A new resistance gene in combination with *Rmg8* confers strong resistance against *Triticum* isolates of *Pyricularia oryzae* in a common wheat landrace

Shizhen Wang, Soichiro Asuke, Trinh Thi Phuong Vy, Yoshihiro Inoue, Izumi Chuma, Joe Win, Kenji Kato, and Yukio Tosa.

First, second, third, fourth, fifth, and eighth authors: Graduate School of Agricultural Sciences, Kobe University, Kobe 657-8501, Japan; Sixth author: The Sainsbury Laboratory, Norwich Research Park, Norwich NR4 7UH, UK; seventh author: Graduate School of Environmental and Life Science, Okayama University, Okayama 700-8530, Japan.

Corresponding author: Y. Tosa; E-mail address:tosayuki@kobe-u.ac.jp

First and second authors contributed equally to this work.

ABSTRACT

Wang, S., Asuke, S., Vy, T. T. P., Inoue, Y., Chuma, I., Win, J., Kato, K., and Tosa, Y. A new resistance gene in combination with *Rmg8* confers strong resistance against *Triticum* isolates of *Pyricularia oryzae* in a common wheat landrace.

The wheat blast fungus (*Triticum* pathotype of *Pyricularia oryzae*) first arose in Brazil in 1985 and has recently spread to Asia. Resistance genes against this new pathogen are very rare in common wheat populations. We screened 520 local landraces of common wheat collected worldwide with Br48, a *Triticum* isolate collected in Brazil, and found a highly resistant, unique accession, GR119. When F_2 seedlings derived from a cross between GR119 and Chinese Spring (CS, susceptible control) were inoculated with Br48, resistant and susceptible seedlings segregated in a 15:1 ratio, suggesting that GR119 carries two resistance genes. When the F_2 seedlings were inoculated with Br48ΔA8 carrying a disrupted allele of *AVR-Rmg8* (an avirulence gene corresponding to a previously reported resistance gene, *Rmg8*), however, the segregation fitted a 3:1 ratio. These results suggest that one of the two genes in GR119 was *Rmg8*. The other, new gene was tentatively designated as *RmgGR119*. GR119 was highly resistant to all *Triticum* isolates tested. Spikes of GR119 were highly resistant to Br48, moderately resistant to Br48ΔA8 and a hybrid culture carrying *avr-Rmg8* (nonfunctional allele), and highly resistant to its

transformant carrying *AVR-Rmg8*. The strong resistance of GR119 was attributed to the combined effects of *Rmg8* and *RmgGR119*.

Wheat blast is a devastating disease caused by the *Triticum* subgroup or pathotype (Cruz and Valent 2017) of *Pyricularia oryzae* (*Magnaporthe oryzae*) (Zhang et al. 2016) which recently evolved through host jumps (Inoue et al. 2017). It was first reported in Brazil in 1985 (Urashima et al. 1993) and then spread to neighboring countries such as Argentina, Paraguay, and Bolivia (Cruz et al. 2012; Kohli et al. 2011), but was contained within South America for 30 years. In 2016 a severe outbreak of wheat blast occurred in eight southwestern districts in Bangladesh (Callaway 2016). This was the first outbreak of wheat blast in Asia or Eurasia. It affected approximately 15% of the wheat area in those districts with up to 100% yield losses (Islam et al. 2016). Phylogenetic analysis suggested that the outbreak in Bangladesh was caused by blast strains that most likely originated from South America (Islam et al. 2016: Malaker et al. 2016). In 2017 wheat blast spread to India, the second largest wheat-producing country in the world (Bhattacharya and Mondal 2017). It is an urgent task to stop its spread to other countries before it becomes pandemic.

The symptoms of wheat blast appear mainly on the spikes. Fungicides are not effective in controlling wheat spike blast if warm, rainy weather occurs during the heading stage (Cruz and Valent 2017). This may be explained by the fact that conditions favoring spike infection are high temperatures (25-30^{o}C) coupled with a wetting-period of more than 25 h (Cardoso et al. 2008). Resistance to QoI fungicides is already widespread in Brazilian populations of the wheat blast fungus

(Castroagudin et al. 2015).

Breeding for resistance is an alternative, environmentally-friendly method to control blast. Four genes have been identified in wheat for resistance to the wheat blast fungus. Zhan et al. (2008) identified *Rmg2* and *Rmg3* in common wheat cultivar "Thatcher", but those genes were ineffective at high temperature or at the heading stage (Inoue et al. unpublished). Tagle et al. (2015) identified *Rmg7* in an accession of *Triticum dicoccum*. Although *Rmg7* was expressed at the heading stage (Tagle et al. 2015), it was not effective at higher temperatures (26^{o}C) (Anh et al. 2018). Subsequently, Anh et al. (2015) identified *Rmg8* in common wheat cultivar "S-615". *Rmg8* was expressed at the heading stage and was effective even at 26^{o}C (Anh et al. 2018) although some blown flecks developed under some conditions. Cruz et al. (2016b) recently reported that a 2NS chromosomal segment from *Aegilops ventricosa* conferred resistance in wheat spikes. However, the resistance conferred by 2NS was ineffective against a highly aggressive new isolate, B-71, in most wheat genetic backgrounds. Additional resistance genes are urgently needed to win the arms race with the wheat blast fungus.

In the present study, we screened 520 local landraces of common wheat collected worldwide, and found a highly resistant accession, GR119, in a collection from Albania. GR119 carried two resistance genes that acted additively to confer resistance to the wheat blast fungus; one was *Rmg8* whereas the other was a new gene that was tentatively designated as *RmgGR119*.

MATERIALS AND METHODS

Plant materials. A total of 520 common wheat (*Triticum aestivum*) landraces maintained at Okayama University were used in a preliminary screening for resistant accessions. *T. aestivum* 'S-615' (abbr. S615) was used as a resistant control carrying *Rmg8* alone, and *T. aestivum* 'Chinese Spring' (abbr. CS), 'Hope', and *T. carthlicum* (6x) accession KU-140 (laboratory code, St55) were used as susceptible controls carrying no resistance gene.

Fungal materials. Wild isolates used were 14 *Triticum* isolates of *P. oryzae* which were collected in Brazil in 1990-1992 and have been preserved at Kobe University. One of them, Br48, was used as a representative *Triticum* isolate for screening and segregation analysis of wheat F_2 populations. 200R54 (an F_1 culture derived from MZ5-1-6 (*Eleusine* isolate) x Br48) and 200R54+A8 (a transformant of 200R54 carrying a 2.2kb fragment with *AVR-Rmg8* derived from Br48) (Anh et al. 2018) were used for a rapid check of presence/absence of *Rmg8*. *AVR-Rmg8* is an avirulence gene corresponding to *Rmg8* (Anh et al. 2018). 200R54 carries *avr-Rmg8*, a nonfunctional allele of *AVR-Rmg8* derived from MZ5-1-6. If an accession is susceptible to 200R54 and resistant to 200R54+A8, the accession is considered to carry *Rmg8*. For further analyses, *AVR-Rmg8* of Br48 was disrupted (described below),

and the disruptant was designated as Br48ΔA8.

Disruption of *AVR-Rmg8*. Targeted knockout of *AVR-Rmg8* was performed using a split marker strategy (Fig. 1A). A 1.2kb DNA fragment upstream of the *AVR-Rmg8* ORF was amplified from total DNA of Br48 with primers, Up_F_XhoI and Up_R_HindIII (Table 1) with KOD-plus- Ver.2 (TOYOBO, Osaka). The PCR product was digested with *Xho*I and *Hin*dIII and ligated with pSP72-HPH (Inoue et al. 2016) digested with *Xho*I and *Hin*dIII, generating pSP72-AVR-Rmg8_UP-HPH. A 0.65kb DNA fragment downstream of the *AVR-Rmg8* ORF was amplified from total DNA of Br48 with primers, Down_F_BamHI and Down_R_SacI (Table 1). The PCR product was digested with *Bam*HI and *Sac*I, and ligated with pSP72-HPH digested with *Bam*HI and *Sac*I, generating pSP72-HPH-AVR-Rmg8_DOWN. Then, the upstream fragment containing a 5' part of the HPH (hygromycin phosphotransferase) cassette was amplified with primers, Up_F_XhoI and HY (Catlett et al. 2003) (Table 1) using pSP72- AVR-Rmg8_UP-HPH as a template. The downstream fragment containing a 3' part of HPH cassette was amplified with primers, YG (Catlett et al. 2003) (Table 1) and Down_R_SacI using pSP72-HPH-*AVR-Rmg8*_DOWN as a template. These two PCR products were mixed and introduced into protoplasts of Br48 to replace the *AVR-Rmg8* locus with the HPH cassette. The preparation of protoplasts, introduction of DNA fragments into the protoplasts, and regeneration of transformants were performed as described by Tosa et al. (2005). Regenerated colonies were subjected to colony PCR with primers, KO_F and KO_R (Table 1), for

picking up knockout mutants (disruptants).

Infection assays with primary leaves. Wheat seeds were pregerminated on moistened filter papers for 24h, sown in vermiculite supplied with liquid fertilizer in a seeding case (5.5 × 15 × 10 cm), and grown at 22°C in a controlled-environment room with a 12h photoperiod of fluorescent lighting for 7 days. Primary leaves of the 7-day-old seedlings were fixed onto a hard plastic board, the bottom 1/4 of which was inserted into vermiculite in a seedling case, with rubber bands just before inoculation. A conidial suspension (1×10^5 conidia/ml) prepared as described by Tagle et al (2015) was sprayed onto fixed leaves by an air compressor. The inoculated seedlings were kept in darkness in humid trays at 22-25°C for 24h, then returned to dry conditions with fluorescent lighting, and incubated for additional 3-4 days at 22-25°C. Four to five days after inoculation, symptoms (infection types) were evaluated on the basis of the size and color of lesions (Vy et al. 2014). The size was rated on six progressive grades from 0-5: 0 = no visible infection; 1 = pinhead spots; 2 = small lesions (<1.5mm); 3 = scattered lesions of intermediate size (<3mm); 4 = large typical lesion; and 5 = complete blighting of leaf blades. A disease score comprised a number denoting the lesion size and a letter indicating the lesion color: 'B' for brown, and 'G' for green. For example, infection type 1B indicated brown pinhead spots. Infection types 0-5 with brown lesions were considered to be resistant whereas infection types 3G, 4G, and 5G were considered to be susceptible. All infection assays at the seedling stage were conducted at least twice.

142 In addition to the infection assay with intact seedlings as described above, an
143 infection assay based on detached leaves was developed. Pregerminated wheat seeds
144 were sown in soil in 5cm pots (one seed/pot), and grown in a greenhouse for ~2 weeks
145 until the second leaf was fully developed. Primary leaves were detached from the
146 2-week-old seedlings, and fixed onto a hard plastic board with their bottoms inserted
147 into water in test tubes (13 mm in diameter and 75 mm in length). Inoculation,
148 incubation, and evaluation of symptoms were performed as describe above.

149 **Infection assays with spikes.** For inoculation at the heading stage, accession
150 IL191 (collected in Armenia) was used as a control carrying *Rmg8* instead of S615
151 because its spikes emerged simultaneously with those of GR119. Seeds of IL191 and
152 GR119 were sown in soil in large pots (30cm in diameter) and grown in a greenhouse
153 for 5 months. Stems with spikes at the stage of full head emergence were cut at the
154 base, brought back to the laboratory, trimmed to ~50 cm (including spikes), and put
155 into test tubes with water. The spikes were inoculated with conidial suspensions
156 ($1.5\text{-}2.0\times10^5$ conidia/ml), covered with a plastic bag, and incubated in darkness at
157 25°C for 24h. The plastic bag was then removed, and the inoculated spikes were
158 further incubated at 25°C with a 12 h photoperiod. The appearance and development
159 of symptoms were checked daily. When symptom development stopped (7-10 days
160 post-inoculation), infection types were rated with six progressive grades from 0 to 5
161 (Tagle et al. 2015): 0 = no visible infection; 1 = pinhead spots; 2 = small lesions
162 (<1.5mm); 3 = scattered lesions of intermediate size (<3mm); 4 = mixture of green

and white tissues with no apparent browning caused by hypersensitive reaction; and 5 = complete blighting of the spike. Infection types 0 to 3 were considered resistant and 4 to 5 were susceptible. All infection assays at the heading stage were conducted at least twice.

Sequencing of *AVR-Rmg8* homologs. Regions containing *AVR-Rmg8* were amplified from genomic DNA of *Triticum* isolates with primers, AVR-Rmg8_F1 and AVR-Rmg8_R1 (Table 1). PCR products were checked by electrophoresis on a 0.7% agarose gel 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.

Expression analysis of *AVR-Rmg8* in wheat blast field samples from Bangladesh. To confirm the expression of *AVR-Rmg8* in fields, RNA-seq data from symptomatic wheat samples collected in fields in Bangladesh (Islam et al. 2016) were used. Short reads were mapped to the region of *AVR-Rmg8* (Anh et al. 2018) in the Br48 whole genome sequence (Yoshida et al. 2016) using TopHat 2.1.1 (Kim et al. 2013), and the alignments of short read were visualized by IGV 2.4.9 (Thorvaldsdóttir et al. 2013). *PWT3* (Inoue et al. 2017) was used as a negative control.

RESULTS

Preliminary screening with seedling leaves. After screening 520 landraces collected in Eurasia, Africa, and the Americas with *Triticum* isolate Br48, we found 18 resistant accessions (Table 2). To test if the resistance of these accessions is conferred by *Rmg8*, primary leaves of those accessions were inoculated with 200R54 carrying *avr-Rmg8* and 200R54+A8 carrying *AVR-Rmg8*. Interestingly, most (17 of 18) were susceptible to 200R54 and resistant to 200R54+A8, like S615 which carries *Rmg8* alone (Table 2), suggesting that resistance in these accessions was conferred by *Rmg8*. The remaining accession, GR119, was resistant to 200R54, indicating that GR119 carries at least one unknown resistance gene. Against 200R54+A8, GR119 showed complete resistance (infection type 0) that was stronger than the resistance to 200R54 (infection type 1B). However, we could not derive a conclusion on presence/absence of *Rmg8* in GR119 because the difference was quite small.

200R54 is a hybrid derived from a cross, MZ5-1-6 (*Eleusine* isolate) x Br48, so there remained a possibility that the 17 accessions carried additional wheat blast resistance genes corresponding to avirulence genes that 200R54 has not inherited from Br48. To check this possibility, F_2 populations derived from crosses between the 17 accessions and susceptible controls (Hope, CS, or St55) were inoculated with Br48. In all populations resistant and susceptible seedlings segregated in a 3:1 ratio (Table 3), indicating that the resistance of the 17 accessions against Br48 was controlled by *Rmg8* alone.

Identification of *RmgGR119*. When an F_2 population derived from GR119 x

CS was inoculated with Br48, resistant and susceptible seedlings segregated in a 15:1 ratio (Table 4), indicating that the resistance of GR119 against Br48 was controlled by two genes. When the same population was inoculated with 200R54 carrying *avr-Rmg8* (nonfunctional allele), the segregation fitted a 3:1 ratio (Table 4), indicating that GR119 carried an unknown resistance gene other than *Rmg8*. However, there remained a possibility that this resistance gene might correspond to an avirulence gene derived from MZ5-1-6. If this is the case, this should be a gene for resistance to the *Eleusine* isolate, not to the *Triticum* isolate. To exclude this possibility, *AVR-Rmg8* in Br48 was disrupted by the split marker strategy as described in Materials and Methods (Fig. 1A), resulting in a disruptant (Br48ΔA8_d6) in which *AVR-Rmg8* was replaced with the *HPH* gene (Fig. 1B). When the F_2 population from GR119 x CS was inoculated with Br48ΔA8_d6, resistant and susceptible seedlings segregated in a 3:1 ratio (Table 4), indicating that one of the two resistance genes in GR119 against Br48 was *Rmg8*. The new gene detected with Br48ΔA8_d6 was tentatively designated as *RmgGR119*.

Infection assays at the heading stage. To check whether the resistance of GR119 was effective at the heading stage, spikes of IL191 and GR119 were inoculated with Br48, 200R54, 200R54+A8, and Br48ΔA8_d6. Spikes of IL191 were resistant to Br48, susceptible to Br48ΔA8_d6 and 200R54, and again resistant to 200R54+A8 (Fig. 2). This was expected because *Rmg8* had been shown to be effective at the heading stage (Anh et al. 2015). On the other hand, GR119 showed

high resistance to Br48, intermediate resistance to Br48ΔA8_d6 and 200R54, and again high resistance to 200R54+A8 (Fig. 2). Clear differences were recognized in symptoms between Br48 and Br48ΔA8_d6 and between 200R54 and 200R54+A8. These results suggested that (i) the strong resistance of GR119 is effective not only in seedlings but in spikes, and (ii) may be attributed to additive effects of *Rmg8* and *RmgGR119*.

Distribution of avirulence genes corresponding to *Rmg8* and *RmgGR119*. The Kobe University collection of the wheat blast fungus was composed of *Triticum* isolates collected in Brazil in the early period of the wheat blast emergence (1990-1992). Br48 was a part of this collection. To examine whether *Triticum* isolates other than Br48 also carried *AVR-Rmg8*, we selected 13 isolates that carried the Atm or B types of *PWT3* (Inoue et al. 2017) and therefore were virulent on wheat cultivars/accessions irrespective of genotypes at the *Rwt3* locus conditioning the host specificity at the genus level (Inoue et al. 2017). Anh et al. (2015) has already reported that the 13 isolates were all avirulent on S615. They were also avirulent on IL191, another *Rmg8* carrier found in the present study (Table 5). These results suggest that these isolates in the early stage of wheat blast evolution carry functional homologs of *AVR-Rmg8*.

When the *AVR-Rmg8* region was amplified with primers shown in Table 1, fragments with an expected size (1.45kb) were obtained from all of the 13 isolates (Fig. 3A). Sequence analyses revealed that the *AVR-Rmg8* homologs were divided

246 into three types (Fig. 3B). Type eI ("e" stands for eight) represented by Br48
247 (LC223814) were shared by three isolates (Table 5). Type eII had 8 nucleotide and 5
248 amino acid substitutions in comparison with eI (Fig. 3B) and was shared by 9 isolates
249 (Table 5). Type eII' had one nucleotide and one amino acid substitutions in
250 comparison with eII (Fig. 3B) and was found in one isolate (Table 5). There was no
251 correlation between the *AVR-Rmg8* types of these isolates and the degree of their
252 avirulence on IL191 (Table 5).

253 Isolates spreading now in Bangladesh are all carriers of the Atc type of *PWT3*,
254 in which ORF is disrupted through multiple insertions of transposable elements
255 (Inoue et al. 2017). To check whether the Bangladeshi isolates carry and express
256 *AVR-Rmg8*, we used RNA-seq data reported by Islam et al. (2016). Short reads from a
257 symptomatic field sample (wheat plant with blast symptoms) (No.12) collected in
258 Bangladesh during the outbreak of wheat blast in 2016 were mapped to regions of
259 *AVR-Rmg8* and *PWT3* in the whole genome sequence of Br48 (Yoshida et al. 2016).
260 No reads were mapped to the *PWT3* region as expected (Fig. 4B). By contrast, many
261 reads with a 100% match were mapped to the *AVR-Rmg8* region (Fig. 4A). These
262 results indicate that Bangladeshi isolates carry the Br48 type (eI type) of *AVR-Rmg8*
263 and express it during the infection in fields.

264 GR119 was resistant to the 13 isolates collected in Brazil, and its resistance
265 tended to be stronger than IL191 as a whole (Table 5). However, there was no direct
266 way to examine whether these isolates carried an avirulence gene corresponding

RmgGR119 (tentatively designated as *AVR-RmgGR119*) because *AVR-RmgGR119* has not yet been cloned. To obtain a clue to the distribution of *AVR-RmgGR119* in the population of *Triticum* isolates, five isolates were arbitrarily chosen from the 13 isolates, and sprayed onto F_2 populations derived from GR119 x CS. With all isolates resistant and susceptible seedlings segregated in a 15:1 ratio (Table 6), suggesting that these isolates carry *AVR-RmgGR119* in addition to *AVR-Rmg8*.

Development of a selection system for breeding. To introduce *Rmg8* and *RmgGR119* from GR119 to elite varieties simultaneously, progenies carrying both genes must be distinguished from those carrying *Rmg8* alone or *RmgGR119* alone in each backcross generation. To check if it is possible, detached primary leaves of GR119 (*Rmg8*/*RmgGR119*), S615 (*Rmg8*/-), and CS (-/-) were inoculated with Br48 and Br48ΔA8_d6. These cultivar-strain combinations include all of the four cases from the viewpoint of resistance genes involved in the interactions: *Rmg8*+*RmgGR119* (GR119 – Br48), *RmgGR119* alone (GR119 – Br48ΔA8_d6), *Rmg8* alone (S615 – Br48), and none (the others). In this infection assay with detached leaves the effects of *Rmg8* alone and *RmgGR119* alone were intermediate (Fig. 5). Detailed observation revealed that there were some differences between the two genes in their modes of action on fungal infection. In the GR119 − Br48ΔA8_d6 interaction conditioned by *RmgGR119* alone, the number of lesions was much reduced, but once small lesions were formed, they developed to typical diamond-shaped lesions (Fig. 5). In the S615 – Br48 interaction conditioned by *Rmg8* alone, the

number of lesions was not reduced to the same extent as in the case with *RmgGR119*, but lesion development was prevented, resulting in brown flecks (Fig. 5). On the other hand, resistance in the GR119 – Br48 interaction conditioned by both genes was complete with no flecks or lesions (Fig. 5). These results suggest that (i) *Rmg8* and *RmgGR119* act additively in protecting plants against wheat blast and (ii) progenies carrying both genes can be distinguished from others in segregating populations and therefore are amenable to selection during backcrossing.

DISCUSSION

Wheat blast, which first arose in Brazil in 1985, has recently spread to Asia; Bangladesh in 2016 (Islam et al. 2016: Malaker et al. 2016) and India in 2017 (Bhattacharya and Mondal 2017). Duveiller et al. (2011) earlier identified India, Bangladesh, and Ethiopia as areas with high risk of wheat blast outbreaks. Cruz et al. (2016a) suggested that there are significant risks of wheat blast in some areas in U.S, for example, Louisiana, Mississippi, and Florida. Global warming will increase the areas at risk because wheat blast is enhanced by high temperature and humidity. Thus, new resistance genes are needed to control wheat blast in affected countries and to prevent its spread to unaffected countries.

In the present study, we screened 520 landraces of common wheat collected worldwide, and found 18 resistant accessions. This result gave us an initial impression

309 that new resistance genes might be easily found in the population of landraces.
310 However, all 18 accessions recognized *AVR-Rmg8* (Table 2, Fig. 2). Although
311 *AVR-Rmg8* corresponds to two resistance genes *Rmg8* and *Rmg7* (Anh et al. 2018), we
312 infer that the 18 common wheat accessions carry *Rmg8* because *Rmg8* and *Rmg7* were
313 identified in hexaploid wheat and tetraploid wheat, respectively (Anh et al. 2015, Tagle
314 et al. 2015). *Rmg8* seems to be the most common gene among very rare wheat blast
315 resistance genes in common wheat populations. The 18 accessions were from Europe,
316 the Middle East, and West Asia (Portugal, Albania, Ukraine, Russia, Turkey, Iran,
317 Armenia, Azerbaijan, and Tajikistan) (Table 2), where wheat blast has not been
318 reported. It is not clear why *Rmg8* was maintained in common wheat populations in
319 those countries.

320 One of the 18 accessions, GR119 collected in Albania, carried a previously
321 unknown resistance gene in addition to *Rmg8* (Table 4). The new gene was tentatively
322 designated as *RmgGR119*. Br48ΔA8_d6 gave clearer segregation than 200R54 (Table
323 4) probably because the use of Br48ΔA8_d6 excluded effects of minor genes that
324 200R54 inherited from MZ5-1-6. Among wild isolates and hybrid strains of *P. oryzae*
325 preserved in our laboratory, there was no culture virulent on GR119 (data not shown).

326 *Rmg8* and *RmgGR119* acted additively to confer an enhanced level of
327 resistance relative to either gene alone, and the combination conferred complete
328 resistance (Fig. 5). The infection assay with detached leaves made it possible to detect
329 the additive effect more clearly, probably because the leaf detachment compromised

resistance in some way. These two genes seem to act additively even at the heading stage. In an infection assay with spikes, GR119 inoculated with Br48 showed higher resistance than IL191 inoculated with Br48 and GR119 inoculated with Br48ΔA8_d6 (Fig. 2). The resistance of IL191 spikes to Br48 is apparently conditioned by *Rmg8* alone because this resistance is ineffective against Br48ΔA8_d6 (Fig. 2). Also, it is reasonable to infer that the resistance of GR119 spikes to Br48ΔA8_d6 is controlled by *RmgGR119* alone although there is no direct evidence such as infection assay with a double disruptant of *AVR-Rmg8* and *AVR-RmgGR119*. To produce such a disruptant, *AVR-RmgGR119* must be cloned. To clone this gene, a screening of mutants virulent on GR119 is under way.

All Brazilian isolates tested carried *AVR-Rmg8* homologs. They were divided into three types, eI (the Br48 type), eII, and eII' (Fig. 3). We have not performed transformation assay to check whether eII and eII' are functional. However, they are inferred to be functional because the carriers of these types are all avirulent on S615 (Anh et al. 2015) and IL191 (Table 5) carrying *Rmg8* alone. If the eII and eII' types were nonfunctional, (i) S615 and IL191 must carry another resistance gene (Rx), (ii) the eII and eII' carriers must carry an avirulence gene (Ax) corresponding to Rx, and (iii) Br48 (an eI carrier) must carry a nonfunctional allele of Ax or be a non-carrier of Ax; a situation fulfilling all of these requirements seems unlikely. It should be noted that transcripts from the eI type of *AVR-Rmg8* was detected in a symptomatic field sample collected in Bangladesh (Fig. 4). Furthermore, mining of whole genome

sequences of 19 isolates collected in Bangladesh (Soanes et al. 2017) revealed that all of the 19 isolates carried the eI type of *AVR-Rmg8* (data not shown). We suggest that *AVR-Rmg8* is widely distributed in the population of the wheat blast fungus, and in turn that *Rmg8* is effective against a wide range of *Triticum* isolates including Bangladeshi isolates. *AVR-RmgGR119* also seems to be common in *Triticum* isolates (Table 6). Further segregation analyses as shown in Table 6 are needed using *Triticum* isolates from various countries.

Breeding for resistance to wheat blast is an urgent requirement because of the likelihood that wheat blast will spread to other countries in Asia and elsewhere. Although this pathogen has not yet appeared in Japan, a "preventive" breeding program has already begun. *Rmg8* and *RmgGR119* are being introduced into Japanese elite varieties through recurrent backcrossing. In each backcross generation, the *Rmg8*/*RmgGR119* genotype (1/4 of the total) can be selected by taking advantage of the knowledge that the resistance response conferred by the combined genes can be distinguished from either gene alone in detached leaf assays. Clearly, robust breeder-friendly genetic markers for *Rmg8* and *RmgGR119* would be preferred, but these are not yet available. Such markers could then be used in countries where tests with the fungus are not permitted.

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474

TABLE 1. Primers used in this study

Name	Sequence (5'-->3')
Up_F_XhoI	TTTctcgagGAGCACTGAGACTACCTCAAACC
Up_R_HindIII	TTTaagcttCAAAGCAACTGACGCAAAGA
Down_F_BamHI	TTggatccTTATCGACGATTGGGAGAGC
Down_R_SacI	TTgagctcTCAAGCCCAGCTTTAAAATGT
HY	GGATGCCTCCGCTCGAAGTA
YG	CGTTGCAAGACCTGCCTGAA
KO_F	TCCACTCAGCGTCTTCACTT
KO_R	ACATCAATGGTGCAAATGGTG
AVR-Rmg8_F1	CGGGCTGTACAACATTTTCA
AVR-Rmg8_R1	GGAGATTTCACGATAGCAAAGC

TABLE 2. Reactions of seedling leaves of selected landraces to *Triticum* isolate Br48, F_1 culture 200R54, and its transformant with *AVR-Rmg8*.

Code of accession[a]	Origin	Donor[b]	Accession No.	Year of collection	Infection type[c] with Br48 (*AVR-Rmg8*)	200R54 (*avr-Rmg8*)	200R54+A8 (*AVR-Rmg8*)	*Rmg8*[d]
S615	-	K. Tsunewaki	-	-	1-2B	4G	0-1B	+
CS	-	K. Tsunewaki	-	-	5G	5G	5G	-
Hope	-	H. Tsujimoto	-	-	5G	5G	5G	-
St55	-	S. Sakamoto	KU-140	1930	5G	5G	5G	-
IL50	Turkey	KU	KU-10302	1971	0-1B	4G	0-1B	+
IL92	Iran	KU	KU-3282	1956	0-1B	4G	0-1B	+
IL131	Turkey	KU	KU-10316	1971	1B	4G	0-1B	+
IL132	Turkey	KU	KU-10337	1971	1B	4G	0-1B	+
IL186	Armenia	KU	KU-1588	1967	0-1B	4-5G	0-1B	+
IL191	Armenia	KU	KU-1649	1967	0-1B	5G	0	+
CP19	Azerbaijan	VIR	WIR-39310	1950	0-1B	4-5G	0	+
CP20	Azerbaijan	VIR	WIR-39311	1950	0-1B	4-5G	0-1B	+
CP21	Azerbaijan	VIR	WIR-39844	1951	0-1B	5G	0-1B	+
CP26	Azerbaijan	VIR	WIR-40184	1951	1B	4-5G	0	+
CP27	Azerbaijan	VIR	WIR-24592	1928	1B	4-5G	0	+
CP30	Tajikistan	VIR	WIR-24599	1928	0-1B	2-3G	0	+
CP61	Ukraine	VIR	WIR-10124	1924	2-3B	4G	0-1B	+
CP71	Russia	VIR	WIR-23900	1928	0-1B	4-5G	0	+
CP73	Russia	VIR	WIR-23924	1928	0-1B	4-5G	0-1B	+
GR119	Albania	JIC	W7880	1941	0-1B	1B	0	ND
GR246	Portugal	CGN	WAG6097	-	0-1B	3-4G	0	+
GR250	Portugal	CGN	CGN06373	1969	0-1B	3-4G	0-1B	+

[a] S615, CS, and Hope, *T. aestivum* cv. S-615, Chinese Spring, and Hope, respectively; St55, an accession of *T. carthlicum* (6x); the others, local landraces of *T. aestivum*.

[b] KU, Kyoto University (https://shigen.nig.ac.jp/wheat/komugi/strains/queryFormNbrp.jsp);
VIR, The N.I.Vavilov All-Russian Institute of Plant Genetic Resources (http://91.151.189.38/virdb/maindb);
JIC, John Innes Centre (https://www.seedstor.ac.uk/index.php);
CGN, Centre for Genetic Resources, the Netherlands (https://cgngenis.wur.nl/ZoekGewas.aspx?ID=52z2dfrj&Cropnumber=01).

[c] After 5-day incubation at 25°C. 0 = no visible infection; 1 = pinhead spots; 2 = small lesions (<1.5 mm); 3 = scattered lesions of intermediate size (<3 mm); 4 = large typical lesions; and 5 = complete blighting of leaf blades. Brown (B), Green (G) lesions.

[d] +, present; -, absent; ND, not determined.

TABLE 3. Segregation of reactions of seedling leaves to *Triticum* isolate Br48 in F_2 populations derived from crosses between resistant accessions and susceptible lines

	No. of F_2 seedlings				
Cross	R[a]	S[a]	Total	$\chi 2$ (3:1)	P
IL50 × Hope	125	33	158	1.43	0.23
IL92 × St55	117	41	158	0.08	0.78
IL131 × Hope	123	37	160	0.30	0.58
IL132 × Hope	121	37	158	0.21	0.65
IL186 × Hope	112	41	153	0.26	0.61
IL191 × Hope	120	31	151	1.61	0.21
CP19 × Hope	126	34	160	1.20	0.27
CP20 × CS	127	31	158	2.44	0.12
CP21 × Hope	118	40	158	0.01	0.93
CP26 × Hope	116	43	159	0.35	0.55
CP27 × Hope	121	36	157	0.36	0.55
CP30 × Hope	124	35	159	0.76	0.34
CP61 × Hope	105	34	139	0.02	0.88
CP71 × Hope	123	28	151	3.36	0.07
CP73 × Hope	114	44	158	0.68	0.41
GR246 × Hope	120	40	160	0.00	1.00
GR250 × Hope	123	35	158	0.68	0.41

[a] R, resistant (infection type 0 to 5B); S, susceptible (infection type 3G to 5G). Infection types were evaluated after 5-day incubation at 22°C.

TABLE 4. Segregation of reactions of seedling leaves to *Triticum* isolate Br48 with *AVR-Rmg8* , F_1 culture 200R54 with *avr-Rmg8* , and Br48ΔA8 with disrupted *AVR-Rmg8* in F_2 populations derived from GR119 × CS

	Infection type of parents		Number of F_2 seedlings											χ^2	
Strain	GR119	CS	0/1B[a]	2B	3B	4B	5B	3G	4G	5G	R[b]	S[b]	Total	3:1	15:1
Br48	0-1B	5G	196	13	9	0	0	2	4	8	218	14	232	44.5 *	0.02
200R54	1B	5G	32	52	28	9	0	2	8	26	121	36	157	0.36	74.58 *
Br48ΔA8	2B	5G	108	64	14	2	0	4	19	26	188	49	237	2.36	84.19 *

[a] Infection type, after 4-day incubation at 25°C. 0 = no visible infection; 1 = pinhead spots; 2 = small lesions; 3 = scattered lesions of intermediate size (<3mm); 4 = large typical lesion; 5 = complete blighting of leaf blades. Brown (B), Green (G).

[b] R, resistant (Infection type 0 to 5B); S, susceptible (Infection type 3G to 5G).

* Significant at P =0.05

Table 5. Reaction of seedling leaves of representative wheat lines to *Triticum* isolates collected in Brazil

Isolate	Origin	Year of collection	Infection type[a]			*AVR-Rmg8* type[b]
			Hope	IL191	GR119	
Br48	Itapora, Mato Grosso do Sul, Brazil	1992	5G	0-1B	0-1B	eI
Br2	Londrina, Parana, Brazil	1990	5G	0-1B	0-1B	eI
Br3	Londrina, Parana, Brazil	1990	5G	0-1B	0-1B	eI
Br5	Jagupita, Parana, Brazil	1990	5G	3B	1-2B	eII
Br8	B.V. Praiso, Parama, Brazil	1990	5G	0-1B	0	eII
Br46	Rio Brilhante, Mato Grosso do Sul, Brazil	1990	5G	0	0	eII
Br49	Dourados , Mato Grosso do Sul, Brazil	1992	5G	0	0	eII
Br50	Rio Brilhante , Mato Grosso do Sul, Brazil	1992	5G	1B	1B	eI
Br108.1	Assai, Parana, Brazil	1992	5G	2B	1-2B	eII
Br116.5	Santa Mariana, Parana, Brazil	1992	5G	0-1B	0	eII'
Br117.1	Santa Mariana, Parana, Brazil	1992	5G	0	0	eII
Br127.11	Sertanopolis, Parana, Brazil	1992	5G	0	0	eII
Br128.1	Sertanopolis, Parana, Brazil	1992	5G	0	0	eII
Br130.8	Rolandia, Parana, Brazil	1992	5G	3B	1-2B	eII

[a] After 5-day incubation at 25°C. 0 = no visible infection; 1 = pinhead spots; 2 = small lesions (<1.5 mm); 3 = scattered lesions of intermediate size (<3 mm); 4 = large typical lesions; and 5 = complete blighting of leaf blades. Brown (B), Green (G) lesions.

[b] Refer to Fig. 3.

TABLE 6. Segregation of reactions of seedling leaves to *Triticum* isolates collected in Brazil in F_2 populations derived from GR119 × CS

	Infection type of parents		Number of F_2 seedlings											χ^2	
Isolates	GR119	CS	0/1B[a]	2B	3B	4B	5B	3G	4G	5G	R[b]	S[b]	Total	3:1	15:1
Br3	0-1B	5G	88	2	4	0	0	0	1	4	94	5	99	21.0 *	0.24
Br8	0	5G	90	3	0	0	0	1	1	3	93	5	98	20.7 *	0.22
Br50	1B	5G	81	4	4	0	0	0	1	6	89	7	96	16.1 *	0.18
Br117.1	0	5G	88	2	1	0	0	2	0	3	91	5	96	20.1 *	0.18
Br128.1	0	5G	89	4	1	0	0	0	2	3	94	5	99	21.0 *	0.24

[a] Infection type, after 4-day incubation at 25°C. 0 = no visible infection; 1 = pinhead spots; 2 = small lesions; 3 = scattered lesions of intermediate size (<3mm); 4 = large typical lesion; 5 = complete blighting of leaf blades. Brown (B), Green (G).

[b] R, resistant (Infection type 0 to 5B); S, susceptible (Infection type 3G to 5G).

* Significant at P=0.05

Figure legends

Fig. 1. Targeted gene disruption of *AVR-Rmg8*. (A) Split marker strategy used for the deletion of *AVR-Rmg8.* Regions demarked by crossover lines represent the regions used for facilitating homologous recombination. Primers used to amplify targeted fragments are indicated by arrow heads. Truncated (5'HPH, 3'HPH) or complete HPH cassettes are shown as rectangles. Length of the overlapping region was 466 bp. (B) Verification of *AVR-Rmg8* deletion by PCR using primers indicated in A (KO_F and KO_R). PCR fragments of 986bp and 2,059bp were predicted to be amplified from the wild type and mutant genomic DNA, respectively. Marker sizes are shown at the left. Br48, wild-type Br48; Br48ΔA8_d6, a Δ*AVR-Rmg8* mutant (*AVR-Rmg8* disruptant); Br48E_d16, an ectopic mutant.

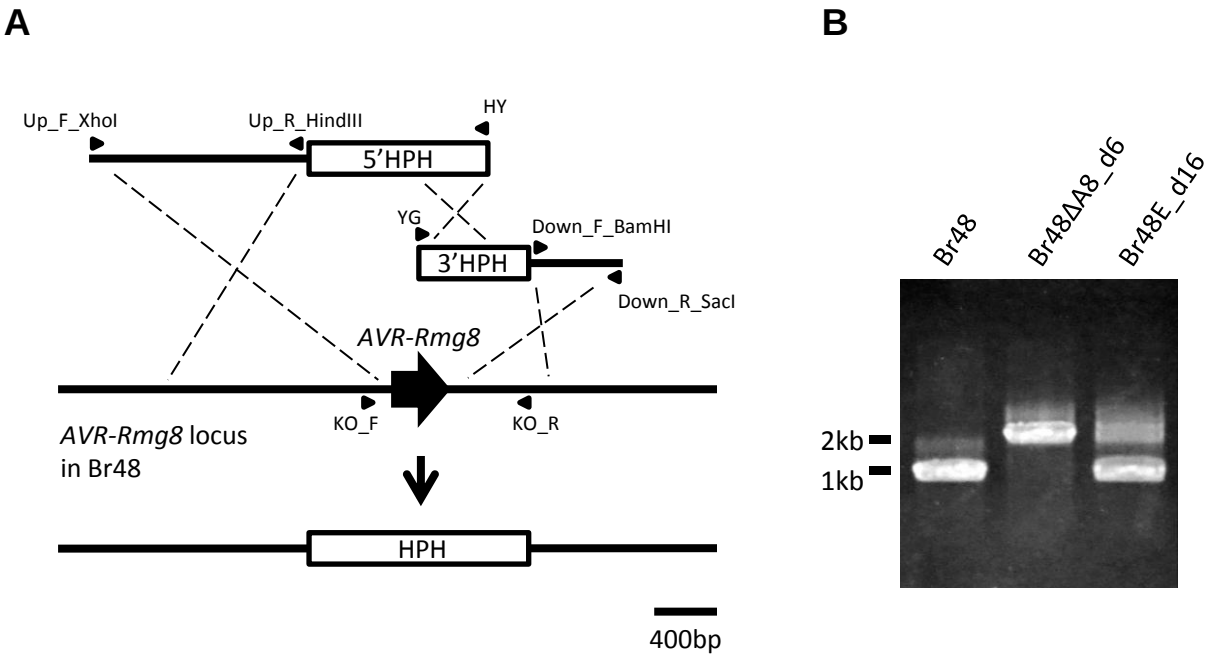
Fig. 2. Reactions of detached spikes of IL191 (*Rmg8/rmgGR119*) and GR119 (*Rmg8/RmgGR119*) to *Triticum* isolate Br48 carrying *AVR-Rmg8*, F_1 hybrid 200R54 carrying *avr-Rmg8*, transformant 200R54+A8 carrying transgene *AVR-Rmg8,* and disruptant Br48ΔA8_d6 carrying disrupted *AVR-Rmg8*, 8 days after inoculation.

Fig. 3. *AVR-Rmg8* homologs in *Triticum* isolates collected in Brazil. A 1.45kb fragment containing *AVR-Rmg8* was amplified (A) and sequenced. Amino acid sequences of three types (eI, eII, eII') detected in these isolates are shown in (B). Refer to Table 5 for the *AVR-Rmg8* type of each isolate.

Fig. 4. The alignment view of short reads from symptomatic field sample No.12 collected in Bangladesh around *AVR-Rmg8* (A) and *PWT3* (B) regions in Br48 genome. Open reading frames of these effector genes are shown as black bars. A black dot in the read alignments indicates one nucleotide insertion against the

reference genome. Read counts in "Coverage" are shown on the right vertical axis.

Fig. 5. Reactions of 14-day-old detached primary leaves of GR119, S615, and CS to Br48 and Br48ΔA8_d6 (*AVR-Rmg8* disruptant), after 5-day inoculation at 25˚C. Square brackets indicate genotypes of the wheat lines. Resistance genes conferring resistant reactions are shown in red.



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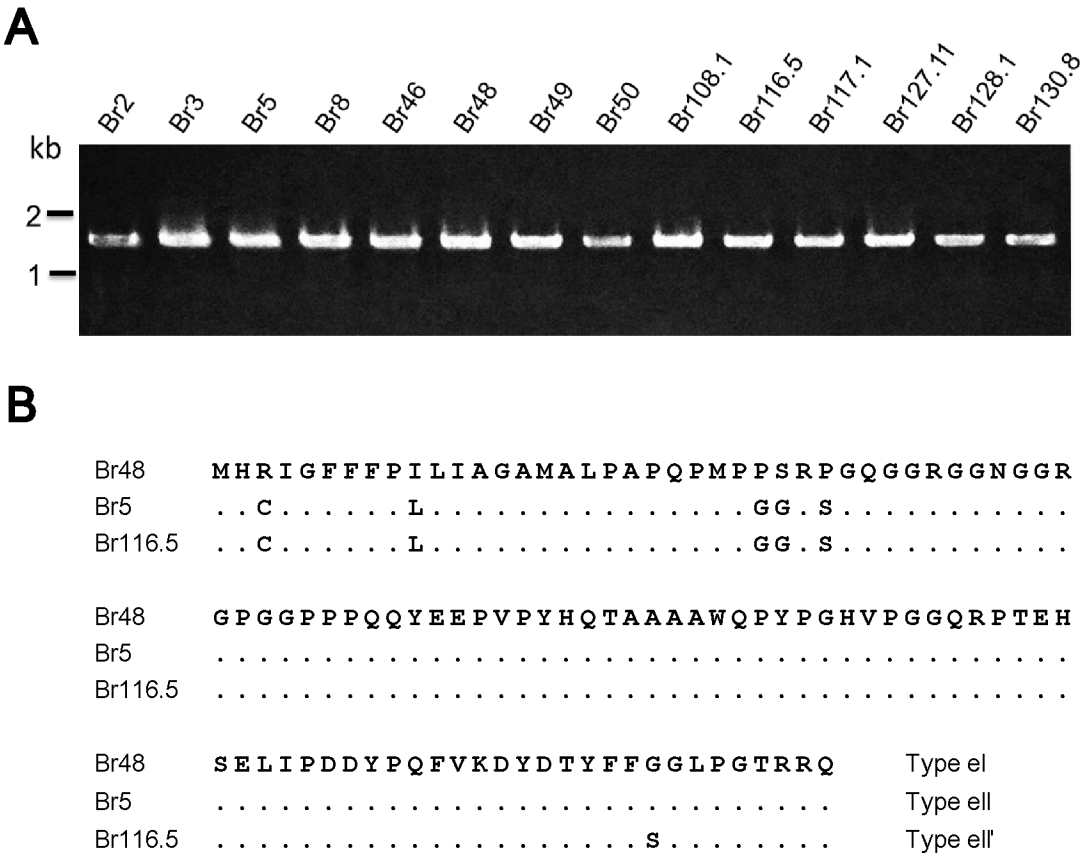

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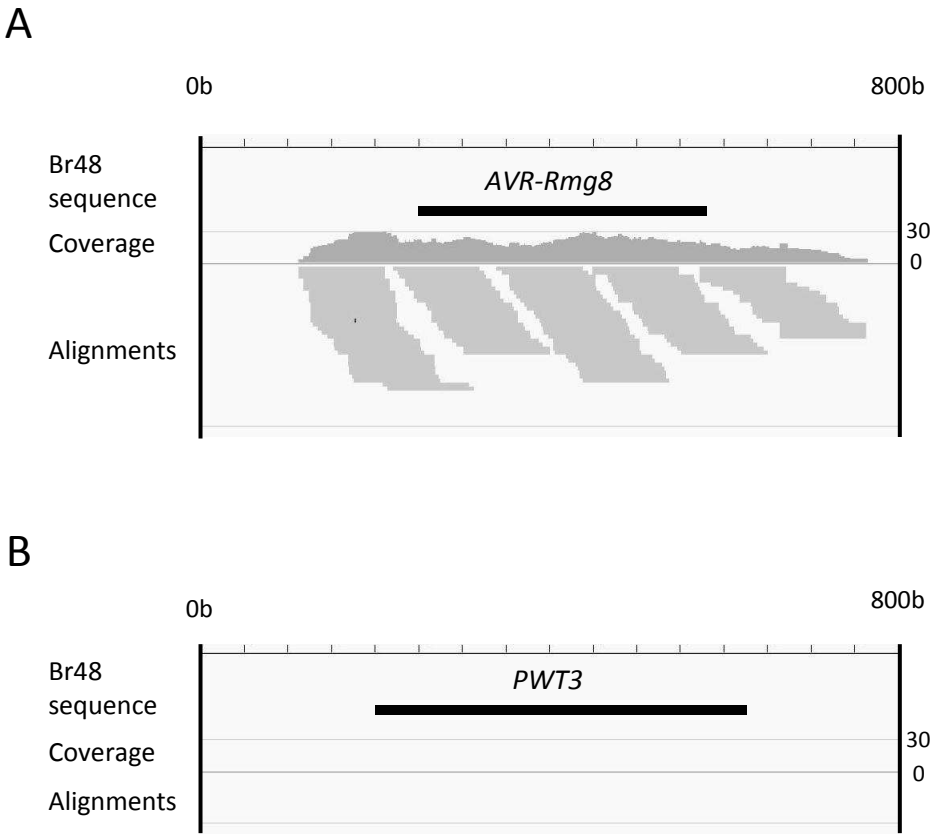

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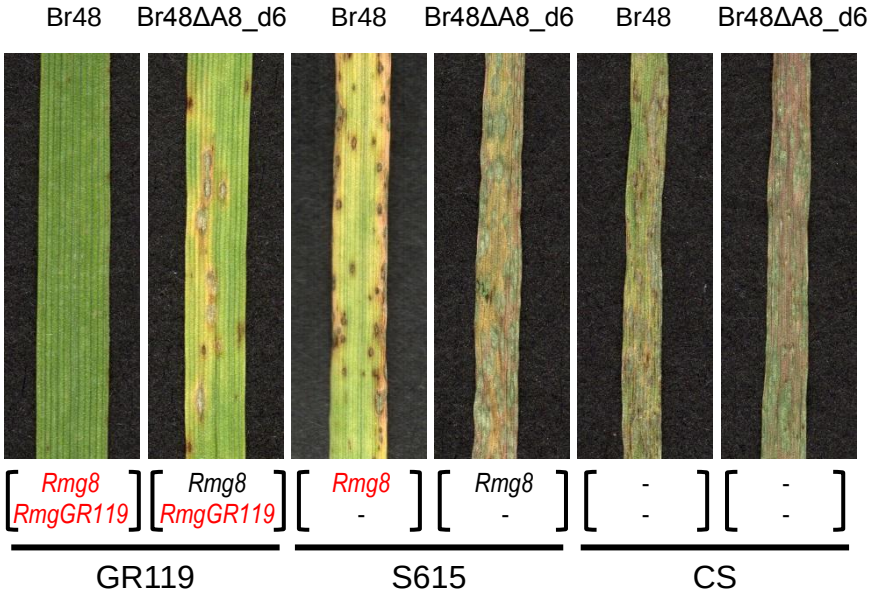

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