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Doctoral dissertation

**Identification and utilization of resistance genes
against the wheat blast fungus in common wheat**

コムギいもち病抵抗性遺伝子の同定と利用

June, 2018

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Chapter1

General Introduction

Plant disease disturbs growth and development of plants and causes various symptoms such as necrosis, stunting, wilting, and so on. Plant diseases caused by biotic agents, such as bacteria, fungi and viruses, are called infectious diseases while those caused by abiotic factors, such as inappropriate temperature, air pollution, or fungicides, are called noninfectious diseases.

According to the report of FAO, there are about 795 million people who are still unable to have enough food to support their daily life in 2015 and we need additional food production by 50% till 2030, and 70% by 2050, respectively. More than 10% of potential food production has been lost due to plant diseases every year. (FAO 2000; Strange 2005) There are some famous examples of famines in the history. For example, potato has been the main food resource for European peoples between 1750 and 1850. In 1844, however, an outbreak of potato late blight was caused by *Phytophthora infestans* in Ireland, which resulted in death of many people or immigration to U.S. (Kamoun et al. 2015; Zadoks 2008). Downy mildew of grape caused by *Plasmopara viticola* resulted in a loss of production and decrease of grape quality since it became widely distributed in 19th century (Amati et al. 1996; Reuveni and Reuveni 1995; Kamoun et al. 2015). Brown spot of rice caused by *Bipolaris oryzae*, which happened in Bangladesh, caused 90% yield losses and notable Bengal famine in 1942 (Nghiep and Gaur 2004; Kumar et al. 2011). A huge decline of food production caused by plant disease is emphasized by pathologists. To solve the

problem brought by plant diseases, many solutions have been developed and used such as cultural control and pesticide applications (Strange 2005). An alternative, and probably the most effective, low-cost, and environment-friendly way for disease management is breeding of resistant cultivars.

Resistance of plants against pathogens has been divided into two categories. If all or most accessions/cultivars of a plant species cannot be infected by a pathogen, this type of resistance is called nonhost resistance. By contrast, if at least some accessions or cultivars of a plant species are infected by a pathogen, the resistance shown by the other accessions/cultivars are called host resistance (Antonovics et al. 2012). Considering data on characteristics of various types of host-parasite interactions, Heath (1981) proposed a generalized concept of host-parasite specificity. In her concept, host-parasite specificity is divided into two levels, plant species specificity and cultivar specificity. In plant species specificity compatibility is specific, and resistance involved (nonhost resistance) is genetically complex. In cultivar specificity, incompatibility is specific, and resistance involved (host resistance) follows gene-for-gene concept which was first proposed by Flor (1956). The gene-for-gene concept is a fundamental theory explaining a mode of genetic interactions between plant cultivars and pathogen races. It indicates that, for each resistance gene (R gene) in a host, there is a corresponding avirulence gene (AVR gene) in the parasite. When a resistance gene matches its corresponding avirulence gene, defense reactions are activated, resulting in host resistance.

Molecular studies on plant-pathogen interactions have elucidated that plants have their own immunity system. Boller and He (2009) suggested that the first layer of this system is based on recognition of pathogen/microbe-associated molecular patterns (PAMPs or MAMPs) by pattern recognition receptors (PRR) at the plant's cell surface. This is called PAMP-triggered immunity (PTI). The second layer is based on recognition of effectors, which is produced by pathogens to inhibit PTI, by resistance genes. This is called effector-triggered immunity (ETI) and follows the gene-for-gene concept. ETI usually results in hypersensitive response (HR) with cell death at infection site. HR usually involves two phases. The first is an increase of extra cellular calcium and hydrogen ions influx into the triggered cell. The second is production of reactive oxygen species to go through HR with killing invading pathogens or preventing them from spreading to health cells. Some pathogen-related (PR) protein could also bring about defense responses. (Orlandi et al. 1992; Mehdy 1994).

Pyricularia oryzae (syn. *Magnaporthe oryzae*) is one of the most important fungal pathogens that cause blast diseases on monocot species. Rice blast disease is now found in more than 85 countries and threaten the world's food security. Incidences of blast is related to environmental conditions such as high humidity. *P. oryzae* prefers such circumstances and often been found in lowland, irrigated or deep-water rice field (Latif et al. 2011; Sharma et al. 2012). Yield losses caused by rice blast disease could be up to 50%, and annual losses of rice production are comparable to foods for more than 60 million people. (Scheuermann et al. 2012). In

addition to rice, *P. oryzae* causes blast diseases on about 50 species of grass family, including wheat, barley, millet, and oat. It can be divided into several host-specific subgroups, including *Avena* pathotype pathogenic on oat (*Avena sativa*), *Panicum* pathotype pathogenic on common millet (*Panicum miliaceum*), *Setaria* pathotype pathogenic on foxtail millet (*Setaria italica*), and *Oryza* pathotype pathogenic on rice (*Oryza sativa*), based on host specificity, mating ability, and genetic relatedness (Urashima et al. 1993; Kato et al, 2000)

In 1985 a new pathotype of *P. oryzae* - *Triticum* pathotype pathogenic on wheat - was first observed in Brazil, and caused a serious outbreak of wheat blast. Then this new pathogen 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). In 2017 wheat blast spread to India, the second largest wheat-producing country in the world. 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).

The evolution of new races of rice blast fungus was supposed to be caused by mutations of AVR genes, such as base substitutions, insertion of transposons, or deletions. Also, the chromosomal position of AVR genes were considered to be associated with the frequency of deletions. For example, when AVR genes were located on chromosomal regions close to telomeres, they were prone to be suffered

from chromosomal rearrangements including translocation and deletion, resulting in increased mobility (Chuma et al. 2011; Seidl and Thomma 2014; Orbach et al. 2000; Robberecht et al. 2013). Recently, similar mechanisms were suggested to be involved in the evolution of the host genus-specific pathotypes of the blast fungus. Yoshida et al. (2016) revealed that *Oryza* isolates lost the largest number of genes from the genome while *Triticum* and *Avena* isolates have suffered from the smallest number of gene losses compared with other host-specific pathotypes. Inoue et al. (2017) revealed that the *Triticum* pathotype evolved through loss of function of an AVR gene, *PWT3*, caused by the insertion of transposable elements and inheritance of a chromosomal segment carrying a nonfunctional allele.

Wheat is one of the most important and widely cultivated crop plants worldwide. In 2016, wheat is the second most-produced crop after maize with its production reaching to 749 million tonnes (<http://www.fao.org/faostat/zh/#data/QC>). As mentioned above, wheat blast, first detected in Brazil, spread from South America to Bangladesh in 2016, and attacked approximately 15% of cultivated wheat area with 100% yield losses in heavily damaged areas (Callaway 2016; Islam et al. 2017; Cruz and Valent 2017). This was the first outbreak of wheat blast in Asia or Eurasia (Callaway E. 2016). There is a possibility that wheat blast may soon become a pandemic disease with serious effects on wheat production worldwide. To prevent this serious disease, cultivars with durable resistance are needed. The first step for breeding for resistance is to identify resistance genes against the wheat blast fungus. At present, however, there are only one resistance gene that may be used for breeding – *Rmg8* reported by

Anh et al. (2015). In this study, therefore, I aimed at identification of novel resistance genes. First, we screened 520 local landraces of common wheat accessions collected worldwide, and found a highly resistant accession, GR119, in a collection from Albania. I found that GR119 carried two resistance genes, *Rmg8* and a new resistance gene. Second, I established a system for introducing these genes into elite varieties through infection assay in each backcross generation.

Chapter 2

Identification of a new resistance gene against *Triticum* isolates of *Pyricularia oryzae* in a common wheat landrace

2.1 Introduction

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). 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°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°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°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 this chapter I aimed to identify new resistance genes in common wheat accessions collected worldwide.

2.2 Material and Methods

2.2.1 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.

2.2.2 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 F₂ populations. 200R54 (an F₁ 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.

2.2.3 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 *Hind*III and ligated with pSP72-HPH (Inoue et al. 2016) digested with *Xho*I and *Hind*III, 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).

2.2.4 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.

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

2.2.5 Infection assays with spikes.

For inoculation at the heading stage, accession IL191 (collected in Armenia) was used as a control carrying *Rmg8* instead of S615 because its spikes emerged simultaneously with those of GR119. Seeds of IL191 and GR119 were sown in soil in large pots (30cm in diameter) and grown in a greenhouse for 5 months. Stems with spikes at the stage of full head emergence were cut at the base, brought back to the laboratory, trimmed to ~50 cm (including spikes), and put into test tubes with water. The spikes were inoculated with conidial suspensions ($1.5\text{-}2.0 \times 10^5$ conidia/ml), covered with a plastic bag, and incubated in darkness at 25°C for 24h. The plastic bag was then removed, and the inoculated spikes were further incubated at 25°C with a 12 h photoperiod. The appearance and development of symptoms were checked daily. When symptom development stopped (7-10 days post-inoculation), infection types

were rated with six progressive grades from 0 to 5 (Tagle et al. 2015): 0 = no visible infection; 1 = pinhead spots; 2 = small lesions (<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.

2.2.6 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.

2.2.7 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.

2.3 Result

2.3.1 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, F₂ 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.

2.3.2 Identification of *RmgGR119*.

When an F₂ 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 F₂ 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*.

2.3.3 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*.

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

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

GR119 was resistant to the 13 isolates collected in Brazil, and its resistance

tended to be stronger than IL191 as a whole (Table 5). However, there was no direct 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 F₂ 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*.

2.3.5 Additive effects of *Rmg8* and *RmgGR119*.

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.

2.4 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

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

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

Rmg8 and *RmgGR119* acted additively to confer an enhanced level of resistance relative to either gene alone, and the combination conferred complete resistance (Fig. 5). The infection assay with detached leaves made it possible to detect 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 Br48 Δ A8_d6 (Fig. 2). The resistance of IL191 spikes to Br48 is apparently conditioned by *Rmg8* alone because this resistance is ineffective against Br48 Δ A8_d6 (Fig. 2). Also, it is reasonable to infer that the resistance of GR119 spikes to Br48 Δ A8_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.

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, F₁ culture 200R54 and its transformant with *AVR-Rmg8*.

Code of accession ^a	Origin	Donor ^b	Accession No.	Year of collection	Infection type ^c with			<i>Rmg8</i> ^d
					Br48 (<i>AVR-Rmg8</i>)	200R54 (<i>avr-Rmg8</i>)	200R54+A8 (<i>AVR-Rmg8</i>)	
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=52z2dfjr&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 F₂ populations derived from crosses between resistant accessions and susceptible lines

Cross	No. of F ₂ seedlings			χ^2 (3:1)	P
	R ^a	S ^a	Total		
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*, F₁ culture 200R54 with *avr-Rmg8*, and Br48ΔA8 with disrupted *AVR-Rmg8* in F₂ populations derived from GR119 × CS

Strain	Infection type		Number of F ₂ seedlings											3:1	15:1
	of parents		0/1B ^a	2B	3B	4B	5B	3G	4G	5G	R ^b	S ^b	Total		
	GR119	CS													
Br48	0-1B	5G	196	13	9	0	0	2	4	8	218	14	232	44.51	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, Parana, 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 F₂ populations derived from GR119 × CS

Isolates	Infection type		Number of F ₂ seedlings											3:1	15:1
	of parents		0/1B ^a	2B	3B	4B	5B	3G	4G	5G	R ^b	S ^b	Total		
	GR119	CS													
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 $P=0.05$

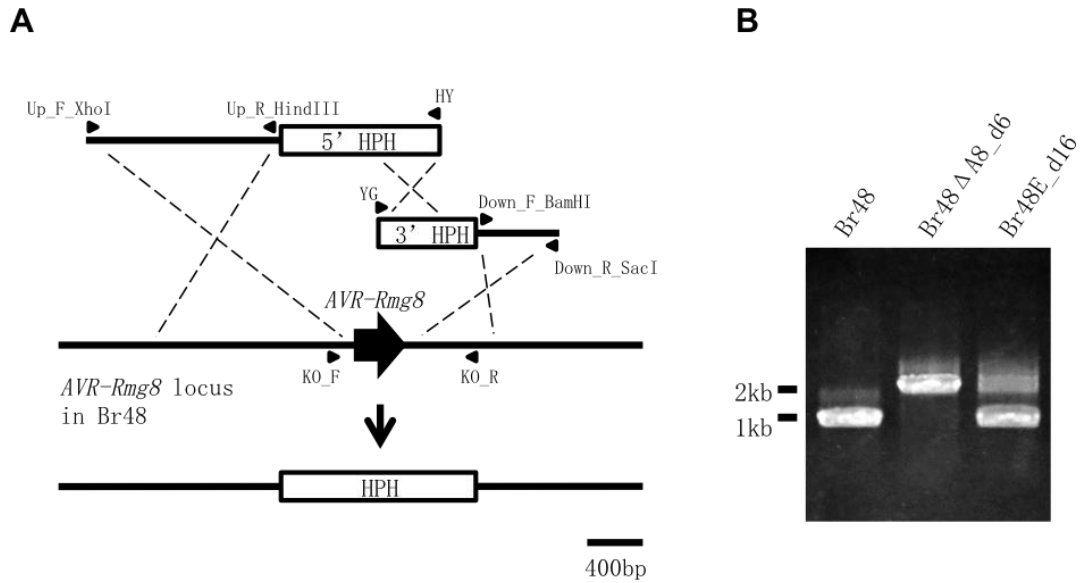


Fig. 1. Targeted gene disruption of *AVR-Rmg8*. (A) Split marker strategy used for the deletion of *AVR-Rmg8*. Regions demarked by crossover lines represent the regions used for facilitating homologous recombination. Primers used to amplify targeted fragments are indicated by arrow heads. Truncated (5'HPH, 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.

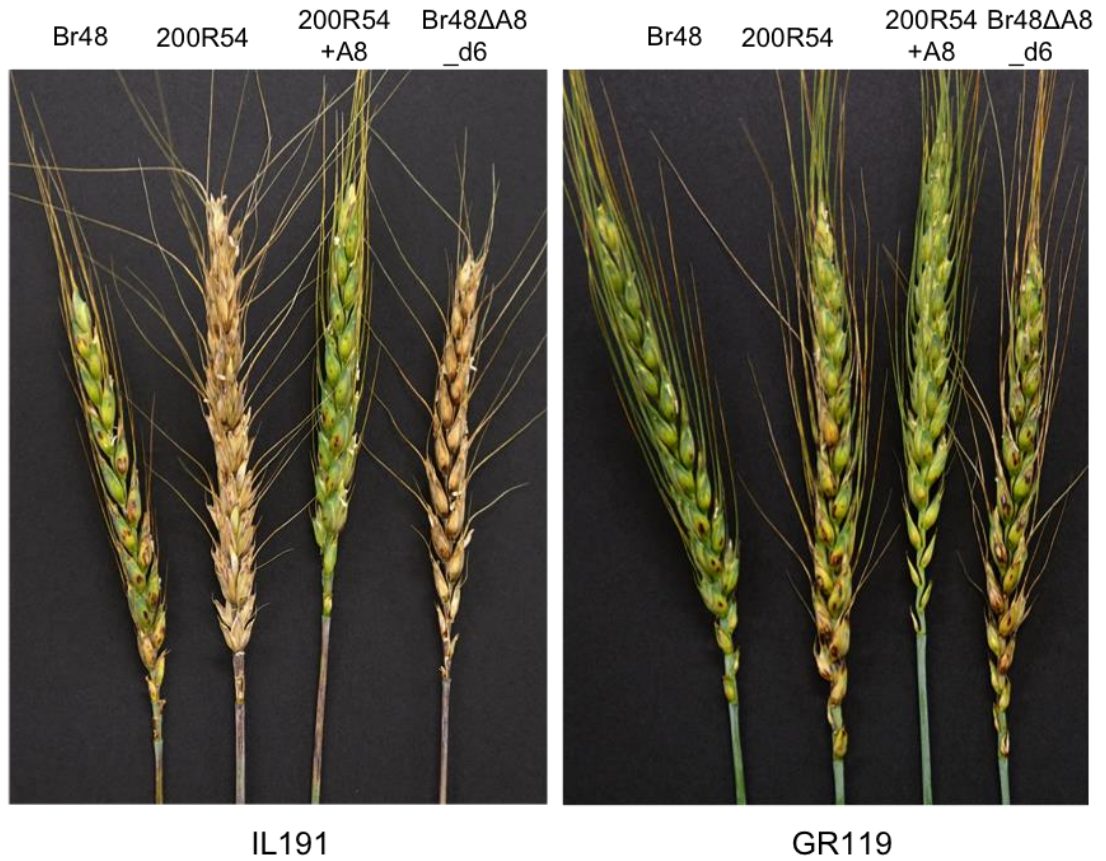


Fig. 2. Reactions of detached spikes of IL191 (*Rmg8/rmgGR119*) and GR119 (*Rmg8/RmgGR119*) to *Triticum* isolate Br48 carrying *AVR-Rmg8*, F₁ hybrid 200R54 carrying *avr-Rmg8*, transformant 200R54+A8 carrying transgene *AVR-Rmg8*, and disruptant Br48ΔA8 carrying disrupted *AVR-Rmg8*, 8 days after inoculation.

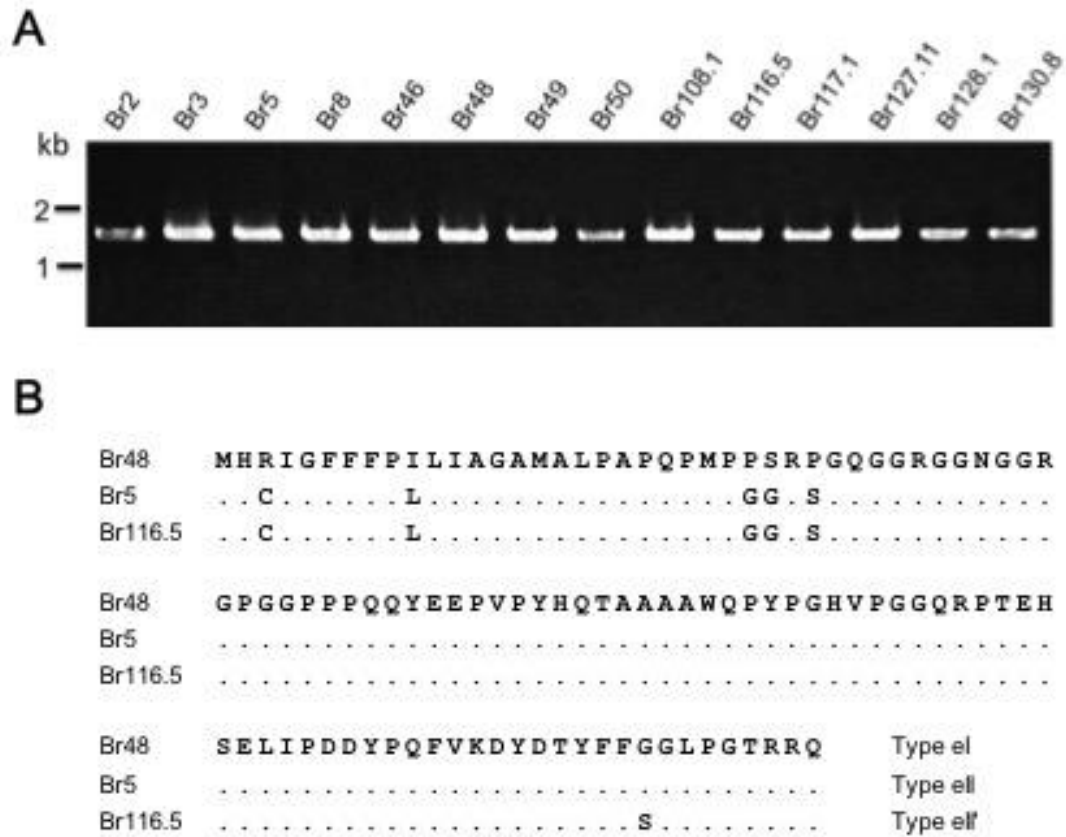


Fig. 3. *AVR-Rmg8* homologs in *Triticum* isolates collected in Brazil. A 1.45kb fragment containing *AVR-Rmg8* was amplified (A) and sequenced. Amino acid sequences of three types (eI, eII, eII') detected in these isolates are shown in (B). Refer to Table 5 for the *AVR-Rmg8* type of each isolate.

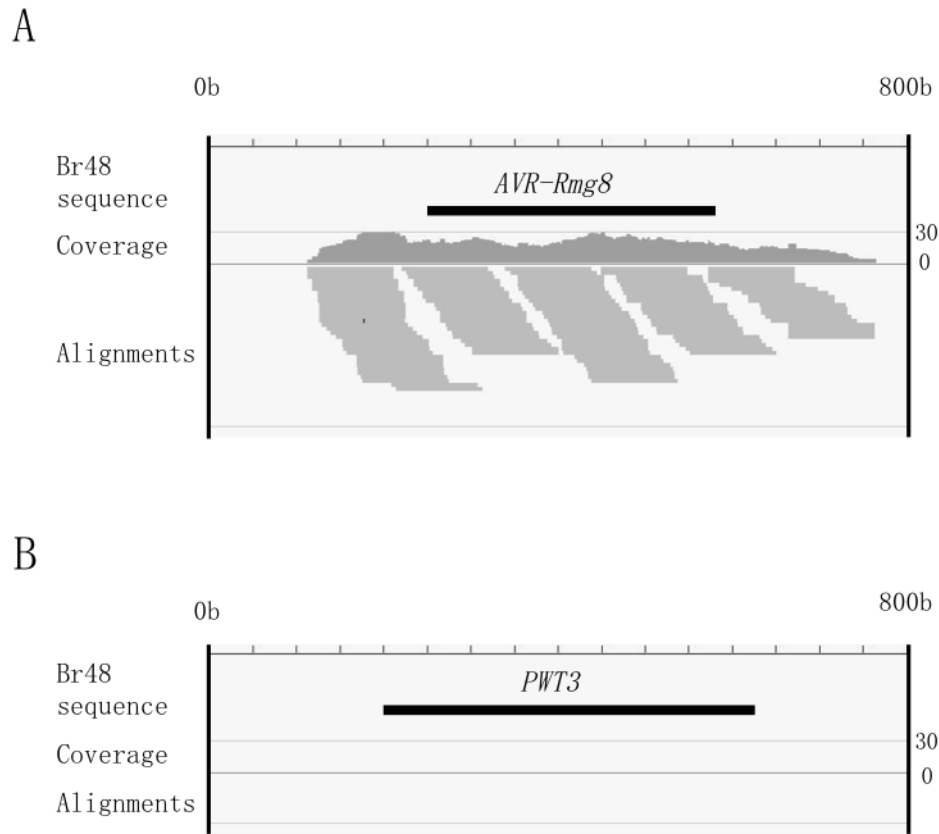


Fig. 4. The alignment view of short reads from symptomatic field sample No.12 collected in Bangladesh around *AVR-Rmg8* (A) and *PWT3* (B) regions in Br48 genome. Open reading frames of these effector genes are shown as black bars. A black dot in the read alignments indicates one nucleotide insertion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

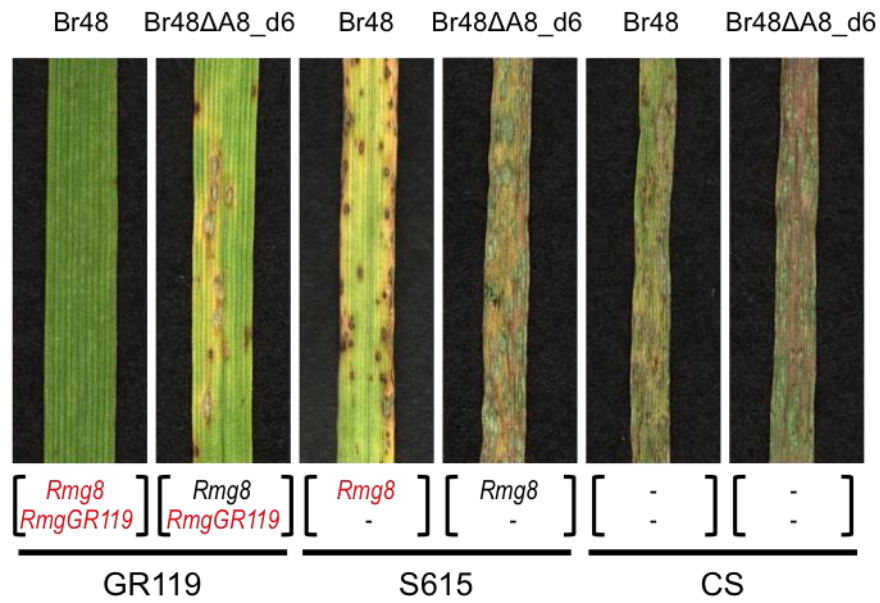


Fig. 5. Reactions of 14-day-old detached primary leaves of GR119, S615, and CS to Br48 and Br48ΔA8_d6 (*AVR-Rmg8* disruptant), after 5-day inoculation at 25°C. Square brackets indicate genotypes of the wheat lines. Resistance genes conferring resistant reactions are shown in red.

Chapter 3

Establishment of a system for selecting carriers of two resistance genes in backcrossing

3.1 Introduction

The final goal of breeding for resistance is to produce cultivars with broad-spectrum and durable resistance. Broad-spectrum resistance refers to the resistance against multiple pathogens (Wisser et al. 2005). Durable resistance refers to resistance that remains effective over a prolonged period of widespread use under conditions conducive to disease. There are some examples of the success of resistance breeding in controls of plant diseases. Forty years ago, powdery mildew was a serious disease of barley and wheat in the United Kingdom, but its risk has recently been reduced largely by using varieties with mildew resistance (HGCA 2014).

In this chapter, we aimed to introduce the two resistance genes, *Rmg8* and *RmgGR119*, of GR119 into a Japanese elite variety, ‘Chikugo-izumi’. To make it possible, individuals with both genes must be distinguished and selected in each backcross generation. We tested two methods, double inoculation of a first leaf and inoculation of secondary and third leaves with different fungal strains. The result

indicated that the double inoculation made it easier to differentiate genotypes of backcross progenies.

3.2 Materials and Methods

3.2.1 Plant materials

Plant material used were two BC₂F₁ populations derived from backcrosses with *Triticum aestivum* cv. Chikugo-izumi (ChI), which is recommended variety in Fukuoka prefecture and Nagasaki prefecture but susceptible to the wheat blast fungus. One population was ChI x (ChI/GR119//Ch-I) containing *Rmg8* and *RmgGR119*, while the other was ChI x (ChI/(S615 x Sch))//ChI containing *Rmg8* alone. Sch is an abbreviation of *T. aestivum* cv. Shin-chunaga carrying no resistance genes. As a starting material for producing backcross populations containing *Rmg8* alone, I used a susceptible F₃ line derived from S615 x Sch (Anh et al. 2015) instead of S615, because it was expected to inherit the early-heading characteristics from Sch, and therefore to be easily crossed with ChI. These lines were produced in collaboration with Dr. M. Fujita, Institute of Crop Science, National Agriculture and Food Research Organization, Japan. The pedigree of ChI x (ChI/GR119//Ch-I) is shown in Fig. 6. Briefly, F₁ derived from ChI x GR119 (*Rmg8/RmgGR119*) was backcrossed with ChI, resulting in a BC₁F₁ population. A BC₁F₁ individual carrying both genes was selected through double inoculation described below. Then, ChI was crossed with the BC₁F₁ individual, resulting in the BC₂F₁ population. ChI x (ChI/(S615 x Sch))//ChI was

produced through similar procedures.

3.2.2 Fungal materials

Br48 was used as a representative *Triticum* isolate. To identify individuals carrying *RmgGR119* alone, Br48 Δ A8_d6 (an *AVR-Rmg8* disruptant of Br48) was used. They were maintained on barley seed media under dry condition in a refrigerator. Barley seeds with these strains were transferred to a potato dextrose agar (PDA) slant in a test tube just before use, and incubated at 25°C.

3.2.3 Infection assay

BC₂F₁ seeds were pregerminated on moistened filter paper for 24h, sown in jiffy pots filled with soil (Sakata Prime Mix, Sakata, Yokohama) and grown at 22°C. Conidia for inocula were produced as described by Tagle et al (2015). The produced conidia were suspended in water at 1×10⁵ spores/ml. Tween 20 (0.01%) was added to the suspension just before spray.

3.2.3.1 Double inoculation of primary leaves

Primary leaves of 14-day-old seedlings were detached and fixed onto a hard plastic board with their bottoms inserted into water in test tubes as described in Chapter 2. After their top half was covered with an aluminum foil, Br48 was sprayed onto the bottom half. The inoculated leaves were incubated in a humid box at 25°C in darkness. After 24 hours, they were taken out from the box, and transferred to an

incubator at 25°C. After leaf surfaces were dried, their bottom half was covered with an aluminum foil, and Br48ΔA8_d6 was sprayed onto the uncovered top half. The inoculated leaves were again incubated in a humid box at 25°C in darkness. After 24 hours, they were taken out from the box, transferred to an incubator with a 12h photoperiod of fluorescent lighting, and incubated further at 25°C.

3.2.3.2 Inoculation of secondary and third leaves with different fungal strains

Secondary leaves were detached, fixed onto a hard plastic board with their bottoms inserted into water in test tubes as described above, and inoculated with Br48 (first inoculation). The inoculated secondary leaves were kept in a humid box in darkness at 25°C for 24 hours, then returned to an incubator with fluorescent lighting. Three to four days after the first inoculation (when the fourth leaves completely developed), the third leaves were detached, fixed, and inoculated with Br48ΔA8 (second inoculation). The inoculated third leaves were kept in a humid box as described above, then returned to a dry condition with fluorescent lighting, and incubated for additional 3-4 days at 25°C.

3.3 Results

3.3.1 Double inoculation of primary leaves

To choose individuals that possess two resistance genes, bottom and top halves of primary leaves were inoculated with Br48 and Br48ΔA8, respectively. Phenotypes

of individuals derived from ChI x (ChI/GR119//Ch-I) were divided into four types (Fig. 7). The A type showed strong resistance to Br48 and intermediate resistance to Br48ΔA8, indicating that it possesses two resistant genes, *Rmg8* and *RmgGR119*. The B type showed resistance to Br48 but susceptible to Br48ΔA8_d6, indicating that it possesses *Rmg8* alone. The C type showed intermediate resistance to both, indicating that it possesses *RmgGR119* alone. The type D were susceptible to both, indicating that it possesses no resistance genes.

In the BC₂F₁ individuals derived from ChI x (ChI/(S615 x Sch)//ChI), phenotypes were divided into 2 types because this population inherited only one resistant gene, *Rmg8*, from S615. The type E was resistant to Br48 but susceptible to Br48ΔA8_d6, indicating it possesses *Rmg8*. The type F was susceptible to both, indicating that it does not carry any resistant genes.

3.3.2 Inoculation of secondary and third leaves with different fungal strains

In this alternative method the second and third leaves of each individual were inoculated with Br48 and Br48ΔA8_d6, respectively, with a 4-day interval. In individuals derived from ChI x (ChI/GR119//Ch-I), No.1, 2, 4, 5, 6, 8, 11, 14, 15, 17, 18, and 20 showed strong resistance to Br48 while No. 1, 2, 3, 5, 7, 10, 14, 15, 16, 17, 19, and 20 showed intermediate resistance to Br48ΔA8_d6 (Fig. 8). Considering these results, I chose No. 1, 2, 5, 14, 15, 17 and 20 as individuals possessing *Rmg8* and *RmgGR119*. Similarly, No. 1, 4, 7, 8, 10, 11, 12, 13, 15, 16 and 17 derived from ChI x (ChI/(S615 x Sch)//ChI) were chosen as individuals possessing *Rmg8* alone (Fig. 9).

However, discrimination among those types in this assay was relatively difficult compared with the double inoculation.

3.4 Discussion

GR119 possess two resistance genes, *Rmg8* and *RmgGR119*, whose combination confers strong resistance to *Triticum* isolates both at the seedling and heading stage. To introduce these two genes into elite varieties simultaneously, the *Rmg8/RmgGR119* genotype must be selected in every backcross generation. In this chapter I tested two methods to select the *Rmg8/RmgGR119* genotype. One was double inoculation in which Br48 and Br48 Δ A8_d6 were sprayed onto different parts of the same leaf with a one-day interval. The other was inoculation of the second and third leaves with Br48 and Br48 Δ A8_d6 with a four-day interval. In both methods, it was easy to distinguish different phenotypes against Br48 because *Rmg8* confers strong resistance at the seedling stage. When inoculated with Br48 Δ A8, individuals possessing *RmgGR119* alone was expected to show an intermediate resistance. However, infection types against Br48 Δ A8 in the second/third leaf assay were sometimes not so clear, and their genotypes were difficult to determine. Consequently, I conclude that double inoculation give clearer results for distinguishing genotypes of individuals rather than the second/third leaf assay. It is not clear why the second/third leaf assay showed ambiguous reactions against Br48 Δ A8_d6. One possibility is that, when detached, the texture of the second and third leaves may not have fully developed and weaker than the primary leaves.

Although this pathogen has not yet appeared in Japan, I started introduction of *Rmg8* and *RmgGR119* into a Japanese elite variety, Chikugo-izumi, 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.

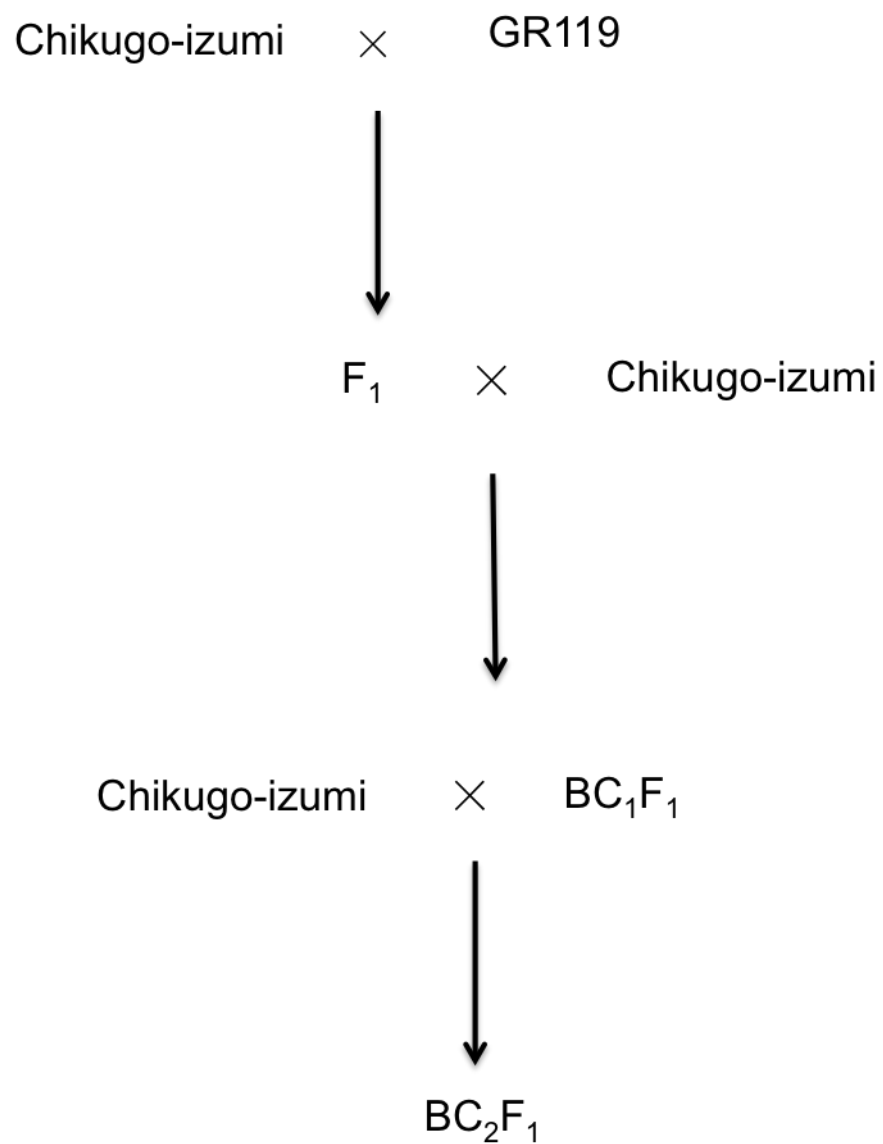


Fig. 6. Pedigree of BC₂F₁ populations derived from backcrosses of GR119 with cv. Chikugo-izumi (ChI).

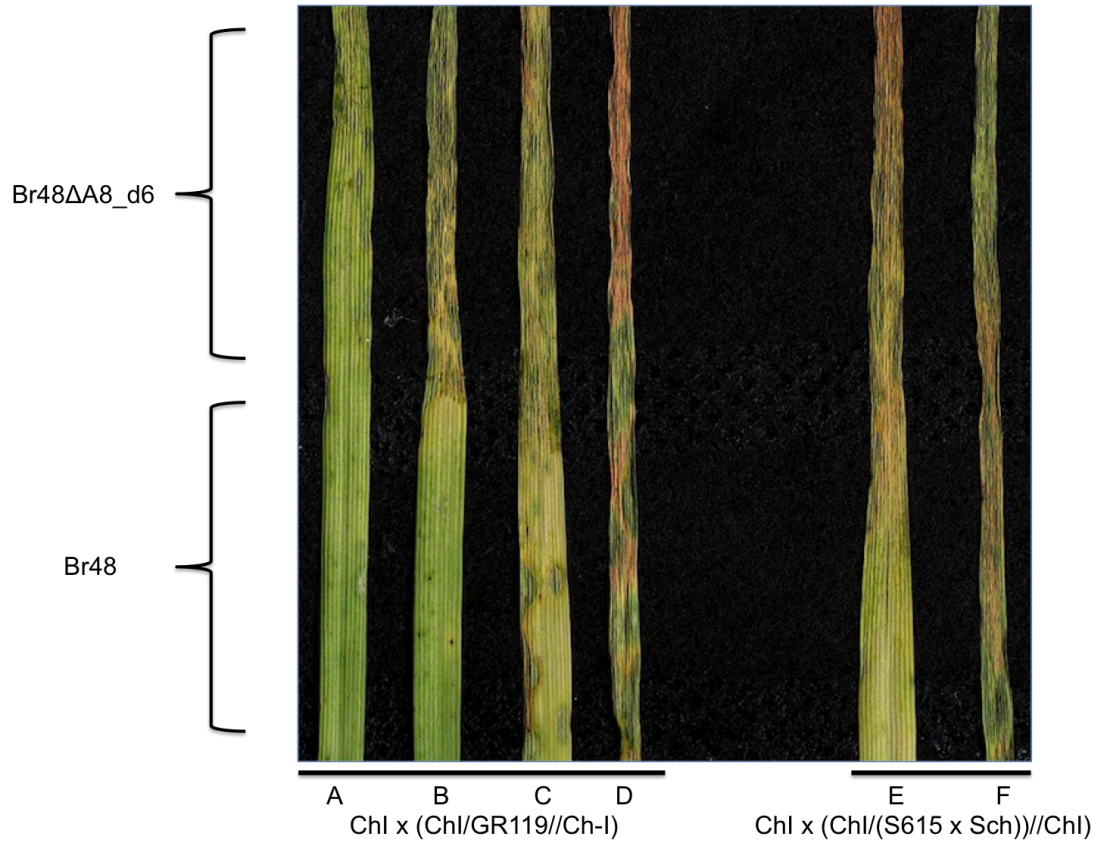
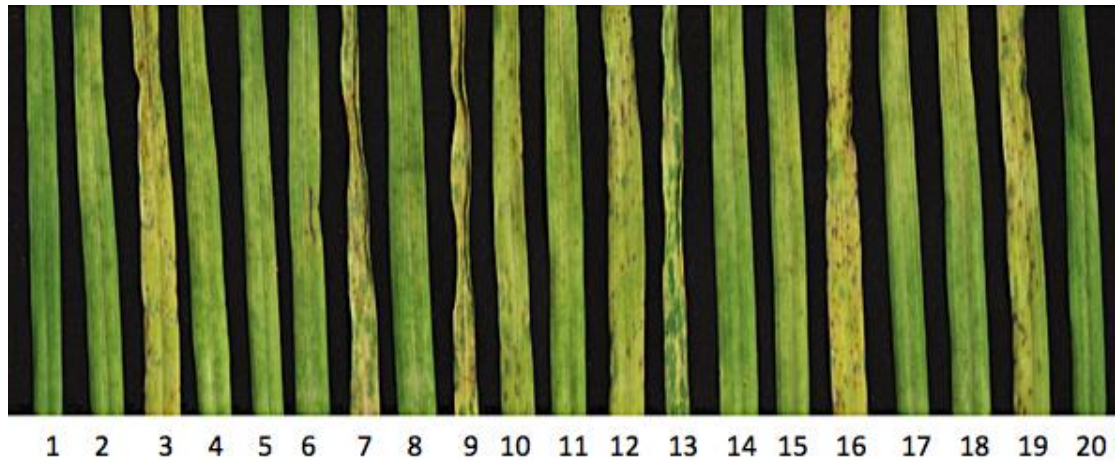


Fig.7. Reactions of 14-day-old detached primary leaves of representative BC₂F₁ individuals inoculated with Br48 (the bottom half) and Br48ΔA8_d6 (the top half), 4 days after the first inoculation. Genotypes of A, B, C, D (ChI x (ChI/GR119//Ch-I)) and E, F (ChI x (ChI/(S615 x Sch))//ChI) were concluded to be *Rmg8/RmgGR119*, *Rmg8/rmgGR119*, *rmg8/RmgGR119*; *rmg8/rmgGR119*, *Rmg8/rmgGR119*, and *rmg8/rmgGR119*, respectively.

Br48



Br48 Δ A8_d6

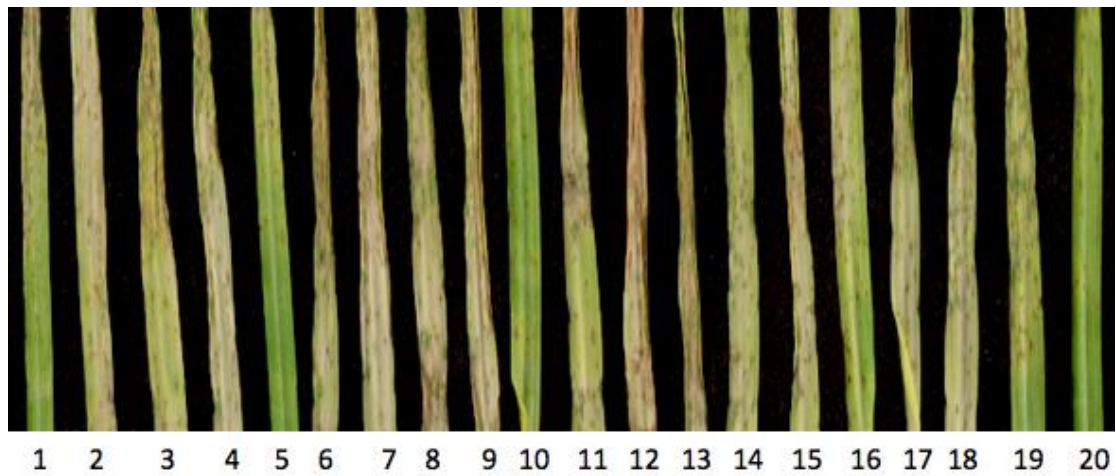


Fig. 8. Reactions of 20 BC₂F₁ seedlings derived from ChI x (ChI/GR119//Ch-I) to Br48 and Br48 Δ A8_d6, 4 days after inoculation. Secondary and third leaves were detached from 20 and 24 days after sowing, and used for inoculation with Br48 and Br48 Δ A8_d6, respectively.

Br48



Br48ΔA8_d6

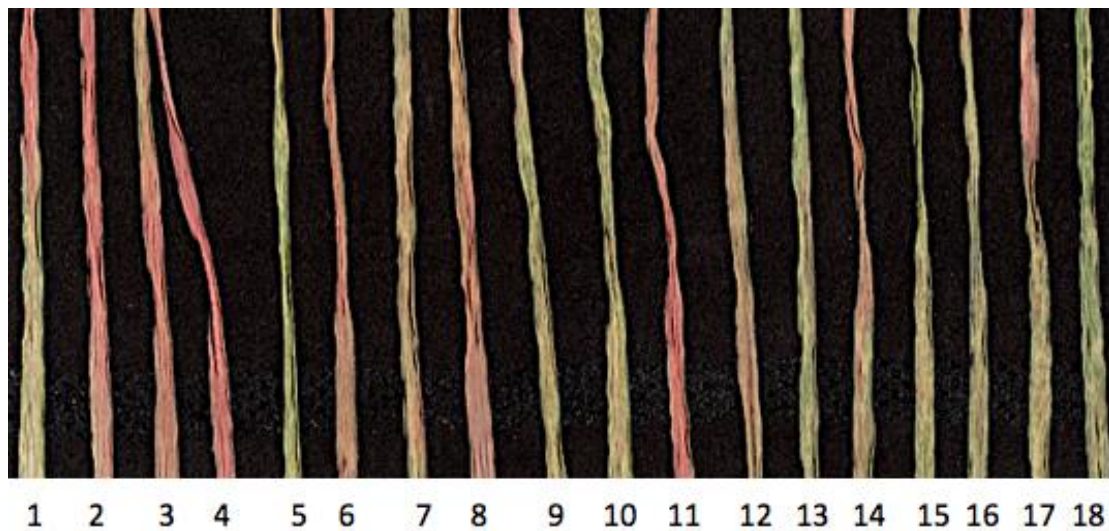


Fig. 9. Reactions of 18 BC₂F₁ seedlings derived from ChI x (ChI/(S615 x Sch))//ChI to Br48 and Br48ΔA8_d6, 4 days after inoculation. Secondary and third leaves were detached from 20 and 24 days after sowing, and used for inoculation with Br48 and Br48ΔA8_d6, respectively.

Chapter 4

General Discussion

Pyricularia oryzae is well known as a pathogen of rice, but recently evolved a new pathotype, *Triticum* pathotype pathogenic on wheat, through host jump (Inoue et al 2017). The new pathotype spread to Asia and is now becoming a pandemic disease causing a serious damage on the food security of the world.

In this study, I found 18 common wheat accessions showing strong resistance to Br48 (Table 2). An infection assay with 200R54 lacking *AVR-Rmg8* and its transformant carrying *AVR-Rmg8* suggested that the resistance of 17 accessions among the 18 accessions was controlled by *Rmg8* alone (Table 2, 3). Genetic analyses of the remaining one accession, GR119, revealed that this accession carries two resistance genes, *Rmg8* and a new R gene designated as *RmgGR119* (Table 4). These genes showed different modes of actions, and their combination gave complete, strong resistance to GR119 (Fig. 5).

GR119 showed strong resistance to 14 *Triticum* isolates collected in Brazil (Table 5). F₂ seedlings from GR119 x CS inoculated with Brazilian isolates segregated in a 15:1 ratio, suggesting that both resistance genes are effective against wheat blast isolates in Brazil. Actually, all of the 14 Brazilian isolates carried *AVR-Rmg8* homologs (Fig. 3). Sequence analyses revealed that those homologs were

divided into 3 types, eI, eII, eII', based on nucleotide and amino acid sequences. Although all of these homologs are inferred to be functional, their function should be confirmed by introducing them into 200R54. Interestingly, a preliminary infection assay with spikes by a colleague in our laboratory suggested that the avirulence conferred by the eII type may be vulnerable to high temperature (28°C) compared with the avirulence conferred by the eI type, and that the eI type carriers (e.g. Br48) showed remarkable avirulence even at the high temperature (personal communication). It should be noted that Bangladeshi isolates are all carriers of the eI type of *AVR-Rmg8*. Thus, we suggest that GR119 should show strong resistance to Bangladeshi isolates even at the high temperature and at the heading stage.

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 order to detect individual possess both of *Rmg8* and *RmgGR119*, we tested two methods and showed that the double inoculation gave clearer results. One reason may be attributable to the textures of leaves. Another may be that, in this assay, infection types against the two fungal strains can be observed simultaneously on the same leaves. I expect that Chikugo-izumi carrying *Rmg8* and *RmgGR119* will be available in Japan within a few years.

Summary

Triticum isolates of *Pyricularia oryzae*, a causal agent of wheat blast, has spread to Asia and caused serious production losses in Bangladesh and India. So, in this study, I aimed to find new resistance genes against the wheat blast fungus, and to introduce them to a Japanese elite variety.

First, in a primary screening of 520 common wheat accessions, we found 18 accessions resistant to the wheat blast fungus. Among them, 17 accessions possessed *Rmg8*, a resistance gene previously identified in common wheat cultivar S-615. The remaining accession, GR119, showed strong resistance to Br48, a *Triticum* isolate collected in Brazil.

Second, genetic analysis of wheat F₂ population suggested that GR119 possessed 2 resistance genes. One was *Rmg8* while the other was a new gene, which was designated as *RmgGR119*. These two genes acted additively in protecting plants against wheat blast, and therefore, GR119 showed strong resistant to Br48 both at seedling and heading stage.

Third, all Brazilian *Triticum* isolates tested carried *AVR-Rmg8* homologs. They were divided into three types, eI (the Br48 type), eII, and eII'. Furthermore, analysis of short reads and genome sequence data indicated that Bangladeshi isolates carried the eI type of *AVR-Rmg8*. These results suggest that *Rmg8* is effective against a wide range of wheat blast isolates.

Last, I compared two methods to select individuals possess *Rmg8/RmgGR119*. Both methods made it possible to distinguish the four genotypes, but double inoculation gave clearer results. This method can be used for choosing individuals that possess the two genes in each backcross generation.

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