGDV.

Trigger_G9 were inoculated with RDV and monitored daily for symptoms. Almost 95% of the Trigger_G9 transgenic plants, irrespective of whether or not they harbored a transgene, developed typical symptoms that were similar to those on RDV-inoculated wild-type rice plants (Table 3). Symptoms developed at the same rate and were as severe as those in the wild-type rice plants (Supplementary Fig. S1).

4. Discussion

Transgenic plants that expressed Trigger_G9, which targeted the RGDV gene for Pns9 viroplasm matrix protein had strong, heritable resistance to RGDV infection. These transgenic rice plants that remained asymptomatic after challenge with RGDV did not contain detectable amounts of the virus, as determined by ELISA. This result is considered to be important for the field level control of viral disease because our transgenic plants will not be sources of RGDV infection.

In the case of animal viruses that belong to the same family *Reoviridae*, evidence is accumulating that suppression of the gene for a viroplasm matrix protein, important in viral replication, is effective in viral control. In animal reoviruses, the reduction of expression of genes for NSP2 and NSP5 (viroplasm matrix proteins of mammalian rotavirus) and of the gene for μNS (a viroplasm matrix protein of mammalian orthoreovirus) by siRNA adversely affected many aspects of the viral replication cycle, including the formation of viroplasms; the synthesis of all viral proteins, plus-strand RNAs, and dsRNAs; and the production of infectious virions (Kobayashi et al., 2006; López et al., 2005; Silvestri et al., 2004). Our previous study of RNAi-mediated resistance to RDV (genus *Phytoreovirus*) demonstrated that strong resistance was induced in transgenic rice plants when the gene for RDV Pns12, a functional orthologue of RGDV Pns9 (Akita et al., 2011), was targeted for RNA silencing (Shimizu et al., 2009). Our results here also showed that strong resistance to RGDV (genus *Phytoreovirus*) was induced in transgenic rice plants that harbored the RNAi construct directed against the gene for viroplasm matrix protein Pns9, suggesting that this protein plays a key role in viral proliferation. Thus, interfering with the expression of a gene for a viroplasm matrix protein seems to be a viable strategy to use RNAi for conferring resistance to viruses that construct viroplasm inclusions to propagate.

Table 3
Absence of RDV resistance of T_1 generation of transgenic rice plants that harbored Trigger_G9 for RNAi and of control plants.

Transgene	Parent line		Reactions of test plants	
	T_0	n[a]	S[b]	R[c]
Trigger_G9	#1	32	30	2[e]
	#2	27	26	1[e]
	#3	33	32	1[e]
Nipponbare (susceptible cv.[d])		34	32	2[e]

[a] Number of rice plants examined.
[b] Typical symptoms were observed 2 weeks after inoculation.
[c] Plants remained symptomless for the 8-week of observation period.
[d] Cultivars used as controls.
[e] Plant escaped from infection by RDV.

In *Arabidopsis thaliana*, DCL2, DCL3 and DCL4 process both replicating viral RNAs and RNA-induced hpRNAs into 22-, 24- and 21-nt siRNAs, respectively (Brodersen and Voinnet, 2006). The 21- and 22-nt siRNAs are generally believed to be involved in antiviral activities (Bouché et al., 2006; Deleris et al., 2006; Fusaro et al., 2006). Our transgenic plants accumulated 21-, 22-, and 24-nt classes of transgene-specific siRNAs, suggesting that the antiviral RNA silencing machinery of the host plant had been activated. No potential off-target candidates were detected in our designed trigger sequence when they were analyzed with a web-based computational siRNA-scanning tool (Xu et al., 2006) using default settings (21 nt identity or complementarity). Moreover, there were no significant differences between the morphology of our transgenic plants and that of non-inoculated wild-type rice plants, suggesting that genes critical for the growth of rice plants were unaffected.

The spectrum of RNAi-mediated virus resistance is highly sequence specific and thus limited to the virus from which the transgene is derived (Hassani-Mehraban et al., 2009). Similarly, our RGDV-resistant rice plants were not resistant to challenge inoculation with RDV, which is transmitted to rice plants by the same vector insect, probably because the nucleotide sequence of the coding region of RGDV Pns9 on S9 genome segment shares only 49% identities with that of RDV Pns12 on S12 segment (Moriyasu et al., 2007), thus the conserved region longer than 21 nt was not found between them. Taken together, our results demonstrate that our designed trigger sequence is functioning normally and that the RGDV resistance of the Trigger_G9 transgenic rice plants is not due to resistance to the vector insect but rather specifically to inhibition of RGDV replication.

The expression of viral dsRNA for targeting the Pns9 sequence seems to be an efficient method for developing lines of RGDV-resistant rice plants. The T_2 generation of Trigger_G9 transgenic rice plants exhibited strong resistance to RGDV infection, suggesting that the observed high level resistance will be stable into future generations. However, the possibility of breakdown of the RNAi-mediated resistance, as described (Hassani-Mehraban et al., 2009; Mitter et al., 2003), cannot be excluded if the transgene silencing was suppressed by following infection with heterologous viruses, since crops are frequently infected with multiple viruses simultaneously in the fields. For example, in southern Vietnam, a mixed infection with *Rice ragged stunt virus* and *Rice grassy stunt virus*, which belongs to the genus *Oryzavirus* in the family *Reoviridae* and to the genus *Tenuivirus*, respectively, has been a serious problem for rice production (Cabunagan and Choi, 2009). Therefore, introduction of a chimeric transgene, in which heterologous virus genome sequences are fused to each other, would be a promising method to engineer broad-resistance.

RNAi is a natural antiviral defense in plants, which can be exploited in transgenic plants for pre-programming virus recognition and ensuring enhanced resistance. However, levels of resistance to several plant viruses have varied widely (Mansoor et al., 2006). Recently, we have demonstrated that the variable levels of resistance to viral infection of plants that harbor RNAi constructs depend on which viral gene was targeted by the individual

construct, suggesting that it is essential to target the expression of a protein that plays an important role in viral propagation (Shimizu et al., 2011b). In this study, we verified that our strategy is a practical and effective way to control serious plant reoviruses. Thus, our strategy should be applicable to viruses that belong to the genera *Phytoreovirus*, *Fijivirus*, and *Oryzavirus* in the family *Reoviridae*.

Acknowledgments

The authors are grateful to Dr. K. Shimamoto (Nara Institute of Science and Technology, Ikoma, Japan) for kindly providing the RNAi vector pANDA. This work was supported by a grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences of the Bio-Oriented Technology Research Advancement Institution (BRAIN) and by a grant from the Ministry of Agriculture, Forestry, and Fisheries of Japan (Genomic for Agricultural Innovation, GMA-0003).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.12.015.

References

Akita, F., Miyazaki, N., Hibino, H., Shimizu, T., Higashiura, A., Uehara-Ichiki, T., Sasaya, T., Tsukihara, T., Nakagawa, A., Iwasaki, K., Omura, T., 2011. Viroplasm matrix protein Pns9 from rice gall dwarf virus forms octameric structure. J. Gen. Virol. 92, 2214–2221.

Baulcombe, D., 2005. RNA silencing. Trends Biochem. Sci. 30, 290–293.

Bernstein, E., Caudy, A.A., Hammond, S.M., Hannon, G.J., 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409, 363–366.

Bonfim, K., Faria, J.C., Nogueira, E.O.P.L., Mendes, É.A., Aragão, F.J.L., 2007. RNAi-mediated resistance to *Bean golden mosaic virus* in genetically engineered common bean (*Phaseolus vulgaris*). Mol. Plant Microbe Interact. 20, 717–726.

Bouché, N., Lauressergues, D., Gasciolli, V., Vaucheret, H., 2006. An antagonistic function for *Arabidopsis* DCL2 in development and a new function for DCL4 in generating viral siRNAs. EMBO J. 25, 3347–3356.

Brodersen, P., Voinnet, O., 2006. The diversity of RNA silencing pathways in plants. Trends Genet. 22, 268–280.

Brogdon, W.G., McAllister, J.C., 1998. Insecticide resistance and vector control. Emerg. Infect. Dis. 4, 605–613.

Cabunagan, R., Choi, I.-R., 2009. Why Outbreaks of Virus Diseases Occurred in South Vietnam? Ricehoppers, http://ricehoppers.net/2009/06/why-outbreaks-of-virus-diseases-occurred-in-south-vietnam/.

Christensen, A.H., Sharrock, R.A., Quail, P.H., 1992. Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol. Biol. 18, 675–689.

Collinge, D.B., Jørgensen, H.J., Lund, O.S., Lyngkjær, M.F., 2010. Engineering pathogen resistance in crop plants: current trends and future prospects. Annu. Rev. Phytopathol. 48, 269–291.

Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K.D., Carrington, J.C., Voinnet, O., 2006. Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. Science 313, 68–71.

Di Nicola-Negri, E., Brunetti, A., Tavazza, M., Ilardi, V., 2005. Hairpin RNA-mediated silencing of *Plum pox virus* P1 and HC-Pro genes for efficient and predictable resistance to the virus. Transgenic Res. 14, 989–994.

Edwards, K., Johnstone, C., Thompson, C., 1991. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res. 19, 1349.

Fahim, M., Ayala-Navarrete, L., Millar, A.A., Larkin, P.J., 2010. Hairpin RNA derived from viral *NIa* gene confers immunity to wheat streak mosaic virus infection in transgenic wheat plants. Plant Biotechnol. J. 8, 821–834.

Fusaro, A.F., Matthew, L., Smith, N.A., Curtin, S.J., Dedic-Hagan, J., Ellacott, G.A., Watson, J.M., Wang, M.B., Brosnan, C., Carroll, B.J., Waterhouse, P.M., 2006. RNA interference-inducing hairpin RNAs in plants act through the viral defence pathway. EMBO Rep. 7, 1168–1175.

Hamilton, A., Voinnet, O., Chappell, L., Baulcombe, D., 2002. Two classes of short interfering RNA in RNA silencing. EMBO J. 21, 4671–4679.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 404, 293–296.

Hassani-Mehraban, A., Brenkman, A.B., van den Broek, N.J.F., Goldbach, R., Kormelink, R., 2009. RNAi-mediated transgenic Tospovirus resistance broken by intraspecies silencing suppressor protein complementation. Mol. Plant Microbe Interact. 22, 1250–1257.

Hibi, T., Omura, T., Saito, Y., 1984. Double-stranded RNA of rice gall dwarf virus. J. Gen. Virol. 65, 1585–1590.

Hibino, H., 1996. Biology and epidemiology of rice viruses. Annu. Rev. Phytopathol. 34, 249–274.

Hiei, Y., Ohta, S., Komari, T., Kumashiro, T., 1994. Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J. 6, 271–282.

Ichimi, K., Kikuchi, A., Moriyasu, Y., Zhong, B.X., Hagiwara, K., Kamiunten, H., Omura, T., 2002. Sequence analysis and GTP-binding ability of the minor core protein P5 of *Rice gall dwarf virus*. Jarq: Jpn. Agric. Res. Q. 36, 83–87.

Kalantidis, K., Psaradakis, S., Tabler, M., Tsagris, M., 2002. The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. Mol. Plant Microbe Interact. 15, 826–833.

Kobayashi, T., Chappell, J.D., Danthi, P., Dermody, T.S., 2006. Gene-specific inhibition of reovirus replication by RNA interference. J. Virol. 80, 9053–9063.

Koganezawa, H., Hibino, H., Motoyoshi, F., Kato, H., Noda, H., Ishikawa, K., Omura, T., 1990. Nucleotide sequence of segment S9 of the genome of rice gall dwarf virus. J. Gen. Virol. 71, 1861–1863.

Liu, J., Carmell, M.A., Rivas, F.V., Marsden, C.G., Thomson, J.M., Song, J.J., Hammond, S.M., Joshua-Tor, L., Hannon, G.J., 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science 305, 1437–1441.

López, T., Rojas, M., Ayala-Bretón, C., López, S., Arias, C.F., 2005. Reduced expression of the rotavirus NSP5 gene has a pleiotropic effect on virus replication. J. Gen. Virol. 86, 1609–1617.

Mansoor, S., Amin, I., Hussain, M., Zafar, Y., Briddon, R.W., 2006. Engineering novel traits in plants through RNA interference. Trends Plant Sci. 11, 559–565.

Maruyama, W., Ichimi, K., Fukui, Y., Yan, J., Zhu, Y., Kamiunten, H., Omura, T., 1997. The minor outer capsid protein P2 of rice gall dwarf virus has a primary structure conserved with, yet is chemically dissimilar to, rice dwarf virus P2, a protein associated with virus infectivity. Arch. Virol. 142, 2011–2019.

Miki, D., Itoh, R., Shimamoto, K., 2005. RNA silencing of single and multiple members in a gene family of rice. Plant Physiol. 138, 1903–1913.

Miki, D., Shimamoto, K., 2004. Simple RNAi vectors for stable and transient suppression of gene function in rice. Plant Cell Physiol. 45, 490–495.

Mitter, N., Sulistyowati, E., Dietzgen, R.G., 2003. *Cucumber mosaic virus* infection transiently breaks dsRNA-induced transgenic immunity to *Potato virus Y* in tobacco. Mol. Plant Microbe Interact. 16, 936–944.

Moriyasu, Y., Ishikawa, K., Kikuchi, A., Imanishi, S., Tomita, S., Akutsu, K., Omura, T., 2000. Sequence analysis of Pns11, a nonstructural protein of rice gall dwarf virus, and its expression and detection in infected rice plants and vector insects. Virus Genes 20, 237–241.

Moriyasu, Y., Maruyama-Funatsuki, W., Kikuchi, A., Ichimi, K., Zhong, B., Yan, J., Zhu, Y., Suga, H., Watanabe, Y., Ichiki-Uehara, T., Shimizu, T., Hagiwara, K., Kamiunten, H., Akutsu, K., Omura, T., 2007. Molecular analysis of the genome segments S1, S4, S6, S7 and S12 of a *Rice gall dwarf virus* isolate from Thailand; completion of the genomic sequence. Arch. Virol. 152, 1315–1322.

Noda, H., Ishikawa, K., Hibino, H., Kato, H., Omura, T., 1991. Nucleotide sequences of genome segments S8, encoding a capsid protein, and S10, encoding a 36K protein, of rice gall dwarf virus. J. Gen. Virol. 72, 2837–2842.

Omura, T., Inoue, H., Morinaka, T., Saito, Y., Chettanachit, D., Putta, M., Parejarearn, A., Disthaporn, S., 1980. Rice gall dwarf, a new virus disease. Plant Dis. 64, 795–797.

Omura, T., Minobe, Y., Matsuoka, M., Nozu, Y., Tsuchizaki, T., Saito, Y., 1985. Location of structural proteins in particles of rice gall dwarf virus. J. Gen. Virol. 66, 811–815.

Ong, C.A., Omura, T., 1982. Rice gall dwarf virus occurrence in peninsular Malaysia. Int. Rice Res. Notes 7, 7.

Patil, B.L., Ogwok, E., Wagaba, H., Mohammed, I.U., Yadav, J.S., Bagewadi, B., Taylor, N.J., Kreuze, J.F., Maruthi, M.N., Alicai, T., Fauquet, C.M., 2011. RNAi-mediated resistance to diverse isolates belonging to two virus species involved in Cassava brown streak disease. Mol. Plant Pathol. 12, 31–41.

Prins, M., Laimer, M., Noris, E., Schubert, J., Wassenegger, M., Tepfer, M., 2008. Strategies for antiviral resistance in transgenic plants. Mol. Plant Pathol. 9, 73–83.

Reyes, C.A., De Francesco, A., Peña, E.J., Costa, N., Plata, M.I., Sendin, L., Castagnaro, A.P., García, M.L., 2011. Resistance to Citrus psorosis virus in transgenic sweet orange plants is triggered by coat protein–RNA silencing. J. Biotechnol. 151, 151–158.

Ribeiro, S.G., Lohuis, H., Goldbach, R., Prins, M., 2007. Tomato chlorotic mottle virus is a target of RNA silencing but the presence of specific short interfering RNAs does not guarantee resistance in transgenic plants. J. Virol. 81, 1563–1573.

Satoh, K., Kondoh, H., Sasaya, T., Shimizu, T., Choi, I.R., Omura, T., Kikuchi, S., 2010. Selective modification of rice (*Oryza sativa*) gene expression by rice stripe virus infection. J. Gen. Virol. 91, 294–305.

Shimizu, T., Nakazono-Nagaoka, E., Akita, F., Uehara-Ichiki, T., Sasaya, T., Omura, T., 2011a. Immunity to *Rice black streaked dwarf virus*, a plant reovirus, can be achieved in rice plants by RNA silencing against the gene for the viroplasm component protein. Virus Res. 160, 400–403.

Shimizu, T., Nakazono-Nagaoka, E., Uehara-Ichiki, T., Sasaya, T., Omura, T., 2011b. Targeting specific genes for RNA interference is crucial to the development of strong resistance to *Rice stripe virus*. Plant Biotechnol. J. 9, 503–512.

Shimizu, T., Satoh, K., Kikuchi, S., Omura, T., 2007. The repression of cell wall- and plastid-related genes and the induction of defense-related genes in rice plants infected with *Rice dwarf virus*. Mol. Plant Microbe Interact. 20, 247–254.

Shimizu, T., Yoshii, M., Wei, T., Hirochika, H., Omura, T., 2009. Silencing by RNAi of the gene for Pns12, a viroplasm matrix protein of *Rice dwarf virus*, results in strong resistance of transgenic rice plants to the virus. Plant Biotechnol. J. 7, 24–32.

Silvestri, L.S., Taraporewala, Z.F., Patton, J.T., 2004. Rotavirus replication: plus-sense templates for double-stranded RNA synthesis are made in viroplasms. J. Virol. 78, 7763–7774.

Takahashi, Y., Omura, T., Shohara, K., Tsuchizaki, T., 1991. Comparison of four serological methods for practical detection of ten viruses of rice in plants and insects. Plant Dis. 75, 458–461.

Takahashi, Y., Tomiyama, M., Hibino, H., Omura, T., 1994. Conserved primary structures in core capsid proteins and reassembly of core particles and outer capsids between rice gall dwarf and rice dwarf phytoreoviruses. J. Gen. Virol. 75, 269–275.

Voinnet, O., 2005. Induction and suppression of RNA silencing: insights from viral infections. Nat. Rev. Genet. 6, 206–220.

Wang, M.B., Abbott, D.C., Waterhouse, P.M., 2000. A single copy of a virus-derived transgene encoding hairpin RNA gives immunity to barley yellow dwarf virus. Mol. Plant Pathol. 1, 347–356.

Waterhouse, P.M., Wang, M.B., Lough, T., 2001. Gene silencing as an adaptive defence against viruses. Nature 411, 834–842.

Wei, T., Shimizu, T., Hagiwara, K., Kikuchi, A., Moriyasu, Y., Suzuki, N., Chen, H., Omura, T., 2006. Pns12 protein of *Rice dwarf virus* is essential for formation of viroplasms and nucleation of viral-assembly complexes. J. Gen. Virol. 87, 429–438.

Wei, T., Uehara-Ichiki, T., Miyazaki, N., Hibino, H., Iwasaki, K., Omura, T., 2009. Association of *Rice gall dwarf virus* with microtubules is necessary for viral release from cultured insect vector cells. J. Virol. 83, 10830–10835.

Xie, L., Lin, Q., Wu, Z., Zhou, Z., Duan, Y., 1994. Diagnosis, monitoring and control strategies of rice virus diseases in China. J. Fijian Agric. Univ. 23, 280–285.

Xie, S., Chang, S., Liu, X., Luan, L., Liu, C., 1983. Primary source of rice gall dwarf virus in the west of Guandong Province. Guangdong Agric. Sci. 41, 46.

Xu, P., Zhang, Y., Kang, L., Roossinck, M.J., Mysore, K.S., 2006. Computational estimation and experimental verification of off-target silencing during post-transcriptional gene silencing in plants. Plant Physiol. 142, 429–440.