Relationship between Resistance to *Rice necrosis mosaic* virus and the Expression Levels of Rice *RNA-dependent RNA polymerase* 6 (OsRDR6) in Various Rice Cultivars

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Abstract

Rice necrosis mosaic virus (RNMV) is a fungus-transmitted bymovirus that leads to losses in rice yield. This research tested ten rice cultivars (cvs) with different levels of resistance to RNMV. The lowest levels of RNMV RNA were found in two high-resistance cvs; the highest levels were found in the two low-resistance cvs. The *RNA-dependent RNA polymerase 6* gene in rice (*OsRDR6*) was found to be the most highly expressed in two high-resistance cvs and the least expressed in two low-resistance cvs although its basal level of constitutive expression was similar among cvs. Plant growth and yields were also tested. The extent of RNMV RNA accumulation affected plant height, panicle/ tiller numbers, and seed weight. The RNMV induced *OsRDR6* expression level in the rest of cvs was more or less inversely correlated to RNMV RNA accumulation. The observed results suggest a close relationship between RNMV resistance and RNMV induced *OsRDR6* expression level in these cvs.

Discipline: Crop Science

Additional key words: crop breeding, gene, RNAi, RNA silencing, virus susceptibility

Introduction

Rice is the most important staple crop of tropical and subtropical areas and feeds more people than any other crop. Serious constraints on rice cultivation are diseases caused by fungi, bacteria, viruses, and nematodes. Viral diseases are the most serious threat to the production of various cereals, including rice (Suzuki et al. 2015). Rice viruses in eastern and southeastern Asia are responsible for repeated outbreaks of known and new viruses that have caused considerable yield loss over the past few decades. Among the viral diseases of rice, *Rice necrosis mosaic virus* (RNMV), transmitted by the fungus *Polymixa graminis,* can reduce yields by 12.7% to 100%, depending upon the variety (Fujii 1967, Ghosh 1980, Inouye & Fujii 1977). RNMV was first reported in

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Japan (Fujii 1967) and then later in India (Ghosh 1980) in rice, causing mosaic symptoms characterized by yellow flecks and streaks on lower leaves (Badge et al. 1997, Fujii 1978, Ghosh 1980). RNMV is in the same genus Bymovirus in the family Potyviridae as Barley yellow mosaic virus (BYMV) and Wheat yellow mosaic virus (WYMV) (Andika et al. 2016, Usugi et al. 1989). It infects only rice and decreases rice grain yields by inhibiting plant growth after inoculation by infested soil (Fujii 1978) and through manual inoculation by sap from infected rice leaves (Fujikawa et al. 1969). However, an Indian isolate infects not only rice but also dicotyledonous weeds, such as Ludwigia perennis and Brachiaria ramose (Ghosh 1981). Later it was found to affect the dicotyledonous plants: jutes (Corchorus olitorius, C. capsularis), roselle (Hibiscus sabdariffa), kenaf

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(*H. cannabinus*), rice bean (*Vignaum bellata*), cotton (*Gossypium hirsutum*), and tomato (*Solanum lycopersicum*) (Ghosh 1985, Ghosh et al. 2012). In all these dicotyledonous plants manually inoculated with the sap of RNMV infected rice leaves, it promoted growth and improved productivity: increased plant height, leaf size, yield, etc. (Ghosh 1982, 1985, Ghosh et al. 2012). The partial and complete genome sequences of RNMV (RNA1 and RNA2) have been published (Badge et al. 1997, Wagh et al. 2016b), but the biological properties and interactions with different rice cvs are not yet known.

RNA silencing is a universal gene regulation mechanism in eukaryotes that affects several functions, including developmental control, epigenetic modifications, and antiviral defense (Baulcombe 2004, Chen 2009). In plants, RNA silencing is a key defense mechanism against viruses (Csorba et al. 2009, Palukaitis 2011) - the mechanism involves several key protein factors to repress transcription, translation, and DNA methylation (Agrawal et al. 2003, Grewal & Rice 2004). The genes involved in transcription leading to RNA degradation are DICER-like proteins, ARGONAUTE proteins, and RNA-dependent RNA polymerases (RDRs) (Baulcombe 2004, Eamens et al. 2008, Voinnet 2009). In this process, RDR 6 with Suppressor of Gene Silencing 3 (SGS3) plays a significant role in synthesizing an RNA strand complementary to an aberrant RNA template, resulting in double-stranded RNA (dsRNA) (Rajamäki et al. 2014). RDRs in plants can be grouped into four phylogenetic clades: RDR1, RDR2, RDR3, and RDR6 (Wassenegger & Krczal 2006, Zong et al. 2009). Regarding RDR6 in Nicotiana glutinosa, NgRDR6 expression was induced by H₂O₂, salicylic acid (SA), methyl jasmonate (MeJA), abscisic acid (ABA), Cucumber mosaic virus (CMV), and fungi (Rhizoctonia solani and Colletotrichum nicotianae) (Yang et al. 2011). GhRDR6 in cotton (Gossypium hirsutum L.) was induced in cotton by a variety of signaling molecules such as ABA, jasmonic acid (JA), and ethylene (ET) but not by H₂O₂ SA, or viruses (Potato virus Y and Tobacco mosaic virus) (Wang et al. 2012). The expression of OsRDR6 in rice has been induced by ABA, kinetin, and viruses (Rice dwarf virus, Rice stripe mosaic virus, CMV, and RNMV) - (Yang et al. 2008, Jiang et al. 2012, Hong et al. 2015, Wagh et al. 2016a). OsRDR6 expression needs to be checked with other signaling molecules including H₂O₂ SA, and MeJA, which have never been tested.

RDR6 is involved in defense against viruses through RNA silencing (Mourrain et al. 2000). Other *RDRs* also contribute to silencing mediated turnover of transcripts encoded by endogenous plant genes and transgenes (Baulcombe 2004, Wang et al. 2010). *RDR1* and *RDR6* are both involved in virus-derived siRNAs in antiviral silencing (Qu et al. 2008, Wang et al. 2010, 2011, 2012). More recently, *OsRDR6* was reported to be involved in defending against viral, bacterial, and fungal pathogens (Wagh et al. 2016a). This paper compares ten rice cvs with various levels of resistance for RNMV RNA accumulation, *OsRDR6* expression, symptoms (including plant height, the numbers of panicle and tiller), and yield; *OsRDR6* expression level is reported to be inversely related to RNA accumulation level and symptoms. The paper also describes the response of *OsRDR6* to signaling molecules, which could contribute to the molecular understanding of disease resistance.

Materials and methods

1. Plants

Ten rice (*Oryza sativa* L.) cvs were used (Table 1). Nine of them were selected from field resistance assay data (Fujii 1978), and the remaining one had been used previously (Alam et al. 2015b), namely, very high resistance (vHR), Kantou 52 and Sensho; high resistance (HR), Tanginbouzu; medium resistance (MR), Nangoku Wase; low resistance (LR), Reihou; and very low resistance (vLR), Akebono, Nipponbare, Kibiyoshi and Yamabiko. There is no data on the RNMV resistance of Asominori. The seeds of ten cvs were surface sterilized using 0.5% (v/v) mercuric chloride (HgCl₂) solution and placed onto a 1/2 MS agar medium plate. The petri dish was cultured at 27°C under a daily cycle of 16 h continuous light and 8 h dark (as described by Alam et al. 2015a).

2. Inoculation with RNMV

Plants were inoculated with RNMV by applying soil infested with *Polymyxa graminis* to transmit the virus as previously described (Alam et al. 2015a). After seven weeks, RNMV-infected rice cvs were used for total RNA isolation. RT-PCR detection of RNMV RNA was conducted as previously described (Alam et al. 2015a).

A total of nine plants per cv was used to test plant height, tiller and panicle numbers, and yield loss. In a parallel investigation, surface sterilized seeds [cv Akebono (vLR)] were grown for seven weeks in big plastic pots containing a commercial soil mixture then used for treating rice with a signaling molecule. Similarly, for RNMV inoculation, the seeds were sown in an infested soil and grown for the same duration. Under these conditions, 100% of plants were successfully infected.

3. Signaling molecule treatments

The seedlings [cv Akebono (vLR)] were sprayed with each of the solutions at an age of 40 days. SA (Code30423-82, Nacalai Tesque, Kyoto, Japan), ABA (100 μ M, Code A110000, Wako Chemical, Tokyo, Japan), gibberellin (GA) (100 μ M, Code 16627-04, Nacalai Tesque, Kyoto, Japan), H₂O₂ (100 μ M, Code 18411-25, Nacalai Tesque, Kyoto, Japan) and MeJA (100 μ M, Code 392707, Sigma-Aldrich Japan, Tokyo, Japan) were applied to the leaves and incubated for 72 h in a growth chamber. SA was dissolved in sterilized water and then diluted appropriately with H₂O. ABA, MeJA, and H₂O₂ were first dissolved in 99.5% (v/v) ethanol and then diluted appropriately with H₂O. Resistance to RNMV and OsRDR6 Expression in Rice Cultivars

4. RNA extraction and RT-PCR /RT-qPCR

Total RNA was extracted as described in Wagh et al. (2016a). RNMV RNA accumulation and *OsRDR6* expression in RNMV-infected rice cvs were detected by RT-PCR/RT-quantitative real-time PCR (RT-qPCR), as explained in Wagh et al. (2016a). For RT-PCR primers, OsRDR6-F and OsRDR6-R (Table 2) were used at 94°C for 2 min, followed by 30 cycles of amplification (94°C for 20 s, 56°C for 20 s, and 72°C for 50 s). RNMV was detected by RT-PCR using primers RNMV (RNMV-R1-F-5' and RNMV-R1-R-3') (Table 2) by PCR amplification (25 cycles at 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s). Actin transcripts served as an internal standard using the primers Actin-5' and Actin-3' (Table 2) and were detected by PCR amplification (23 cycles at

Table 1. List of cultivars used and their resistance against RNMV

Name of cultivar	Туре	Country	Upland/lowland	RNMV resistance*	Reference**
Akebono	Japonica	Japan	Lowland	vLR	1)
Asominori	"	"	"	nd	2)
Kantou 52	"	"	"	vHR	3)
Kibiyoshi	"	"	"	vLR	4)
Nangoku Wase	"	"	"	MR	5)
Nipponbare	"	"	"	vLR	6)
Reihou	"	"	"	LR	7)
Sensho	"	"	Upland	vHR	8)
Tanginbouzu	"	"	Lowland	HR	9)
Yamabiko	"	"	"	vLR	10)

* Resistance (Fujii, 1978): vHR, very high resistance; HR, high resistance; MR, medium resistance; LR, low resistance; vLR, very low resistance; nd, no data

2) Code=SAI01280 3) Code=KAN00520 4) Code=CYG00060 5) Code=KUI00010

() C = 1 + 100001

- 6) Code=AICE2640
- 7) Code=SAI01000 8) Code=AICE0410
- 9) Code=Z0000470
- 10) Code=TOU00070

Table 2. PCR primers

Primer	Nucleotide sequence	
OsRDR6-F	5'-AGGGAATCATCAACCAGCAC-3'	
OsRDR6-R	5'-CTCCACTTCTGGAGGCAGAC-3'	
OsRDR6-QF	5'-GTGCGGGAG GCTGAAGAAGG-3'	
RNMV-R1-F-5'	5'-GCACAAGGTTATTGCACCCGCAT-3'	
RNMV-R1-R1	5'- TTGCGACGGGCGTGCCGTATGAA -3'	
RNMV-R1-R-3'	5'-TTCCGGTGTACGCAAACATGGCGAACC-3'	
Actin-5'	5'-GAGTATGATGAGTCGGGTCCAG-3'	
Actin-3'	5'-ACACCAACAATCCCAAACAGA-3'	

^{** 1)} https://ineweb.narcc.affrc.go.jp/search/ine.cgi?action=inedata_top&ineCode=HIG00620

94°C for 10 s, 55°C for 10 s, and 72°C for 20 s). RT-qPCR analysis with the primer combinations of OsRDR6-QF and OsRDR6-R, and RNMV-R1-F-5' and RNMV-R1-R1 (Table 2) for *OsRDR6* and RNMV, respectively, was carried out by the procedure as described by Alam et al. (2015a).

Results

1. RNMV RNA accumulation and plant growth

To assess the accumulation levels of RNMV RNA, the seeds were sown in a mixture of infested and commercial soils and incubated as reported by Wagh et al. (2016a). RNMV RNA accumulations were analyzed by RT-qPCR. As shown in Fig. 1, of all ten cvs Kantou 52 (vHR) and Tanginbouzu (HR) were found to contain the least RNMV RNA accumulation. Nipponbare (vLR), Yamabiko (vLR), Sensho (vHR), Asominori (nd), Nangoku Wase (MR), and Reihou (LR) were at a moderate level of RNMV RNA accumulation, while Kibiyoshi (vLR) and Akebono (vLR) were at the highest levels of RNMV RNA accumulation. Infected plants showed necrosis and yellowing with higher intensity and wider range in Nipponbare (vLR), Sensho (vHR), Reihou (LR), Kibiyoshi (vLR), and Akebono (vLR), Yamabiko (vLR), Asominori (nd), and Nangoku Wase (MR) showed milder necrosis, except for Kantou 52 (vHR) and Tanginbouzu (HR) which did not have substantial symptoms (Fig. 2).

2. OsRDR6 expression in response to RNMV

To clarify the relation between OsRDR6 and the accumulation of RNMV RNA, the expression of OsRDR6 was analyzed in both mock and RNMV-infected leaves (Fig. 3). There was very little OsRDR6 expression in all mock inoculated leaves and almost no significant difference in OsRDR6 expression among them. However, in the RNMV-infected leaves, there were various high levels of OsRDR6 expression among the ten cvs. The highest OsRDR6 expression was observed in Tanginbouzu (HR) and Kantou 52 (vHR), whereas moderate expression was found in Nipponbare (vLR), Yamabiko (vLR), Sensho (vHR), Asominori (nd), and Nangoku Wase (MR). Surprisingly, a slightly higher moderate expression of OsRDR6 in Reihou (LR) (Fig. 3) corresponded to a somewhat higher moderate level of RNMV RNA accumulation (Fig. 1). The lowest expression of OsRDR6 was observed in Kibiyoshi (vLR) and Akebono (vLR), where the greatest amount of RNMV RNA accumulated (Fig. 3). These results suggest that a higher expression of OsRDR6 in RNMV-infected rice cvs is related to a lower accumulation of RNMV RNA.



Fig. 1. Analysis of RNMV RNA accumulation in various rice cvs using RT-qPCR

Total RNA was extracted from inoculated and control leaves of rice cultivars, namely Nipponbare (vLR), Kantou 52 (vHR), Tanginbouzu (HR), Yamabiko (vLR), Sensho (vHR), Asominori (nd), Nangoku Wase (MR), Reihou (LR), Kibiyoshi (vLR), and Akebono (vLR). At the flowering stage, young lower leaves were used for RNA extraction approximately 7 weeks after being transferred to the infested soil. Data are mean \pm SE (n = 3). Values with different letters differ significantly among inoculated leaves in Tukey's test at P < 0.05. The *actin* gene was used as an internal control.

3. Effect of RNMV infection on growth and yields of rice

Ten cvs were tested and compared for plant height, and panicle and tiller numbers in infected plants with those of mock inoculated from nine individual plants seven weeks after being transferred to infested soils (Fig. 4a, b, c). The yield (Fig. 4d), only in Kantou 52 (vHR) and Tanginbouzu (HR) were not significantly different between the mock and infected plants. Reihou (LR), Kibiyoshi (vLR), and Akebono (vLR) were found to have considerably lower yields (Fig. 4d). These results suggest that the higher accumulation of RNMV RNA in Akebono



Fig. 2. Necrosis symptoms caused by RNMV on young lower leaves of rice

internal control.

RNMV and mock infected rice, from the left - Nipponbare (vLR), Kantou 52 (vHR), Tanginbouzu (HR), Yamabiko (vLR), Sensho (vHR), Asominori (nd), Nangoku Wase (MR), Reihou (LR), Kibiyoshi (vLR), and Akebono (vLR). Pictures were taken 20 days after transfer to the infested soil.



Fig. 3. RT-qPCR analysis of *OsRDR6***expression in RNMV-infected rice** At the flowering stage, young lower leaves were used for RNA extraction approximately 7 weeks after inoculation. Data are mean \pm SE (n = 3). Values with different letters differ significantly among leaves in Tukey's test at P < 0.05. The *actin* gene was used as an

(vLR) and Kibiyoshi (vLR) affected plant growth and yield more severely. However, Tanginbouzu (HR) and Kantou 52 (vHR) showed lower accumulation of RNMV (Fig. 1).

4. *OsRDR6* expression in response to signaling molecules

RT-PCR analysis revealed that *OsRDR6* expression was induced by various signaling molecules such as SA, ABA, GA, MeJA, H_2O_2 , and RNMV (Fig. 5). The expression level of *OsRDR6* was monitored at 0, 24, 48, and 72 h post-treatment. *OsRDR6* expression reached a peak at 48 h then continued to decline at 72 h posttreatment of MeJA, ABA, SA, and H_2O_2 (Fig. 5a, b, d, and e). The GA treatment showed an increased expression of *OsRDR6* at 48 h and it remained at a similar level at 72 h post-treatment (Fig. 5c).

Discussion

This experiment showed that the RNMV RNA accumulation varied among the ten cvs tested (Table 1).

RNMV RNA accumulation was the highest in Akebono (vLR), and Kibiyoshi (vLR). Nipponbare (vLR), Sensho (vHR), Nangoku Wase (MR), Asominori (nd), and Yamabiko (vLR) showed moderate levels of accumulation. The lowest levels were observed in Kantou 52 (vHR) and Tanginbouzu (HR) (Fig. 1). Both Akebono (vLR) and Kibiyoshi (vLR) are in the group of vLR by Fujii (1978). There is a contradiction in that Sensho (vHR) was as vHR (Fujii 1978) whereas our RTqPCR showed its medium resistance (Fig. 2). Their results were obtained from the field experiments and checking resistance was done only by symptoms. Rechecking may need to be done. The obtained results indicate that more RNMV-resistant cvs accumulated less RNMV RNA than less RNMV-resistant cvs did.

There was a significant reduction in plant height, tiller and panicle numbers, and increased yield losses in Akebono (vLR), Kibiyoshi (vLR), and Reihou (LR) (Fig. 4a, b, and c), which are found to be susceptible to RNMV (Fig. 1). Though Reihou (LR) showed a slightly higher moderate level of expression of *OsRDR6* and RNMV RNA accumulation, it is not clear why Reihou (LR)



Fig. 4. Effect of RNMV infection on plant height (a), panicle number (b), tiller number (c), and yield (d)

Nine plants from each cv were analyzed. They were planted in pots 30 cm in diameter. a) Plant height was measured by placing the plants in a pot next to a wall-mounted scale, b) panicle and c) tiller numbers were counted for each plant seven weeks after being transferred to the infested soil and d) yield losses of 13-week old plants were estimated by weighing 100 randomly selected seeds from each plant with three replications. The average (\pm SD) values were obtained from three biological replications, with 3 plants from each cv. Statistical analysis between mock and virus infected leaves was made for each cv. Asterisks denote significant differences between mock and infected leaves for each cv per the Student's t-test at P < 0.05.

showed discrepancies between severe symptoms, a somewhat higher RNMV RNA accumulation, and a slightly elevated moderate level of *OsRDR6* expression. Further investigation will be needed on this point (Figs. 1, 3, 4). Similarly, a reduction in tiller and panicle numbers and increased yield losses by RNMV infection were reported previously (Fujii 1967, Ghosh 1980). Also, a statistically significant reduction in plant height was observed after RNMV infections (Fig. 4a), which agrees with earlier experimental results (Fujii 1967, Fujikawa et al. 1969, Ghosh 1980).

Signaling molecules play an important role in controlling development and signaling networks that participate in plant response to a wide variety of biotic and abiotic stresses (Verma et al. 2013). However, there has been little reporting of direct evidence of the effects of signaling molecules on OsRDR6 – only for ABA and kinetin (Yang et al. 2008). In this study, the transcript

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levels of OsRDR6 were up-regulated by diverse signaling molecules, such as MeJA, ABA, GA, H₂O₂, SA, and RNMV (Fig. 5). These are new and significant except ABA and RNMV. For OsRDR6 expression analysis in response to signaling molecules, only Akebono (vLR) was used, and the timing of sampling was limited to 72 h. after the treatment. Further comparative analysis of OsRDR6 expression in response to signaling molecules between high-and low-resistance cvs or hormonal responses against RNMV infection could contribute to a better understanding of the relationship between signaling molecules and virus resistance.

Hong et al. (2015) also reported an increase in susceptibility to *Rice dwarf phytoreovirus* (RDV) in *OsRDR6* downregulated rice, followed by a reduction in RDV vsiRNA rates. However, *OsRDR6* over-expression did not affect RDV, though it was reported that *OsRDR6* expression was induced by viruses (CMV and RNMV),



Fig. 5. RT-PCR analyses of *OsRDR6* **expression in rice after treatment with signaling molecules** (a) MeJA, (b) ABA, (c) GA, (d) SA, (e) H₂O₂, (f) mock, and (g) RNMV. Numbers represent hours (h) and days after treatment in case of RNMV. The *actin* gene was used as the standard control to show the normalization of the amount of templates in PCR reactions. Total RNA was extracted from leaves 7 weeks after rice [cv Akebono (vLR)] plants were transferred to infested soil. Hpt, hours post-treatment; dpi, days post-infection.

a bacterium (*Xanthomonas oryzae* pv. *oryzae*), and fungus (*Magnaporthe oryzae*) (Wagh et al. 2016a). The induction of *OsRDR6* by these signaling molecules involved in the initiation of a defence cascade shows that *OsRDR6* plays an important role in protecting against pathogens (Hong et al. 2015, Wagh et al. 2016b). These molecules and pathogens affect *OsRDR6* induction efficiently for plant defense.

RDRs play a key role in RNA silencing to produce double-stranded RNAs, which are cleaved into small RNAs (Balcombe 2004, Qi et al. 2009, Wang et al. 2010). OsRDR1 is involved in RNA silencing mediated by double-stranded RNA (Chen et al. 2010, 2013). OsRDR6, also known as SHOOTLESS2 (SHL2) or ROD-LIKE LEMMA (ROL), is involved in shoot morphogenesis and stamen and spikelet development of rice (Nagasaki et al. 2007, Song et al. 2012, Toriba et al. 2010). The level of RNMV RNA accumulation that was found in OsRDR6 mutant *shl2-rol* to be higher than in Nipponbare (vLR) (Wagh et al. 2016a) suggests that OsRDR6 plays an important role in virus accumulation. Thus the OsRDR6 expression in ten cvs with various levels of resistance (Fig. 4) were tested. The results showed that the OsRDR6 expression level inversely correlated with the level of RNMV RNA accumulation (Figs. 1 and 3). The cvs with lower expression of OsRDR6 showed that those are more susceptible to RNMV and showed the reduction in panicle and tiller numbers and increased yield losses (Fig. 1, Fig. 4b, c and d). Whereas A. thaliana RDR6 and RDR1 have different behaviors, such as virus-activated siRNA biogenesis and target specificity for CMV RNA for each other, both are required for antiviral defense (Wang et al. 2010, 2011). The authors' recent studies reported the induction of expression of OsRDR6 by RNMV and CMV infection (Wagh et al. 2016a). Still, there remains an open question as to why and how OsRDR6 expression varies among cvs. The findings presented in this paper should pave the way towards understanding the molecular mechanism of pathogenhost interactions, which would help in crop-breeding programs for developing useful resistant cvs.

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