Research Paper

Evaluation of major Japanese rice cultivars for resistance to bacterial grain rot caused by *Burkholderia glumae* and identification of standard cultivars for resistance

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Bacterial grain rot (BGR), caused by the bacterial pathogen *Burkholderia glumae*, is one of the most destructive rice (*Oryza sativa*) diseases in Japan; however, there are no BGR-resistant cultivars for use in Japan. We previously developed a cut-panicle inoculation method to assess the levels of BGR resistance in the World Rice Collection (WRC). Here, we evaluated major Japanese cultivars for BGR resistance and found that none showed “strong” or “medium to strong” resistance; most were categorized as “medium to weak”. On the basis of the screening results, standard cultivars for BGR resistance were selected according to resistance level and relative maturity. Our results indicate that it is necessary to introduce quantitative trait loci (QTLs) from *indica* or *tropical japonica* resistant cultivars into Japanese *temperate japonica* to develop BGR-resistant cultivars for Japan. We previously developed a near-isogenic line (RBG2-NIL) by introducing the genomic region containing RBG2 from ‘Kele’ (*indica*) into ‘Hitomebore’. In this experiment, we confirmed the resistance level of RBG2-NIL. The resistance score of RBG2-NIL was “medium to strong”, indicating its effectiveness against BGR.

Key Words: *Oryza sativa* L., disease resistance, bacterial grain rot (BGR), bacterial panicle blight, *Burkholderia glumae*, cut-panicle inoculation, rice.

Introduction

Burkholderia glumae causes bacterial grain rot (BGR) and bacterial seedling rot (BSR) in rice (*Oryza sativa* L.), which are increasingly important diseases in global rice production (Ham *et al.* 2011, Mizobuchi *et al.* 2016). Since *B. glumae* was first discovered in 1955 in Japan (Goto and Ohata 1956, it has also been reported in other regions such as the USA (Nandakumar *et al.* 2009), East and South Asia (Ashfaq *et al.* 2017, Chien and Chang 1987, Cottyn *et al.* 1996a, 1996b, Jeong *et al.* 2003, Luo *et al.* 2007, Mondal *et al.* 2015, Trung *et al.* 1993), Latin America (Nandakumar *et al.* 2007b, Zeigler and Alvarez 1989), and South Africa (Zhou 2014). The term “bacterial panicle blight” is also used to refer to BGR, mainly in the USA and Latin America (Ham *et al.* 2011). Primary infection occurs when seeds contaminated with *B. glumae* are sown and then transplanted into

fields. At heading, plants located near the diseased primary-infected plants are also attacked by the pathogen, thus establishing secondary infection. The color of the spikelets changes from the normal green to reddish brown, and BGR becomes apparent. Eventually, the infection may cause unfilled or aborted grains (Ham *et al.* 2011). In 2015, the epidemic area of BGR in Japan covered 30,006 ha (JPPA 2016).

Serious problems are occurring due to BGR and BSR in seed production fields and nursery facilities in Japan (Hasegawa 2012). Seed treatment with oxolinic acid, a quinoline derivative, is a major means of bacterial disease control in Japan (Hikichi 1993a, 1993b, Hikichi *et al.* 1989). However, the occurrence of BGR strains naturally resistant to oxolinic acid has been a serious limitation to this method (Hikichi *et al.* 2001, Maeda *et al.* 2004, 2007). On the other hand, seed disinfection in hot water (for example, 60°C for 10 min) has been established (Hayasaka *et al.* 2001). Although hot-water immersion was as effective as conventional chemical seed disinfection against damage caused by *Pyricularia oryzae* (rice blast) (Hayasaka *et al.* 2001), it was recently found to be somewhat ineffective

Communicated by Hiroshi Kato

Received February 19, 2018. Accepted May 22, 2018.

First Published Online in J-STAGE on August 28, 2018.

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against diseases caused by *B. glumae* (Hasegawa 2012).

One reason for the increase in damage by BGR worldwide is climate change. According to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change in 2014, warming of the climate system is unequivocal, and many of the observed changes are unprecedented over decades to millennia (IPCC 2014). Specifically, the globally averaged combined land and ocean surface temperature data, as calculated using a linear model, show warming of 0.85°C over the period 1880 to 2012 (IPCC 2014). Because the optimal temperature range for the growth of *B. glumae* is relatively high (30–35°C) (Kurita *et al.* 1964, Tsushima *et al.* 1986), global warming may cause BGR to become even more prevalent (Ham *et al.* 2011).

Many studies have been done to enhance resistance to rice blast, and some blast-resistant cultivars have been developed and cultivated in Japan (Nakamura *et al.* 2006, Saka *et al.* 2007). On the other hand, cultivars with BGR resistance have not yet been developed. An urgent necessity for development of BGR resistant cultivars includes the following backgrounds: absence of highly effective pesticide for BGR, sudden outbreak due to environmental changes, and occurrence in both panicle and seedling.

In the past, many field studies have been performed to understand the genetic control of BGR resistance by using spray inoculation at heading or syringe inoculation at booting. Although no source of complete resistance has been discovered (Miyagawa and Kimura 1989, Shahjahan *et al.* 2000), several cultivars show lower disease severity than others and appear to be partially resistant to BGR (Goto and Watanabe 1975, Groth *et al.* 2007, Imbe *et al.* 1986, Mogi and Tsushima 1985, Nandakumar *et al.* 2007a, Nandakumar and Rush 2008, Pinson *et al.* 2010, Prabhu and Bedendo 1988, Saylor *et al.* 2006, Sha *et al.* 2006, Takita *et al.* 1988, Wasano and Okuda 1994, Yasunaga *et al.* 2002). Because BGR tends to be highly affected by environmental conditions such as humidity and temperature, it is difficult to evaluate the resistance of cultivars with different heading dates by using field inoculation (Tsushima 1996).

To minimize environmental variation at the time of inoculation, a “cut-panicle inoculation method” was proposed (Miyagawa and Kimura 1989). This method is based on the “cut-spike” test developed for the evaluation of *Fusarium* head blight resistance in barley (Hori *et al.* 2005, Takeda and Heta 1989). This method entails the collection of panicles from field-grown plants and their inoculation under controlled conditions at flowering (Miyagawa and Kimura 1989). By using a revised cut-panicle inoculation method in which panicles at 3 days after heading are harvested and inoculated, we previously assessed the resistance levels of 84 cultivars, including 62 accessions from WRC (the World Rice Collection of NARO), and found 5 to be relatively resistant to BGR (Mizobuchi *et al.* 2013a, 2016). To elucidate the genetic control of the BGR resistance of ‘Kele’, which proved to be the most resistant (Mizobuchi *et al.* 2013a, 2016), we conducted QTL analysis of BGR resistance using

backcross inbred lines (BILs) derived from a cross between ‘Kele’ (resistant) and ‘Hitomebore’ (susceptible). We successfully mapped a major BGR-resistance QTL on chromosome 1 and named it *RBG2* (Mizobuchi *et al.* 2015). In 2012 (Mizobuchi *et al.* 2013a, 2016), we examined about 10 *temperate japonica* cultivars and found none with definite resistance.

Here, we evaluated major Japanese cultivars for BGR resistance to find out whether any resistant Japanese cultivars exist. No Japanese cultivars showed “strong” or “medium to strong” resistance, indicating that it is necessary to introduce QTLs from *indica* or *tropical japonica* resistant cultivars into Japanese *temperate japonica* cultivars to develop BGR-resistant cultivars for use in Japan. To provide a tool for assessment of BGR resistance levels of current and future cultivars, we selected standard cultivars that covered a range of resistance levels and relative maturities.

Materials and Methods

Plant materials

The names of examined cultivars are listed in **Supplemental Table 1**. Forty-three major Japanese rice (*Oryza sativa* L.) cultivars were grown in paddy fields at NARO in Tsukuba, Japan. Because Hokkaido is a northern area of Japan and the level of damage from BGR is low because of low temperatures, cultivars from Hokkaido were not included in this experiment. Because we have previously assessed WRC cultivars (Ebana *et al.* 2010, Kojima *et al.* 2005) for BGR resistance (Mizobuchi *et al.* 2013a, 2016), we included 12 WRC cultivars for comparison. We previously mapped a QTL (*RBG2*) (Mizobuchi *et al.* 2015) from ‘Kele’, a resistant traditional lowland *indica* cultivar that originated in India. We introduced the genomic region containing *RBG2* (about 30 kb) into ‘Hitomebore’, a susceptible modern lowland *temperate japonica* cultivar from Japan, and named the near-isogenic line *RBG2-NIL*. In this experiment, we analyzed the BGR resistance of *RBG2-NIL*.

Assessment of panicle resistance to BGR

Fifty-five rice cultivars and *RBG2-NIL* were grown in paddy fields in 2017. Agricultural chemicals (‘Helseed’ (Hokko Chemical Industry Co., LTD., Tokyo, Japan) as a fungicide and ‘Stana’ (Sumitomo Chemical Co., LTD., Tokyo, Japan) as a bactericide) were used for seed disinfection under the guidance of the manufacturers. Twenty-three-day-old seedlings were transplanted at a density of one seedling per hill on 10 May 2017. Each cultivar was planted in a single row of 10 hills at 15 cm between hills and 30 cm between rows. Basal fertilizer was applied at 56 kg N, 56 kg P, and 56 kg K ha⁻¹. Cultivars were categorized by days to heading and inoculated on 17 different dates (experiments 1–17, conducted from 21 July to 11 September). Each inoculation test was confirmed by inclusion of ‘Kele’ (resistant) and ‘Hitomebore’ (susceptible) as controls (**Figs. 1, 2**). We used the full set of materials (55 cultivars and *RBG2-NIL*)

Fig. 1. Difference in resistance to bacterial grain rot (BGR) between (A) Hitomebore and (B) Kele.

Fig. 2. Panicle disease scores of control cultivars Hitomebore (susceptible) and Kele (resistant). Error bars indicate SD.

to analyze the relationships between panicle disease score and daily average temperature at heading or days to heading. Four cultivars from the analysis were deleted from **Fig. 3** and **Supplemental Table 1** because we were not allowed to publicize their names. We expanded the flowering time of ‘Kele’ and ‘Hitomebore’ by transplanting once each week for 10 weeks (from 26 April to 28 June).

We measured resistance to BGR in these cultivars by a cut-panicle inoculation method in which panicles at 3 days after heading were harvested and inoculated. Inoculation and measurement were conducted as previously described (Mizobuchi *et al.* 2013a, 2016). *Burkholderia glumae* strain Kyu82-34-2 (MAFF 302744), which has stable virulence (Tsushima *et al.* 1986), was used as a source of inoculum. Panicles were spray-inoculated (~1.7–2 ml per panicle) with a freshly prepared inoculum suspension adjusted to $OD_{620} = 0.2$ corresponding to 10^8 cfu per ml. The inoculated panicles were then placed in a plant growth chamber maintained at 27°C and 100% humidity for 20 h. After the infection period, the panicles were covered with a transparent plastic bag and moved to another growth chamber maintained at 27°C according to a 14-h photoperiod with light intensity of 1000 lux. Five panicles per cultivar were inocu-

lated, and disease symptoms were scored 6 days after inoculation on a 0–10 scale, where 0 indicates 0% infected spikelets per panicle (resistant) and 10 indicates >90% infected spikelets per panicle (susceptible). Each cultivar was analyzed three times.

Because cultivars could not all be assessed in the same experiment, revised data were calculated for each cultivar by comparing the scores of ‘Hitomebore’ and ‘Kele’ in each experiment with their average scores and adjusting the scores for the test cultivars accordingly (**Supplemental Table 1**).

“Daily average temperature at heading stage” of each cultivar was defined as the average of the daily average temperatures of the 4 days from heading to inoculation. We used daily average temperature data for Tsukuba from web page of the Japan Meteorological Agency (<http://www.jma.go.jp/jma/indexe.html>). To compare the resistance between *temple japonica* and *indica*, we used the nonparametric Wilcoxon test provided by JMP version 9.0 software (SAS Institute, Cary, NC, USA).

Selection of standard cultivars for BGR resistance

We selected standard cultivars for BGR resistance according to both resistance level and relative maturity. A disease score of <3 was classified as “strong”, a score of ≥3 and <4 as “medium to strong”, a score of ≥4 and <6 as “medium”, a score of ≥6 and <7.5 as “weak to medium”, and a score of ≥7.5 as “weak”.

Results

Assessment of panicle resistance to BGR

We measured the levels of BGR resistance of 55 rice cultivars and *RBG2-NIL* by the cut-panicle inoculation method. Cultivars were categorized by heading date and inoculated on 17 different dates (experiments 1–17). Each inoculation test was confirmed by inclusion of ‘Kele’ (resistant) and ‘Hitomebore’ (susceptible) as controls. In all experiments, about 6 days after cut-panicle inoculation, many spikelets of ‘Hitomebore’ had brown lesions; in contrast, few spikelets of ‘Kele’ had lesions, and most spikelets had none (**Fig. 1**). The disease score of ‘Hitomebore’ ranged from 6.8 to 8.0 and that of ‘Kele’ ranged from 0.4 to 2.8 (**Fig. 2**). The means and standard deviations of BGR of ‘Hitomebore’ and ‘Kele’ were 7.2 ± 0.4 and 1.6 ± 0.6 , respectively, and their scores from each individual experiment (1–17) were used to revise the scores of cultivars tested in the same experiment (see Materials and Methods and **Supplemental Table 1**). The revised panicle disease scores ranged from 1.6 to 8.2 (*indica*), from 2.7 to 3.1 (*tropical japonica*), and from 4.7 to 7.8 (*temperate japonica*), with ‘Kele’ and ‘Tachisuzuka’ being the most resistant and susceptible cultivars, respectively (**Fig. 3**, **Supplemental Table 1**). Most of the major Japanese cultivars including ‘Koshihikari’, the leading cultivar in Japan, had scores of 6–7.5 (“weak to medium”), and none showed “strong” or “medium to strong”

Fig. 3. Panicle disease scores of cultivars analyzed. Pink bars, *temperate japonica*; a pink diagonal hatching bar, RBG2-NIL; red bars, “top 20” cultivars (*temperate japonica*); blue bars, *indica*; yellow bars, *tropical japonica*. “Top 20” cultivars are Japanese non-glutinous cultivars (excluding Hokkaido) together representing about 80% of the cultivated rice area in Japan in 2016. Four cultivars from the full analysis were omitted from this figure because we were not allowed to publicize their names.

resistance. ‘Kele’ and ‘Jaguary’ (*tropical japonica*) showed “strong” resistance, and four *indica* cultivars (‘ARC 11094’, ‘Jhona 2’, ‘Jarjan’, and ‘Kasalath’) and one *tropical japonica* cultivar (‘Khau Mac Kho’) showed “medium to strong” resistance. The score (U) by nonparametric Wilcoxon test between *temperate japonica* and *indica* was 285.5, indicating that there is a significant difference ($p = 0.0005$) between these groups.

These results indicate that it is necessary to introduce QTLs from *indica* or *tropical japonica* resistant cultivars into Japanese *temperate japonica* cultivars to develop BGR-resistant cultivars for use in Japan. The score of RBG2-NIL was 3.2 (“medium to strong”), which was more resistant than any of the *temperate japonica* cultivars but less resistant than ‘Kele’, the donor cultivar of RBG2. The severity of BGR infection tends to be highly affected by environmental conditions such as temperature (Tsushima 1996). In the cut-panicle inoculation method used in this experiment, temperature was controlled after inoculation. Therefore, we defined the heading stage as the 4 days from heading to inoculation, and analyzed the relationship between panicle disease score and daily average temperature during this period. The daily average temperature at heading for most cultivars ranged from 23.4°C to 26.2°C, but was extremely low (22.1°C) for ‘Tachiaoba’ and ‘Tachisuzuka’ (Supplemental Fig. 1). The heading dates of these cultivars

were so late in Tsukuba (Supplemental Table 1, Supplemental Fig. 2) panicle emergence seemed to be incomplete. The disease scores of these two cultivars were very high (≥ 7.9); thus, the incomplete emergence of panicles probably affected the level of BGR infection.

Selection of standard cultivars for BGR resistance

There was little relationship between panicle disease score and either daily average temperature at heading stage or days to heading. Furthermore, the results for most cultivars obtained in this experiment tended to be similar to those previously obtained by our group (Mizobuchi *et al.* 2013a). Therefore, the cut-panicle method appears to be a reliable method for evaluating BGR resistance. On the basis of the results from the present study, we selected standard cultivars for BGR resistance according to both resistance level and relative maturity (Table 1). Because both ‘Kele’ and ‘Jaguary’, the only two cultivars found to have strong resistance, belong to the ‘Hitomebore’ maturity class (very early) in Tsukuba, there were no “strong” cultivars in the ‘Koshihikari’ (early), ‘Nipponbare’ (intermediate), or ‘Hinohikari’ (late) classes. To represent the “medium to strong” resistance class, we selected ‘ARC 11094’, ‘Kasalath’, ‘Jhona 2’, and ‘Khau Mac Kho’ for the respective ‘Hitomebore’, ‘Koshihikari’, ‘Nipponbare’, and ‘Hinohikari’ maturity classes. To represent the “medium”, “weak to medium”,

Table 1. Standard cultivars for BGR resistance, classified according to resistance level and relative maturity

Relative maturity	Resistance level									
	Strong		Medium to strong		Medium		Weak to medium		Weak	
	Cultivar name	Score ^a	Cultivar name	Score ^a	Cultivar name	Score ^a	Cultivar name	Score ^a	Cultivar name	Score ^a
Hitomebore class (very early)	Kele	1.6	ARC 11094	3.3	Himenomochi	5.4	Akitakomachi	7.0		
	Jaguary	2.7			Hanaechizen	5.9	Hitomebore	7.2		
					Keiboba	5.0	Sasanishiki	6.8		
Koshihikari class (early)			Kasalath	3.6	Nepal 8	5.1	Koshihikari	6.8		
Nipponbare class (intermediate)			Jhona 2	3.3	Asahinoyume	4.7	Nipponbare	6.2	Satojiman	7.5
					Tadukan	5.3	Mochiminori	6.9		
					Hokuriku 193^b	4.5	Takanari^b	6.3		
							Mochidawara^b	6.3		
							Momiroman^b	6.3		
Hinohikari class (late)			Khau Mac Kho	3.1			Hinohikari	7.3	Nishihomare	7.8
							Nikomaru	6.9		
							Yamadanishiki	7.1		
							Mizuhochikara^b	6.9		

^a A score of 0 indicates 0% infected spikelets per panicle (resistant); a score of 10 indicates >90% infected spikelets per panicle (susceptible).

^b Cultivars shown in bold letters are high-yielding cultivars used mainly for forage or processing in Japan.

and “weak” resistance classes, mainly Japanese standard cultivars were selected. In addition to major cultivars with good eating quality, high-yielding cultivars used mainly for forage or processing were also selected as standard cultivars. As expected, panicle symptoms after inoculation differed among the standard cultivars (**Supplemental Fig. 3**).

Discussion

Although BGR caused by *B. glumae* is one of the most common diseases of the Japanese rice crop, there are no BGR-resistant cultivars adapted for use in Japan. Although there were reports about screening Japanese cultivars that (Mizobuchi *et al.* 2016), no common cultivars had been scored as resistant. Here, we evaluated major Japanese rice cultivars for BGR resistance and selected a set of standard cultivars for use in future BGR resistance assessments.

Our results clearly indicate that most of the major Japanese cultivars, including ‘Koshihikari’, the leading cultivar in Japan, were scored as “weak to medium”, and none were scored as “strong” or “medium to strong” (**Fig. 3**). The percentage of planted area represented by the top 20 Japanese non-glutinous cultivars (excluding 3 cultivars from Hokkaido) was about 80% in 2016 (<http://www.komenet.jp/pdf/H28sakutuke.pdf>), and most of these cultivars showed “weak to medium” resistance (**Fig. 3**, **Supplemental Figs. 1, 2**). Similar results were obtained in other studies (Mogi and Tsushima 1985, Takita *et al.* 1988), in which no highly resistant cultivars or lines of Japan were identified, although resistance levels differed among the materials. Wasano and Okuda (1994) reported that ‘Sasanishiki’ was relatively resistant, but our analysis showed that its score was 6.8 (“weak to medium”). The differences between these two studies could be a result of the different inoculation methods used. Here, we assessed resistance by the cut-panicle inoculation method, whereas Wasano and Okuda performed

field evaluation by inoculating a bacterial suspension into the boots (i.e., onto panicles prior to emergence from the stem) with a syringe (Wasano and Okuda 1994). Therefore, it is possible that the influence of environmental conditions after inoculation was lower in our study.

In contrast to the situation with *temperate japonica* cultivars, some *indica* or *tropical japonica* cultivars showed “strong” or “medium to strong” resistance. Thus, it will be necessary to introduce QTLs from *indica* or *tropical japonica* resistant cultivars into Japanese *temperate japonica* to develop BGR-resistant cultivars for use in Japan. We previously finely mapped a QTL (*RBG2*) from ‘Kele’, a resistant traditional lowland cultivar (*indica*) that originated in India (Mizobuchi *et al.* 2015), and developed a near-isogenic line (*RBG2*-NIL) by introducing the genomic region containing *RBG2* into ‘Hitomebore’. The score of *RBG2*-NIL was 3.2, which is classified as “medium to strong”, although the resistance level was less than that of ‘Kele’. Therefore, we expect *RBG2*-NIL to become the first BGR-resistant Japanese *temperate japonica* cultivar in the near future. Besides ‘Kele’, we found several other resistant cultivars. Thus, it is possible that new QTLs from different sources will be detected and utilized for gene pyramiding.

By using cut-panicle inoculation, we could assess disease reactions only 6 days after inoculation; therefore, we are probably measuring the resistance to initial infection. Thus, it will be necessary to confirm the resistance of *RBG2*-NIL to BGR just before harvest, for which we are planning to conduct field evaluation.

We confirmed the resistance of ‘Kele’, ‘Kasalath’, ‘Jhona 2’, ‘Jaguary’, and ‘Khau Mac Kho’, which were also found to be resistant in our previous study (Mizobuchi *et al.* 2013a, 2016). Two late-flowering cultivars (‘Tachiaoba’ and ‘Tachisuzuka’) were observed to be quite susceptible (**Fig. 3**). Because the emergence of panicles from the top internode in these two cultivars seemed to be incomplete

under low temperature at heading (**Supplemental Figs. 1, 2**), panicles seemed to be exposed in a suitable environment for BGR infection under high humidity. At least one cultivar scored as “medium to strong” could be identified in each maturity class, but ‘Kele’ and ‘Jaguary’, which showed “strong” resistance, belong to the ‘Hitomebore’ maturity class (very early heading) in Tsukuba, and there was no “strong” cultivar in any of the other maturity classes. When ‘Kele’ and ‘Jaguary’ are used as controls, it is necessary to delay flowering by late transplanting. Because the whole study was conducted in Tsukuba, it is important that the standard cultivars selected by us (**Table 1**) be evaluated in other areas.

Burkholderia glumae causes not only BGR but also BSR. A previous study found little relationship between resistance to BGR and resistance to BSR among 239 cultivars and 54 breeding lines grown in Japan at that time (Goto 1983). Today, no cultivars of any type have been found to be resistant to both BSR and BGR (Mizobuchi *et al.* 2016). Therefore, the factors associated with resistance to BSR and BGR seem to be different. To date, only one QTL for resistance to BSR has been identified (Mizobuchi *et al.* 2013b). This QTL, *RBG1* (*Resistance to Burkholderia glumae 1*; previously named *qRBS1*), was found on chromosome 10 by analyzing chromosome segment substitution lines (CSSLs) derived from a cross between ‘Nona Bokra’ (BSR-resistant) and ‘Koshihikari’ (BSR-susceptible). Therefore, it will be necessary to combine *RBG1*, *RBG2*, and additional QTLs to achieve stable resistance to both diseases.

Acknowledgments

We thank the large number of researchers at prefectural agricultural institutions and NARO for kindly providing seeds of cultivars used in breeding programs. We are also grateful to Dr. T. Imbe (NARO) for valuable advice. We acknowledge the staff of the technical support section of NARO for field management. This work was supported by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (Project for Climate Change, Rice-2006 and Rice-1201) and by JSPS KAKENHI Grant Number 15K07264. We thank two editors from ELSS (<http://elss.co.jp/en/>) for editing our manuscript before submission.

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