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Innate Vulnerability of *Oryza glaberrima* to *Rice tungro bacilliform virus*

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Abstract

Rice tungro disease (RTD) is a serious threat to rice production in South and Southeast Asia. RTD is caused by *Rice tungro bacilliform virus* (RTBV) and *Rice tungro spherical virus* (RTSV). Co-infection of RTSV and RTBV results in more severe symptoms in African rice *Oryza glaberrima* than in Asian rice *O. sativa*. In this study, we examined whether RTBV alone causes more severe symptoms in *O. glaberrima* than in *O. sativa*. The examination of 18 accessions of *O. glaberrima* for RTBV infection indicated that all the *O. glaberrima* accessions examined were susceptible to RTBV. The reactions to RTBV of three *O. glaberrima* accessions and two RTBV-susceptible varieties of *O. sativa*, Taichung Native 1 (TN1) and IR 64, were compared. RTBV accumulation varied depending on the plants and differences in RTBV accumulation were not evident between the two species. However, the *O. glaberrima* accessions were significantly more stunted by RTBV than IR 64 was. Discoloration of leaves by RTBV was evident in the *O. glaberrima* plants, but not in the *O. sativa* plants. Collectively, these results presumably indicated that *O. glaberrima* is generally more vulnerable to RTBV than *O. sativa* is.

Disciplines: Genetic resources, Plant disease
Additional key words: stunting, agroinoculation

Introduction

Rice tungro disease (RTD) is a significant factor negatively affecting rice production in South Asia and Southeast Asia (Azzam & Chancellor 2002). The major symptoms of RTD are dwarfing and leaf yellowing (Azzam & Chancellor 2002). *Rice tungro bacilliform virus* (RTBV) primarily causes RTD with the assistance of *Rice tungro spherical virus* (RTSV) (Hibino et al. 1987). RTBV is one of the pararetroviruses belonging to the genus *Tungrovirus* in the *Caulimoviridae* family (Hull et al. 2005). RTBV has a circular double-stranded DNA genome of about 8 kbp encapsidated in bacilliform particles (Hay et al. 1991, Hull 1996, Hull et al. 2005). RTSV is a member of the genus *Waikavirus* in the *Secoviridae* family (Sanfaçon et al. 2009, Thompson et al. 2017). RTSV has a single-stranded plus-sense RNA genome of about 12 kbp encapsidated in polyhedral particles (Shen et al. 1993, Hull 1996, Hull et al. 2005). The two viruses are transmitted by green leafhoppers (GLH) such as *Nephotettix virescens* Distant (Cabauatan et al. 1995, Hull 1996). The transmission of RTBV by GLH requires the presence of RTSV or a factor encoded in the RTSV genome (Hibino 1983, Hibino et al. 1987).

Different levels of resistance to tungro disease have been observed among rice cultivars (Encabo et al. 2009, Hibino et al. 1990, Sta Cruz et al. 2003). Utri Merah, which is a cultivar of the Asian rice species *Oryza sativa*, was reported to be one of the rice cultivars most resistant to RTD (Encabo et al. 2009). A majority of *O. sativa*

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plants show dwarfing and leaf yellowing symptoms when affected by RTD; however, usually, the disease does not develop into the necrosis or death of *O. sativa* plants (Hibino et al. 1990, Encabo et al. 2009). In contrast, RTD causes lethal symptoms such as necrosis in African rice *O. glaberrima* (Kobayashi & Ikeda 1992). It was also reported that *O. glaberrima* plants were more stunted than *O. sativa* plants when they were infected with RTSV alone (Budot et al. 2014).

O. glaberrima has been adapted to the African environment where RTBV has not been reported. It was reported that *O. glaberrima* has gone through the domestication bottleneck, and shows significantly less genetic diversity than its progenitor, *O. barthii* (Li et al. 2011, Nabholz et al. 2014). These suggest that plants of *O. glaberrima* would be uniformly susceptible to the virus because they have not been exposed to the same selection pressures as *O. sativa* cultivars have been. To confirm this hypothesis, we examined the reactions of *O. glaberrima* plants to RTBV and compared their reactions with those of RTBV-susceptible *O. sativa* plants.

Materials and methods

1. Plant materials and RTBV inoculation procedures

The seeds of 18 accessions of *O. glaberrima* originating from four countries in Africa were obtained from the T. T. Chang Genetic Resources Center, International Rice Research Institute (IRRI) (Table 1). Two *O. sativa* ssp. indica varieties, Taichung Native 1 (TN1) and IR 64 were included as susceptible control materials (Encabo et al. 2009, Shibata et al. 2007). TN1 is known to be one of the most susceptible rice plants to RTBV (Hibino et al. 1990, Shahjahan et al. 1990). All the seeds were incubated at 28ºC for 2 to 3 days in dark and wet conditions after removing the hull. After germination, the seeds were sown in pots and were grown at 27ºC-29ºC in at the containment facility of IRRI.

2. Inoculation of RTBV

The plants were inoculated with RTBV via the agroinoculation method that has been used to evaluate the reactions of rice plants to RTBV (Dasgupta et al. 1991, Sta Cruz et al. 1999, 2003). Fourteen-day-old plants were

Table 1. Reactions of *Oryza glaberrima* and *O. sativa* plants to *Rice tungro bacilliform virus* (RTBV)

Species	Accession number[1]/variety	Country/region of origin	Number of plants examined	RTBV accumulation[2]	Leaf discoloration[3]	Height reduction rate (%)[4]
Oryza glaberrima	IRGC 86741	Nigeria	4	1.44 ± 0.12	+	49.8 ± 4.7
	IRGC 96718	Senegal	5	1.38 ± 0.23	nd/+	45.5 ± 6.6
	IRGC 96790	Nigeria	7	1.48 ± 0.11	+	31.5 ± 2.6
	IRGC 96793	Nigeria	7	1.07 ± 0.33	+	40.0 ± 4.9
	IRGC 96864	Liberia	3	1.16 ± 0.02	+	48.8 ± 4.8
	IRGC 96868	Liberia	3	1.45 ± 0.31	+	42.1 ± 2.3
	IRGC 100139	Guinea	4	1.33 ± 0.29	+	46.3 ± 6.4
	IRGC 100153	Guinea	3	1.21 ± 0.17	+	41.5 ± 5.9
	IRGC 102569	Liberia	4	0.37 ± 0.10	+	47.3 ± 14.1
	IRGC 102489	Liberia	3	0.72 ± 0.32	+	36.0 ± 8.8
	IRGC 102510	Liberia	3	0.95 ± 0.40	+	46.2 ± 6.7
	IRGC 102556	Liberia	3	1.28 ± 0.25	+	44.0 ± 6.1
	IRGC 103437	Senegal	3	0.86 ± 0.17	+	45.3 ± 4.5
	IRGC 103477	Senegal	3	0.67 ± 0.63	+	43.5 ± 3.1
	IRGC 104038	Senegal	7	1.29 ± 0.17	nd/+	38.5 ± 4.8
	IRGC 104545	Nigeria	8	1.28 ± 0.20	+	30.0 ± 4.1
	IRGC 104914	Nigeria	4	1.07 ± 0.28	+	34.3 ± 5.2
	IRGC 115633	Liberia	3	1.02 ± 0.17	+	49.0 ± 2.7
Oryza sativa	Taichung Native 1	Taiwan	7	1.31 ± 0.07	nd	32.8 ± 2.6
	IR64	Philippines	9	0.72 ± 0.13	nd	31.1 ± 2.7

[1]Accession number according to the International Rice Germplasm Collection (IRGC).
[2]Average ± standard deviation of OD_{405} for RTBV in plant extracts determined by enzyme-linked immunosorbent assay at 21 days post-inoculation.
[3]Leaf yellowing was evaluated as either nd: no distinct leaf discoloration or +: yellow to yellow-orange leaf discoloration observed.
[4]Average ± standard deviation of height reduction rate (100 × [(height of mock-inoculated plant − height RTBV infected plant)/ height of mock-inoculated plant]%).

inoculated with *Agrobacterium tumefaciens* GV3850 containing the RTBV infectious clone (qRTRB1162) (Dasgupta et al. 1991). Mock-treated plants were inoculated with *A. tumefaciens* carrying the empty vector (pBin19) (Sta Cruz et al. 2003). *Agrobacterium* carrying the RTBV infectious clone were cultured on Luria-Bertani agar (LBA) plates supplemented with kanamycin (50 μg/ml) and rifampicin (25 μg/ml) at 28ºC for 48 h; *Agrobacterium* used for mock inoculation was cultured on LBA plates with rifampicin. Bacterial cells were scraped from the plates, and were resuspended in 50 mL LB broth with kanamycin (50 μg/mL) and rifampicin (25 μg/mL). The bacterial cells were cultured overnight in an incubator at 28ºC with shaking. Three mL of the overnight culture of the bacterial cells was transferred into 250 mL LB broth with kanamycin (50 μg/mL) and rifampicin (25 μg/mL) that was then grown overnight in the same conditions. After overnight incubation, the bacterial cells were harvested by centrifugation in a Beckman J-14 rotor at 5000 rpm for 15 minutes. The bacterial pellet was resuspended in 1.5 mL distilled water per 250 mL culture. Using a 1.0 mL disposable syringe with a 25-gauge (0.5 × 16 mm) needle, the bacterial suspension (200 μl) was injected into each plant at the base of the stem. After inoculation, the plants were maintained under natural lighting conditions and temperatures ranging from 27°C to 29°C at the containment facility of IRRI.

3. Evaluation of reactions to RTBV

An enzyme-linked immunosorbent assay (ELISA) with an RTBV-specific antibody was used to determine the presence of RTBV in plants (Bajet et al. 1985). Ten-fold diluted extracts from the second youngest leaves were used for the ELISA. A plant was considered to be infected with RTBV if the OD_{405} value obtained with the ELISA using the plant extract was higher than 0.1 (Shibata et al. 2007). The percentage reduction in plant height was calculated as 100 × [(height of mock-inoculated plant − height RTBV infected plant)/height of mock-inoculated plant]. The symptoms and the plant heights were recorded at 7 to 21 days post-inoculation (dpi). The data were examined by analysis of variance and the significance of differences among the means was examined by the least significant difference (LSD) test ($\alpha = 0.05$).

Result and Discussion

Based on the narrow genetic diversity of *O. glaberrima* (Li et al. 2011, Nabholz et al. 2014), the observation that most *O. glaberrima* accessions show necrosis upon infection with RTSV and RTBV (Kobayashi & Ikeda 1992), and the fact that *O. glaberrima* plants originated from Africa where RTBV has not been reported, we hypothesized that *O. glaberrima* plants would be more vulnerable to RTBV than *O. sativa* plants would be. To determine whether *O. glaberrima* plants show common vulnerability to RTBV, 18 accessions of *O. glaberrima* and two *O. sativa* varieties, TN1 and IR 64, were examined for disease symptoms after RTBV inoculation (Table 1). TN1 was included because it is one of the *O. sativa* varieties that show the most severe symptoms with RTD (Cabauatan et al. 1995, Encabo et al. 2009). IR 64 was included because it is susceptible to RTD but still remains one of the most popular varieties in tropical Asia (Shibata et al. 2007). Three to eight infected plants per *O. glaberrima* accession were examined for height reduction, leaf discoloration, and the accumulation of RTBV by ELISA at 21 dpi (Table 1). The average height reduction rates of *O. glaberrima* accessions ranged from approximately 30% to 50% (Table 1). The height reduction rates for 15 of 18 accessions were more than 35%. In contrast, the average height reduction rates for TN1 and IR 64 were about 33% and 31%, respectively. All the accessions of *O. glaberrima* showed yellow discoloration of leaves by RTBV infection, although the degree of discoloration varied among the accessions (Table 1). In contrast, evident discoloration of leaves was not observed in TN1 and IR 64 plants infected with RTBV. Therefore, the 18 *O. glaberrima* accessions examined appeared to be more sensitive to RTBV infection than the *O. sativa* plants were. The accumulation of RTBV was also examined in the 18 *O. glaberrima* accessions and the two varieties of *O. sativa* by ELISA. The average OD_{405} values for the extracts from the 18 *O. glaberrima* accessions at 21 dpi varied, ranging from 0.37 to 1.48 (Table 1). The average OD_{405} values for the extracts from 13 of 18 accessions were higher than 1.0 while those for the extracts from TN1 and IR 64 were 1.31 and 0.72, respectively. Therefore, it appeared that the levels of RTBV accumulation in a majority of the *O. glaberrima* accessions were similar to the levels in TN1 that is the most susceptible *O. sativa* plant (Hibino et al. 1990, Shahjahan et al. 1990), and higher than those in IR 64.

Based on the symptoms and RTBV accumulation levels observed in the 18 accessions of *O. glaberrima* (Table 1), we selected three accessions of *O. glaberrima* (International Rice Germplasm Collection (IRGC) accession numbers 96790, 96864, and 115633) for which we assessed more plants for height reduction and RTBV accumulation to confirm whether their reactions to RTBV were significantly different from those of the *O. sativa* plants. *O. glaberrima* IRGC 96790 was selected because it was the accession least stunted by RTBV infection (average height reduction rate of 31.5%)

despite showing the highest level of RTBV accumulation (average OD_{405} value of 1.48). *O. glaberrima* IRGC 96864 and IRGC 115633 were selected since they were two of the most stunted accessions (average height reduction rates of 48.8% and 49.0% for IRGC 96864 and 115633, respectively). Seventeen to twenty RTBV infected plants of the three accessions of *O. glaberrima* were examined for their symptoms (Figs. 1 and 2) and their RTBV accumulation (Table 2). For comparison, two varieties of *O. sativa*, TN1 and IR 64 were also included in the evaluation. Consistent with the initial evaluation (Table 1), the three accessions of *O. glaberrima* were more stunted by RTBV infection than the *O. sativa* varieties (Fig. 1), although the differences in height reduction rate were significant only between the *O. glaberrima* accessions (the height reduction rates of approximately 33 to 38%) and IR 64 (16.7%). Yellow to orange color discoloration of leaves was also observed in the three accessions of *O. glaberrima* by 21 dpi, but not in the two varieties of *O. sativa* (Fig. 2). The levels of RTBV accumulation varied among the three accessions of *O. glaberrima*, and also among the two varieties of *O. sativa* (Table 2) and the difference in the level of RTBV accumulation was not evident between the two species. The levels of RTBV accumulation in *O. glaberrima* IRGC 96790 and 115633 were significantly higher than those in *O. glaberrima* IRGC 96864 and in *O. sativa* TN1 and IR 64 (Table 2). Collation of the data for height reduction (Fig. 1) and RTBV accumulation (Table 2) indicated that the level of RTBV accumulation in the plants did not correlate with the severity of symptoms. The lack of correlation between the level of virus accumulation and the severity of symptoms was also observed when the reactions of *O. glaberrima* and *O. sativa* plants to RTSV were compared

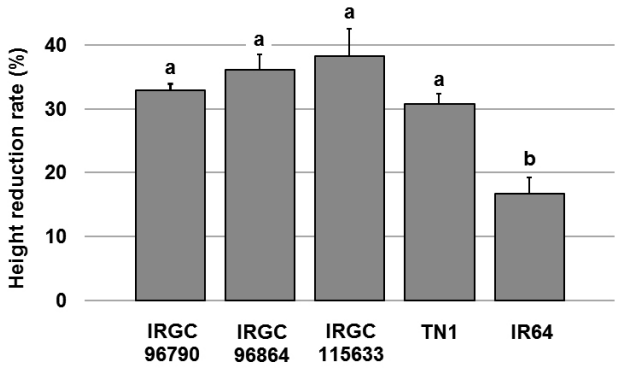

Fig. 1. Height reduction rate for *Oryza glaberrima* and *O. sativa* plants infected with *Rice tungro bacilliform virus*.
Bars with different letters are significantly different as determined by an LSD test ($\alpha = 0.05$). Vertical lines above the bars represent the standard deviation. TN1: Taichung Native 1.

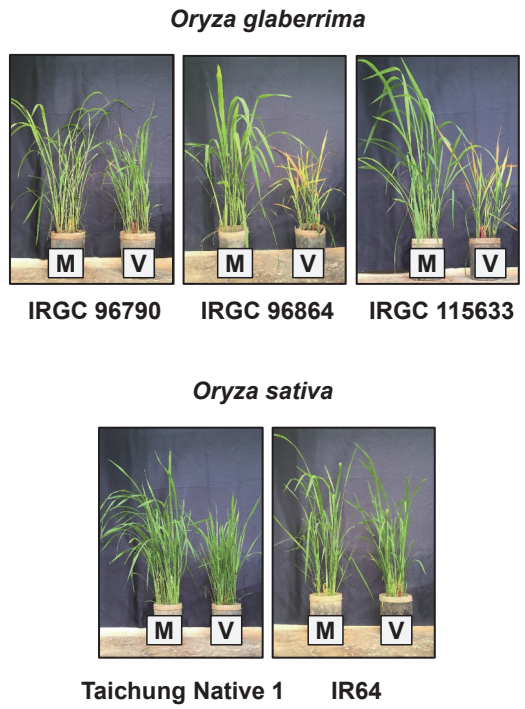

Fig. 2. Representative symptoms in *Oryza glaberrima* and *O. sativa* plants infected with *Rice tungro bacilliform virus* (RTBV) at 21 days post-inoculation.
M: Mock-inoculated plant, V: Plant inoculated with RTBV.

Table 2. Comparison of *Rice tungro bacilliform virus* (RTBV) accumulation in *Oryza glaberrima* and *O. sativa* plants

Species	Accession number[1]/variety	Number of plants examined	Accumulation of RTBV at 21 days post-inoculation[2]
Oryza glaberrima	IRGC 96790	20	1.12 ± 0.07^{c}
	IRGC96864	16	0.47 ± 0.04^{a}
	IRGC 115633	18	0.99 ± 0.06^{c}
Oryza sativa	Taichung Native 1	18	0.68 ± 0.06^{b}
	IR64	17	0.42 ± 0.05^{a}

[1]Accession number according to the International Rice Germplasm Collection (IRGC).
[2]Average ± standard deviation of OD_{405} for RTBV in plant extracts determined by an enzyme-linked immunosorbent assay at 21 days post-inoculation. Values followed by different letters are significantly different as determined by an LSD test ($\alpha = 0.05$).

(Budot et al. 2014).

Due to the practical difficulty in inoculating a large number of plants by the RTBV agroinoculation method in a contained facility, the number of *O. glaberrima* accessions examined for the reactions to RTBV in this study was very limited. Therefore, to confirm whether the high susceptibility to RTBV is a typical reaction of *O. glaberrima* plants, it is desirable to examine more accessions of *O. glaberrima*. However, considering the previous observations that most *O. glaberrima* accessions were hypersensitive to RTD (Kobayashi & Ikeda, 1992) and that *O. glaberrima* has a very narrow genetic diversity (Li et al. 2011, Nabholz et al. 2014), the results presented in this study presumably indicated that the *O. glaberrima* plants were generally more sensitive to RTBV infection than the *O. sativa* plants were.

The greater sensitivity of *O. glaberrima* than *O. sativa* to RTBV observed in this study are consistent with the observation by Kobayashi and Ikeda (1992) that simultaneous infection with RTBV and RTSV caused whole plant necrosis in *O. glaberrima*; however, it did not induce any obvious necrosis in *O. sativa*. The genomic elements underlying the different reactions of *O. glaberrima* and *O. sativa* to RTBV are of interest as they could be associated with a common genetic background in *Oryza* species tolerant to RTBV. Both *O. sativa* and *O. glaberrima* are AA-genome species and their genomic structures are generally similar (Wang et al. 2014). However, there are many genomic differences between these two species (Wang et al. 2014). One genomic difference, which might be associated with their different reactions to RTBV, is the occurrence of endogenous RTBV-like sequences (eRTBVLs) (Kunii et al. 2004, Liu et al. 2012, Chen and Kishima 2016). The distribution of eRTBVLs differs between the two genomes; the *O. sativa* genome contains more than 100 copies of eRTBVLs, while the *O. glaberrima* genome has only a few copies (Kunii et al. 2004). The differences in the eRTBVL copy number in these genomes and the presence or lack of particular eRTBVLs might be associated with the different reactions of these two species to RTBV (Chen & Kishima 2016). Genetic mapping of susceptible factors in the *O. glaberrima* genome and comparison of the reactions of various *Oryza* species differing in eRTBVL copy numbers for the reactions to RTBV may help to confirm the involvement of eRTBVLs in the severity of reactions to RTBV.

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