Effectiveness of the Wheat Blast Resistance Gene *Rmg8* in Bangladesh Suggested by Distribution of an *AVR-Rmg8* Allele in the *Pyricularia oryzae* Population

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Accepted for publication 5 June 2020.

ABSTRACT

Wheat blast caused by the *Triticum* pathotype of *Pyricularia oryzae* was first reported in 1985 in Brazil and recently spread to Bangladesh. We tested whether *Rmg8* and *RmgGR119*, recently identified resistance genes, were effective against Bangladeshi isolates of the pathogen. Common wheat accessions carrying *Rmg8* alone (IL191) or both *Rmg8* and *RmgGR119* (GR119) were inoculated with Brazilian isolates (Br48, Br5, and Br116.5) and Bangladeshi isolates (T-108 and T-109). Br48, T-108, and T-109 carried the eI type of *AVR-Rmg8* (the avirulence gene corresponding to *Rmg8*) while Br5 and Br116.5 carried its variants, eII and eII' types, respectively. Detached primary leaves of IL191 and GR119 were resistant to all isolates at 25°C. At a higher temperature (28°C), their resistance was still effective against the eI carriers but

was reduced to a low level against the eII/eII' carriers. A survey of databases and sequence analyses revealed that all Bangladeshi isolates carried the eI type which induced a higher level of resistance than the eII/eII' types. The resistance of IL191 (*Rmg8*–) to the eI carriers was maintained even at the heading stage and at the higher temperature. In addition, GR119 (*Rmg8/RmgGR119*) displayed higher levels of resistance than IL191 at this stage. These results suggest that *Rmg8* combined with *RmgGR119* will be useful in breeding for resistance against wheat blast in Bangladesh.

Keywords: avirulence gene, Bangladesh, genetics and resistance, *Pyricularia oryzae*, resistance gene, wheat blast

Wheat blast caused by the *Triticum* pathotype (MoT) (Cruz and Valent 2017) of *Pyricularia oryzae* (Zhang et al. 2016) is one of the most destructive wheat diseases in South America and Asia. This disease was first observed in 1985 in Brazil (Urashima et al. 1993), then spread to adjacent countries such as Bolivia (in 1996), Paraguay (in 2002), and Argentina (in 2011), causing significant yield losses (Ceresini et al. 2018). In 2016, an outbreak of wheat blast occurred in Bangladesh (Callaway 2016; Islam et al. 2016; Malaker et al. 2016) and affected 15% of the country's total wheat area (Malaker et al. 2016). This was the first report of a wheat blast epidemic in a country other than in South America. The inoculum for this outbreak in Bangladesh was considered to originate from South America (Islam et al. 2016; Malaker et al. 2016).

Breeding for resistance is the most economic and labor-saving method for disease control. In the past 30 years, efforts have been made to breed wheat cultivars with improved blast resistance. Cruz et al. (2016) found that a 2NS chromosomal fragment derived from *Aegilops ventricosa* conferred resistance to head blast. Recently, BARI Gom33, a new wheat cultivar carrying the 2NS fragment was released in Bangladesh (Mottaleb et al. 2019). However, the

resistance conferred by the 2NS fragment is not effective in some genetic backgrounds or against some fungal strains (Cruz et al. 2016). As such, it is crucial to identify new resistance genes against the wheat blast fungus.

Resistance genes against wheat blast must be effective at high temperatures and at the heading stage of the plant, as wheat blast is a spike disease that is favored by high temperature. We have identified five resistance genes against the wheat blast fungus, i.e., *Rmg2* and *Rmg3* (Zhan et al. 2008), *Rmg7* (Tagle et al. 2015), *Rmg8* (Anh et al. 2015), and *RmgGR119* (Wang et al. 2018). Among these, *Rmg2* and *Rmg3*, identified in common wheat, were temperature sensitive and not effective against spike infection. *Rmg7*, identified in emmer wheat, was effective against spike infection but not effective at high temperature. On the other hand, *Rmg8*, identified in common wheat cultivar S-615, was effective at both seedling and heading stages and at relatively high temperature (Anh et al. 2015; Wang et al. 2018). Recently, Wang et al. (2018) identified a new resistance gene, *RmgGR119*, in common wheat accession GR119 and proved that *Rmg8* combined with *RmgGR119* conferred strong resistance to *Triticum* isolates collected in Brazil.

The final goal for breeders is to develop a cultivar with broad-spectrum and durable resistance. In this context, the spectrum and durability of a resistance gene may be evaluated on the basis of distribution and stability of its corresponding avirulence gene (Tosa and Chuma 2014). Anh et al. (2018) cloned *AVR-Rmg8*, an avirulence gene corresponding to *Rmg8*, and found that it was recognized not only by *Rmg8* but also by *Rmg7* (Anh et al. 2018). Wang et al. (2018) analyzed nucleotide sequences of *AVR-Rmg8* homologs in *Triticum* isolates collected in Brazil and observed that it was divided into three types, eI, eII, and eII'.

In this study, we examined whether *Rmg8* and *RmgGR119* were effective against Bangladeshi isolates of *P. oryzae*. Infection assays coupled with analyses of the avirulence gene suggest that *Rmg8*, and probably *RmgGR119*, are effective against wheat blast in Bangladesh.

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Funding: This research was supported by Grants-in-aid for scientific research from the Japan Society for the Promotion of Science (17H01462), and a grant, "International collaborative research project for solving global issues", from Agriculture, Forestry and Fisheries Research Council Secretariat, Ministry of Agriculture, Forestry and Fisheries (MAFF), Japan.

First and second authors contributed equally to this work.

*The e-Xtra logo stands for "electronic extra" and indicates there are supplementary materials published online.

The author(s) declare no conflict of interest.

MATERIALS AND METHODS

Fungal materials. Fungal materials used in the study comprised 11 *Triticum* isolates (isolates from *Triticum* spp. or isolates of the *Triticum* pathotype) of *P. oryzae*. Eight of these isolates (Supplementary Table S1) were collected in Bangladesh in 2016–17 and provided by Md Tofazzal Islam, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Bangladesh, while another three isolates (Br48, Br5, and Br116.5) were collected in Brazil in 1990–92 (Wang et al. 2018). Br48ΔA8_d6, an *AVR-Rmg8* disruptant of Br48 produced by Wang et al. (2018), was used as a recipient of *AVR-Rmg8* homologs.

Plant materials. *T. aestivum* accession IL191 was selected as a representative of 18 different cultivar/accessions that carry *Rmg8* alone (Wang et al. 2018) as it displayed the highest resistance among them at the seedling stage. *T. aestivum* accession GR119, carrying both *Rmg8* and *RmgGR119* (Wang et al. 2018), and cultivar Hope, carrying no resistance gene, were also employed in the study.

Seedling infection assay. Wheat seeds were sown in vermiculite supplied with liquid fertilizer in a seedling case and grown in a controlled-environment room with a 12-h photoperiod of fluorescent lighting at 22°C. Primary leaves of 8-day-old seedlings were fixed onto a hard-plastic board using rubber bands immediately prior to inoculation. In the case of detached leaf inoculations, seedlings were grown in vermiculite in a controlled-environment room as described above for 8 days or in soil (Sakata Prime Mix, Sakata Seeds Corporation, Yokohama, Japan) in a greenhouse (18 to 26°C) for 17 days. The primary leaves of the 8-day-old seedlings were used for initial evaluations of resistance/susceptibility while the third leaves of the 17-day-old seedlings were used for precise evaluations of the degree of resistance. Leaves were cut at the base, inserted into Eppendorf tubes (2 ml) containing distilled water, and fixed onto a hard-plastic board.

Fungal isolates/strains were grown on oatmeal agar media (5 g of sucrose, 40 g of oat meal, and agar at 20 g/liter) for 7 days. Aerial mycelia from the 7-day-old cultures were removed by rubbing the mycelial surfaces with cotton balls. The cultures were incubated further under blue light to induce sporulation. Three to four days later, conidia were harvested and a suspension (1×10^5 conidia/ml) containing 0.01% Tween 20 was evenly sprayed onto fixed leaves using an air compressor. Inoculated leaves were placed in a humid tray and incubated in the dark at 25 and 28°C. After 24 h, they were transferred to dry conditions with a 12-h photoperiod and further incubated at 25 and 28°C for 3 to 4 days. Infection types were recorded based on the size and color of lesions. The size was rated on six progressive grades from 0 to 5: 0 = no visible infection; 1 = pinhead spots; 2 = small lesions (<1.5 mm); 3 = scattered lesions of intermediate size (<3 mm); 4 = large lesion; and 5 = complete shriveling of leaf blades. The color was designated as B for brown and G for green. A disease score (infection type) was composed of a number denoting the lesion size and a letter indicating the lesion color. For example, infection type 1B indicated brown pinhead spots. Infection types 0, 1B, and 2B were considered resistant (avirulent) while infection types 3G, 4G, and 5G were considered susceptible (virulent). Intermediate types, 3B and 3BG, were categorized as resistant because the browning of leaf tissues in these types had been shown to be caused by hypersensitive reactions (HR) (Hyon et al. 2012). In infection assays with primary leaves,

infection types 4B and 5B were observed in some isolate/cultivar combinations. They were also categorized as resistant because the blighting or shriveling of whole leaves in these types had been shown to be caused by extensive HR (Murakami et al. 2000). Four leaves were employed for each cultivar/isolate combination in one replication. The infection assays with primary leaves and third leaves were replicated three times and twice, respectively.

Spike infection assay. Test plants were grown for 4 to 5 months in a field or greenhouse. At the stage of full head emergence (before flowering), a stem with a spike was cut at the base, trimmed to ~50 cm (including the spike), and put into a test tube (150 mm in length and 10 mm in diameter) with water containing antibiotics (MISAKI solution for cut flowers, OAT Agrio, Tokyo, Japan). The spikes were inoculated with conidial suspension (1.5×10^5 conidia/ml), covered with a black plastic bag, and incubated in the dark at 25 and 28°C. After 24 h, the plastic bag was removed, and the spikes were incubated further under a 12-h photoperiod at the same temperature as in the dark. Seven to eight days postinoculation, infection was rated with six progressive grades from 0 to 5: 0 = no visible infection; 1 = brown pinhead spots; 2 = brown lesions of intermediate size (<3 mm); 3 = large lesions surrounded by brown tissues; 4 = extensive necrosis or chlorosis; and 5 = complete blighting of the spike. Infection types of 0 to 3 were considered resistant, and 4 to 5 were considered susceptible. Two spikes were employed for each cultivar/isolate combination in one replication. The infection assay with spikes was repeated three times.

Extraction of fungal genomic DNA. *P. oryzae* strains were cultured in liquid medium (3 g of yeast extract, 3 g of casamino acid, and 5 g of sucrose/liter) in a 200-ml Erlenmeyer flask for 4 to 5 days. Genomic DNA was extracted from harvested mycelia using a modified version of a protocol described by Nakayashiki et al. (1999). In short, 1 g of a fungal sample (mycelial powder) in 1 ml of DNA extraction buffer (100 mM LiCl, 100 mM EDTA, 10 mM Tris-HCl, pH 7.5, and 0.5% sodium dodecyl sulfate) was incubated in a water bath at 65°C for 1 h. After centrifuging at 15,000 rpm, the supernatant was applied to PCI (phenol/chloroform/isoamyl alcohol = 25:24:1) to remove protein, and then to 1 ml of isopropanol to precipitate DNA. After removing RNA with 1 µl of RNase, DNA was precipitated, washed with ethanol, and resuspended in 25 µl of TE.

Sequencing of *AVR-Rmg8* homologs. A 550-bp fragment containing *AVR-Rmg8* was amplified from genomic DNA of Bangladeshi isolates of *P. oryzae* with primers A8_ORF_Seq_F

Fig. 1. Reactions of detached primary leaves of Hope (–/–), IL191 (*Rmg8*/–), and GR119 (*Rmg8*/*RmgGR119*) to *Pyricularia oryzae* isolates from Bangladesh (T-108 and T-109) and Brazil (Br48, Br5, and Br116.5) at 25 and 28°C, 5 days after inoculation. Infection types are shown in red (resistant) or green (susceptible) under each picture. The assay was repeated three times with similar results. Results of the third replication are shown.

TABLE 1. Primers used in this study

Name	Sequence (5'→3')
A8_ORF_Seq_F	TCTAGTTGCATTTCTCACTCCA
A8_ORF_Seq_R	GGCCGTTTAACGTTTTTGG
AVR-Rmg8_multi_cloning_F	ATAAAAGCAATACGGTCGGA
AVR-Rmg8_multi_cloning_R	CACGTATAATTACGTTCTGTGTG
AVR-Rmg8_F2	ACCGCATCGGCTTTTCTT
AVR-Rmg8_R2	TGCCTTCTAGTACCGGGAAGT

and A8_ORF_Seq_R (Table 1). PCR products were checked by electrophoresis on a 0.7% agarose gel, purified by ethanol precipitation, and then sequenced using the ABI BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and the ABI 3100 Genetic Analyzer following the manufacturer's instructions. Obtained nucleotide sequences were aligned with published sequences of eI (in Br48), eII (in Br5), and eII' (in

Br116.5) types of *AVR-Rmg8* and those in databases (Supplementary Table S2). Based on these nucleotide sequences, maximum likelihood tree was constructed using the MEGA7 software (Kumar et al. 2016). Bootstrap values were generated from 1,000 replications.

Transformation of *P. oryzae* with *AVR-Rmg8* homologs. A 1.8-kb fragment containing the *AVR-Rmg8* ORF, together with a

Fig. 2. Relationship of *AVR-Rmg8* homologs in *Triticum* isolates used in this study and in databases. The dendrogram was constructed based on the nucleotide sequences of *AVR-Rmg8* open reading frames using the maximum likelihood method. The number at the node represents a percentage of 1,000 bootstrap replicates. The scale bar represents 0.005 base substitutions per site. Asterisks indicate the three representative isolates.

1,000-bp upstream region and a 500-bp downstream region, was amplified from Br48 (carrying the eI type), Br5 (carrying the eII type), and Br116.5 (carrying the eII' type) using AVR-Rmg8_multi_cloning_F and AVR-Rmg8_multi_cloning_R primers (Table 1) and KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan). The thermal cyclor setup comprised 94°C for 2 min, and 35 cycles of 94°C for 30 s, 60°C for 30 s, and 68°C for 2 min. The amplicon was run on a 2% agarose gel and extracted from the gel using QIAEX II Gel Extraction Kit (QIAGEN, Hilden, Germany). The purified fragment was ligated with pBluescriptII SK(+) digested with *EcoRV* and introduced into *Escherichia coli* DH5- α . The recombinant plasmids were introduced into Br48 Δ A8_d6 following the PEG-mediated cotransformation protocol (Tosa et al. 2005) with pII99 (carrying a geneticin resistance gene) (Namiki et al. 2001) as a selection marker. Positive colonies were selected on media containing G418 sulfate and confirmed by PCR using the primers mentioned above.

Southern blot analysis of transformants. Genomic DNAs of Br48, Br48 Δ A8_d6, and transformants were digested with *EcoRI* (Takara, Kusatsu, Japan), electrophoresed on a 0.7% agarose gel in the TBE buffer, and transferred to a nylon membrane (Hybond-N+, GE Healthcare, Buckinghamshire, England) through capillary blotting. The DNA fragments were fixed to a membrane by UV

Fig. 3. Southern blot analysis of Br48, Br48 Δ A8 (Δ A8), and its transformants carrying the eI type (Δ A8+eI -3, 4, and 5), eII type (Δ A8+eII -8, 9, and 12), and eII' type (Δ A8+eII' -2, 3, and 5) of AVR-Rmg8 isolated from Br48, Br5, and Br116.5, respectively. A fragment containing the endogenous AVR-Rmg8 in Br48 is indicated by a triangle.

radiation. A 322-bp AVR-Rmg8 fragment amplified from the genomic DNA of Br48 with primers, AVR-Rmg8_F2 and AVR-Rmg8_R2 (Table 1), was labeled following the instruction of the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). The membrane was hybridized for 12 h with the labeled probe in the Gold hybridization buffer (GE Healthcare) at 42°C. After hybridization, the membrane was washed twice in the primary washing buffer (0.4% sodium dodecyl sulfate and 0.5 $\times$ SSC) for 10 min at 55°C, and then twice in 2 $\times$ SSC for 5 min at room temperature. Signals were detected following the manufacturer's instructions.

RESULTS

Infection assay at the seedling stage. In a preliminary experiment, intact primary leaves of Hope (–/–), IL191 (*Rmg8*–/–), and GR119 (*Rmg8*/*RmgGR119*) were inoculated with the eight Bangladeshi isolates of *P. oryzae* and incubated at 25 and 28°C. IL191 and GR119 were resistant to all Bangladeshi isolates at both temperature (Supplementary Table S1). We chose T-108 and T-109 as representatives of the Bangladeshi isolates for further analyses.

When the two Bangladeshi isolates and three representative Brazilian isolates of *P. oryzae* (Br48, Br5, and Br116.5) were sprayed on detached primary leaves of the three wheat lines, these isolates were divided into two groups, group A (T-108, T-109, and Br48) and group B (Br5 and Br116.5). At 25°C, group A was almost completely avirulent on IL191 and GR119 while group B produced some brown flecks (Fig. 1). At 28°C, group A was still avirulent on IL191 and GR119 while group B caused leaf shriveling (Fig. 1). It should be noted that these shriveled leaves were accompanied by tissue browning (infection types 5B and 4B–resistant reactions) in contrast to shriveled leaves of Hope (infection type 5G–susceptible reactions).

Nucleotide sequences of AVR-Rmg8 in various isolates. Br48 in group A carries the eI type AVR-Rmg8 while Br5 and Br116.5 in group B carry its variants (the eII and eII' types) (Wang et al. 2018). To determine AVR-Rmg8 types of Bangladeshi isolates, an AVR-Rmg8 region (550 bp) was amplified from genomic DNA of the eight Bangladeshi isolates and sequenced. Nucleotide sequences of AVR-Rmg8 in these isolates were aligned with those from databases and literatures (Supplementary Table S2). A dendrogram showed that AVR-Rmg8 in *Triticum* isolates worldwide was divided into two groups, eI and eII/eII' (Fig. 2). Isolates from South America harbored all the three types whereas Bangladeshi isolates harbored the eI type alone. The nucleotide sequences of the eI type in Bangladeshi isolates were 100% identical to the eI type in Br48.

Function of the three types of AVR-Rmg8 as avirulence genes. To clarify the association of the AVR-Rmg8 types with the phenotypes directly, 1.8kb fragments containing the eI, eII, and eII'

Fig. 4. Reactions of detached third leaves of IL191 (*Rmg8*–/–) at 28°C to Br48 Δ A8 (Δ A8) and its transformants carrying the eI type (Δ A8+eI -3, 4, and 5), eII type (Δ A8+eII -8, 9, and 12), and eII' type (Δ A8+eII' -2, 3, and 5) of AVR-Rmg8 isolated from Br48, Br5, and Br116.5, respectively, 4 days after inoculation. Infection types are shown in red (resistant) or green (susceptible) under each picture. The assay was repeated twice with similar results. Results of the second replication are shown.

types of *AVR-Rmg8* were isolated from Br48, Br5, and Br116.5, respectively, and introduced into Br48ΔA8_d6 (disruptant of *AVR-Rmg8*) which was virulent on both IL191 and Hope. Consequently, 11, 14, and 12 transformants were obtained from the transformation with the eI, eII, and eII' types, respectively. When sprayed on intact primary leaves of IL191 and Hope at room temperature (~22°C), six with the eI type (Br48ΔA8+eI), six with the eII type (Br48ΔA8+eII), and five with the eII' type (Br48ΔA8+eII') showed avirulence on IL191 (infection types 0 or 1B) without losing the high virulence on Hope (infection type 5G), indicating that all of these types are functional and recognized by *Rmg8*.

Three transformants were arbitrarily chosen as representatives from each of the three groups. The copy number of the transgenes in these transformants varied in each of Br48ΔA8+eI, Br48ΔA8+eII, and Br48ΔA8+eII' (Fig. 3). Nevertheless, the three transformants in each group showed similar tendency in virulence on IL191; when they were sprayed on detached third leaves of IL191 and incubated at 28°C, the eI transformants were completely avirulent while the eII and eII' transformants produced some brown spots or flecks (Fig. 4, Supplementary Fig. S1). These results suggest that the eI type induces a higher level of resistance than the eII/eII' types in *Rmg8* carriers.

Spike infection assay. To evaluate the effect of *Rmg8* and *RmgGR119* at the heading stage, spikes of Hope, IL191, and GR119 were inoculated with the representative Bangladeshi (T-108 and T-109) and Brazilian (Br48 and Br5) isolates of *P. oryzae*. IL191 was resistant to the eI carriers (T-108, T-109, and Br48) but susceptible to the eII carrier (Br5) (Fig. 5). Infection assays with the transformants suggested that these contrastive reactions were attributed to the *AVR-Rmg8* types they harbored (Supplementary Fig. S2). GR119 displayed higher levels of resistance than IL191 against T-109 and Br48 (Fig. 5). Furthermore, GR119 was resistant even against Br5 to which IL191 was susceptible.

DISCUSSION

Wang et al. (2018) reported three types of *AVR-Rmg8*, i.e., eI, eII, and eII', in *Triticum* isolates collected in Brazil in 1990 to 1992. In their infection assay with intact leaves at 25°C, a correlation was not recognized between the *AVR-Rmg8* types of these isolates and the

degree of their avirulence on IL191 (carrying *Rmg8*). However, careful examination with detached leaves and spikes at 25 to 28°C revealed that group A carrying the eI type was highly avirulent on IL191 while group B carrying the eII/eII' types was more aggressive or virulent on this accession (Figs. 1 and 5). Infection assays with the transformants revealed that the eI type induced higher levels of resistance in comparison with the eII/eII' types in this *Rmg8* carrier (Fig. 4, Supplementary Fig. S2). In contrast to the Brazilian isolates, Bangladeshi isolates harbored the eI type with no exception (Fig. 2). The wheat blast population in Bangladesh is considered to be clonally derived from a single strain of one mating type (Cruz and Valent 2017), and therefore, is inferred to be composed of eI type carriers alone. These results suggest that *Rmg8* will be effective against wheat blast in farmer's fields in Bangladesh.

GR119 carrying two genes (*Rmg8/RmgGR119*) displayed higher levels of resistance than IL191 (*Rmg8*–) against two of the three eI carriers tested at the heading stage (Fig. 5). In addition, GR119 was resistant even against the eII carrier to which IL191 was susceptible (Fig. 5). We suggest that the combination of *Rmg8* with *RmgGR119* will make the resistance long-lasting in Bangladesh, and also may be effective in South America where both of the eI and eII/eII' types are prevailing (Fig. 2).

Wang et al. (2018) observed brown flecks on detached leaves of S-615 inoculated with Br48 at 25°C. On detached leaves of IL191 inoculated with the same isolate at 25°C, however, no lesions were produced (Fig. 1). IL191 also showed similar immune responses against the other eI carriers at 25°C (Fig. 1). IL191 was selected as an accession displaying the highest level of resistance to Br48 at the seedling stage from the 18 lines carrying *Rmg8* alone, i.e., cultivar S-615 and 17 accessions found by Wang et al. (2018). It is unlikely that IL191 carries another resistance gene because it is susceptible to Br48ΔA8_d6, an *AVR-Rmg8* disruptant of Br48 (Fig. 4). IL191 and S-615 seem to carry different *Rmg8* alleles. For resistance breeding it is advisable to choose the most effective allele of *Rmg8*.

Jensen et al. (2019) reported that S-615 carrying *Rmg8* was resistant to a Bangladesh isolate but susceptible to a Brazilian isolate. This Brazilian isolate may carry an *AVR-Rmg8* allele other than the eI type. The current global population of the wheat blast fungus is composed of strains that differ with respect to virulence on *Rmg8* (Fig. 2). Group A and group B are regarded as physiological races differentiated on an *Rmg8* carrier although their difference becomes obvious only at high temperature or at the heading stage. When a system of race identification is constructed (Crupe et al. 2020), therefore, an *Rmg8* carrier should be included in the differential cultivars. It should be noted that this variation of *AVR-Rmg8* was already present in the population of wheat blast isolates collected in Brazil in 1990 to 1992, only 5 to 7 years after the first detection of this fungus. This variation does not seem to be caused by an adaptation to *Rmg8*. Further studies are needed to clarify the process of evolution of *AVR-Rmg8*.

ACKNOWLEDGMENTS

We thank Md Tofazzal Islam, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Bangladesh, for providing the wheat blast isolates collected in Bangladesh.

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Fig. 5. Reactions of detached spikes of Hope (–/–), IL191 (*Rmg8*–), and GR119 (*Rmg8/RmgGR119*) to *Pyricularia oryzae* isolates from Bangladesh (T-108 and T-109) and Brazil (Br48 and Br5) at 25 and 28°C, 7 days after inoculation. Infection types are shown in red (resistant) or green (susceptible) under each picture. The assay was repeated three times with similar results. Results of the third replication are shown.

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