Identification of a resistance gene against the wheat blast fungus in common wheat and cloning of its corresponding avirulence gene

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いもち病抵抗性遺伝子の普通系コムギにおける同定と

それに対応する非病原力遺伝子のクローニング

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CHAPTER I

General Introduction

Plant diseases are caused by living agents or nonliving environmental factors. The living agents (pathogens) include fungi, bacteria, mycoplasmas, spiroplasmas, viruses, viroids, nematodes, protozoans, and parasitic higher plants (Agrios 1988). Each has a unique mode of pathogenicity. Among those agents, fungi cause a majority of plant diseases and threaten agricultural production all over the world.

Among integrated pest management practices, use of resistance varieties is one way to restrict the growth and development of a specified pathogen or the damage they cause. Resistance of plants to pathogen has been conventionally classified into host and non-host forms. If at least some accessions or cultivars of a plant species is susceptible to a pathogen, the plant species is called host-plant, and resistance shown by other accessions/cultivars is called host-resistance. Host resistance is divided into two categories: race-specific type and race-nonspecific type. Race-specific resistance is highly effective, but specific to only some pathogen genotypes (race, strains), and known to follow the gene-for-gene concept (Flor 1956). Race-nonspecific resistance is weak but effective to the whole pathogen genotype. By contract, if all accessions/cultivars of a plant species is resistant to a pathogen, the plant species is called nonhost, and the resistance involved is called non-host resistance. Heath (1985) suggested that nonhost resistance to fungi may be under a complex genetic control and often involve a variety of protection factors that individually may segregate within the species without compromising overall resistance. Mechanisms of host and non-host resistance are considered to be basically different (Heath 1980). The expression of both host and nonhost resistance involve many inducible defense responses that can be caused by parasite-specific or nonspecific signals.

To find the most suitable breeding methods and selection methodology, understanding the type of resistance and interactions between plant and pathogen are necessary.

Plants use their innate immunity system in reaction to a wide range of pathogenic microorganisms. Their innate immune system can be divided into two layers including pathogen/microbe-associated molecular pattern (PAMP/MAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Liu et al. 2013; Dodds and Rathjen 2010). Recognition of PAMPs by the corresponding pattern recognition receptors (PRR) in plants lead to PTI, a plant basal defense (Nurnberger and Brunner 2002; Medzhitov and Janeway 1997). It mediates a relatively weaker immune response with a broad-spectrum against pathogens. PTI is suppressed by effectors produced by pathogens. To recognize these effectors, plant evolved corresponding resistance proteins, leading to ETI (Jones and Dangl 2006). Hypersensitive response (HR), a form of localized programmed cell death (PCD) is usually accompanied with ETI (Dangl et al. 1996; Keen et al. 1993).

According to this definition, PTI is multigenically controlled while ETI is controlled by gene-for-gene interactions, which was established by Flor (1971). His study showed that the relationship between flux and its fungal pathogen *Melampsora lini* was controlled by a paired cognate gene. Mechanisms of this interaction was first explained with a simple model that a recognition of a fungal elicitor (encoded by an avirulence allele) by a specific receptor (encoded by R allele) in the plant host leads to HR and plant resistance (Keen 1990).

Blast caused by the filamentous fungus *Pyricularia oryzae* Cavara (*Magnaporthe oryzae* B. C. Couch) (Couch et al. 2002) is one of the most devastating plant diseases of monocot species worldwide (Ou 1980). *P. oryzae* is composed of several genus-specific subgroups or pathotypes (Kato et al. 2000), such as the *Oryzae* isolates pathogenic on rice (*Oryzae sativa*), *Setaria* isolates pathogenic on foxtail millet (*Setatia italica*), *Eleusine*

isolates pathogenic on finger millet, and *Triticum* isolates pathogenic on wheat (*Triticum aestivum*). This pathogen attacks all stages of crops, and symptoms appear on leaves and spikes.

In rice, blast disease occurs in at least 85 countries (Greer and Webster 2001) and all foliar tissues are subject to infection. Especially, infection of the panicle leads to complete loss of grains. Although using host resistance is the most economically, efficiency and environmental friendly method to manage this disease, the fungus overcomes the resistance quickly, and the resistant cultivars typically become ineffective within 2–3 years (Ou 1985; Zeigler et al. 1994). In wheat, *Triticum* isolates also significantly reduces wheat yields and grain quality (Urashima et al. 1993). Therefore, the development of the blast resistant cultivars is necessary.

The first step for developing blast-resistant wheat varieties is identification of resistance genes (Urashima et al. 2004). In our previous study seven blast R genes have been identified in wheat. The first R gene designated *Rmg1* (*Rwt4*) was effective against an *Avena* isolate but not against *Triticum* isolates (Takabayashi et al. 2002). *Rmg4* and *Rmg5* conditioned resistance to a *Digitaria* isolate, but not to *Triticum* isolates (Nga et al. 2009). *Rmg6* was effective against a *Lolium* isolate but not against *Triticum* isolates (Vy et al. 2014). These four R genes governed resistance to appropriate subgroups. On the other hand, the other three genes were effective against isolates of an appropriate subgroup (*Triticum* isolates), including *Rmg2* and *Rmg3* identified in common wheat cultivar 'Thatcher' (Zheng et al. 2008) and *Rmg7* identified in tetraploid accessions, St24, St17, and St25 (Tagle et al. 2015). These genes may be useful for wheat breeding program.

Today, many strategies for breeding for resistance have been developed, but often such attempts have been negated after a few years of commercial production by pathogen races that overcame the resistance. This "breakdown of resistance" was caused by

mutations of avirulence (AVR) genes, leading to loss of recognition by the corresponding resistance (R) gene. The mutation includes insertion of transposons into AVR genes or promoter sequence, point mutations, deletion, or translocation of AVR genes (Chuma et al. 2011)

If stable or durable resistance genes can be selected in advance, it will be useful for resistance breeding. As mentioned above, cultivar specificity is controlled by gene-for-gene interactions between R genes and their corresponding AVR genes (Flor 1971). This relationship has been found in many plant-pathogen systems including biotrophic and hemibiotrophic fungi, nematodes, bacteria, and viruses (Keller et al. 2000). Based on this relationship, some methods have been proposed to predict durability of R genes. One approach is to evaluate fitness cost imposed by mutation of AVR genes (Vera Cruz et al. 2000; Leach et al. 2001). If an AVR gene encodes a virulence effector whose loss of function causes a significant fitness reduction, its corresponding R gene would be durable. Another is to estimate stability of AVR genes based on their chromosomal location and molecular structure around them (Chuma et al. 2011; Tosa and Chuma 2014). If an AVR gene is located on a region with a stable structure, its corresponding R gene would be more durable than genes located on unstable regions. In plant – virus pathosystems, Janzac et al. (2009) proposed that durability of R genes could be predicted by estimating selective constraints on amino acid substitutions in viral avirulence factors. This may be regarded as a method for evaluating fitness cost that has been imposed on AVR factors.

In this study, I first analysed an AVR gene – R gene pair involved in the genus-specific parasitism of *P. oryzae*. During this analysis, I happened to find a resistance gene that may have been hidden during the coevolution of plants and parasites. Second, I tried to identify a new resistance gene that may be useful for breeding of resistant wheat

cultivars. To evaluate its durability, I also identified and cloned an AVR gene corresponding to the resistance gene.

CHAPTER II

Identification and characterization of a hidden resistance gene in tetraploid wheat

2.1. Introduction

As we mentioned before, the use of resistant wheat cultivars is essential to control blast disease. The highest levels of resistance are in general conferred by one or a few major genes that are efficient only toward avirulent *P. oryzae* isolates. To date, significant progress has been achieved through the genetic analysis of wheat resistance and *P. oryzae* avirulence.

The cultivar specificity of the *Oryza* isolates of *Pyricularia oryzae* has been analyzed by many researchers. Kiyosawa (1974) identified 13 rice genes conditioning the resistance to races of the *Oryza* type (rice pathogen). Silue et al. (1992) showed that the cultivar specificity between rice cultivars and races of *Oryza* isolates was conditioned by gene-for-gene relationships. Bryan et al. (2000) established an interaction between resistance gene *Pi-ta* and its corresponding avirulence gene *Avr-pita* at the molecular level.

To elucidate genetic mechanisms controlling the species specificity in *P. oryzae*, our laboratory produced two hybrid populations of *P. oryzae*. One was derived from a cross between *Triticum* isolate Br48 and a *Setaria* isolate GFSI1-7-2. Using this population, Murakami et al. (2000) showed that the specific parasitism of the parental isolates on wheat is conditioned by two loci, *Pwt1* and *Pwt2*. The other hybrid population was derived from a cross between Br48 and an *Avena* isolate, Br58. Using this F_1 population, Takabayashi et al. (2002) identified two other loci, *Pwt3* and *Pwt4*, conditioning the avirulence of the *Anena* isolate on wheat. They designated avirulence/virulence alleles at these loci as *PWT3/pwt3* and *PWT4/pwt4*, respectively, and

suggested that a gene-for-gene interaction underlies the avirulence of the *Avena* isolate on wheat.

The initial objective of this chapter was to identify the putative resistance gene (*Rwt3*) corresponding to *PWT3,* which is assumed to be a fundamental gene that ubiquitously controls avirulence of *Avena* isolates on wheat. To establish the functionality of *PWT3*, attempts were made to produce a near isogenic strain of *Triticum* isolate Br48 that carries *PWT3* by continuous backcrosses. However, we found a new fungal gene associated with avirulence and mycelial color. If this is an avirulence gene under the control of gene-for-gene interactions, there should be its corresponding resistance gene. I found this "hidden" resistance gene, and designated it as *RmgTd*(*t*). Genetic behavior of these unique "complementary" genes is reported here.

2.2. Materials and methods

2.2.1. Fungal materials

Parental isolates of *P. oryzae* were *Avena* isolate Br58 (*MAT1-2*) and *Triticum* isolate Br48 (*MAT1-1*), which were isolated from oat and wheat, respectively, in Brazil in 1990s. Genotypes of Br58 and Br48 are [*PWT3*; *PWT4*] and [*pwt3; pwt4*], respectively. They were maintained on sterilized barley grains under dry conditions at 4°C for long-term storage at the Laboratory of Plant Pathology, Kobe University, as described previously (Hirata et al. 2007). They were transferred to a potato dextrose agar slant (PDA, 24g of potato dextrose broth powder, Difco, Maryland, and 15g of agar powder, Nacalai Tesque, Kyoto, in 1L of water) in a test tube and incubated at 25°C. Slant cultures, 1 week to 3 months old, were used for experiments.

2.2.2. Plant materials

Plant materials used were *Triticum aestivum* cv. Norin 4 (N4) (AABBDD), *T. dicoccoides* accession KU109 (Tat4) (AABB), and *T. paleocolchicum* accession KU156 (Tat14) (AABB). These cultivars/accessions were provided by Dr. K. Tsunewaki and Dr. S. Sakamoto, emeritus professors at Kyoto University. Tat14 was used as a susceptible control. These seeds were sown in vermiculite in a seedling case (5.5 x 15 x 10 cm), and grown at 22° C with 12h photoperiod for 7 days.

2.2.3. Genetic crosses of fungal strains

Pyricularia isolates/strains were crossed on an oatmeal agar medium (20 g of oatmeal, 10 g of agar, and 2.5 g of sucrose in 500 ml of water) as described by Murakami et al. (2000). Resulting progenies were transferred to and grown on sterilized barley seeds in vials at 25°C for 1 month and kept in a case with silica gel at 5°C (Fig. 2.1)**.**

2.2.4. Infection assay

Wheat seeds were pregerminated on moistened filter papers for 2 days, sown in vermiculite (Asahi-Kogyo, Okayama, Japan), supplied with liquid fertilizer in a seedling case (5.5 by 15 by 10 cm), and grown at 22°C in a controlled environment room with a 12-h photoperiod of fluorescent lighting for 7 days.

Br48, Br58 and their progenies were used for pathogenicity tests. Mycelial plugs of fresh slant cultures (1 week to 1 month old) were grown on oatmeal agar medium in petri dishes and incubated in darkness at 22°C for 7 days. Aerial mycelia on the 7-day old cultures were washed off by rubbing mycelial surfaces with cotton balls. The colonies were exposed to near-ultraviolet light (360 nm, 40W) at 22°C for 4 days to induce sporulation (Murakami et al. 2000). The conidia produced were suspended in distilled water and adjusted to a concentration of 1-2 × 10^5 spores/ml. Ten milliliters of the spore suspension with 0.01% Tween 20 were sprayed on the adaxial surface of primary leaves in a plastic case with an air compressor. The cases were sealed to maintain high humidity and

placed in darkness for 24 h. Then, they were transferd to a growth chamber and incubated further at 22°C with a 12-h photoperiod. Four to five days after inoculation, symptoms were evaluated based on the color of lesions and the affected area on the leaves. The affected area was rated by 6 progressive grades from 0 to 5: 0 = no visible evidence of infection; 1, pinpoint spots; 2, small lesion (<1.5mm); 3, lesions with an intermediate size (<3mm); 4, large typical lesions; and 5, complete blighting of leaf blades. These lesions were classified into two categories on the basis of their color; that is, brown (B) and green (G). Infection types were represented by the combination of the 0-5 score and the color code. Infection types 0, 1B, 2B, 3B, 4B, 5B were considered as resistant, and infection type 3G, 4G and 5G as susceptible. Sometimes lesions of size 2 and 3 were accompanied by both brown (B) and green tissues (G). These types of lesions, designated as 2BG and 3BG, respectively, were considered resistant according to Hyon et al. (2012). Each infection assay was repeated at least three times. The inoculation test was repeated at least three times.

For analysis of F_2 and F_3 seedlings derived from a cross between Tat4 x Tat14, 40 F_2 seeds and twenty seeds of each of 86 F_3 lines were sown, grown at 22°C for 7 days, then, inoculated with a conidial suspension (1-2 x 10^5 spores/ml) and incubated at 22°C for four to five days. The infection assay was repeated twice. Numbers of seedling showing each infection type, which were obtained from the two replications, were summed up, and the resulting frequency distribution was subjected to a χ^2 test.

2.2.5. Molecular mapping with SSR markers

For mapping of *PWT3* marker loci were amplified from genomic DNA in a reaction mixture containing 20-50 ng genomic DNA, 1x Quick Taq® HS DyeMix (Toyobo), 0.5µM each of forward and reverse primers. All SSR markers reported by Zheng et al. (2008) and some makers designed using the database of *M. oryzae* 70-15

genomic sequence (*http://www.broadinstitute.org/annotation/fungi/magnaporthe/*) were tested with a routine PCR program: 2 min at 94°C; 35 cycles of 30s at 94°C, 30s at 55°C, and 1 min/kb at 68°C .

For mapping of *RmgTd(t)* twenty seeds of each of 86 F_3 lines derived from a cross Tat4 x Tat14 were sown in vermiculite in a seedling case, and grown at 22°C in a controlled-environment room with a 12-h photoperiod of fluorescent lighting for 7 days. The twenty leaves of each of 86 F_3 lines were harvested for DNA extraction.

Bulked segregant analysis (BSA) (Michelmore et al., 1991) was used to identify markers linked to the resistance gene. Resistant and susceptible DNA bulks were prepared by mixing equal amounts of genomic DNA from 10 resistant and 10 susceptible homozygous F_3 lines, respectively.

Simple sequence repeat (SSR) loci were selected at equal intervals from genetic maps of common wheat constructed by Somers et al. (2004) and Torada et al. (2006). They were amplified from genomic DNA of two parents (Tat4 and Tat14), the resistant bulk, and the susceptible bulk.

Amplified products were fractionated by electrophoresis through 6% polyacrylamide gels and stained using SILVER SEQUENCE DNA Staining Reagents (Promega Corp., Madison, WI). Molecular weight size markers V and VIII (Biehringer Mannheim, Germany) were used as a standard size marker. Markers showing possible links to the resistance gene in BSA were further subjected to segregation analysis with individuals comprising the mapping population.

Segregation data were analyzed using MAPMAKER Macintosh V2.0. Parameters for map construction were a minimum log of the likelihood ratio of 3.0 and a maximum recombination fraction of 0.4. The Kosambi mapping function was employed to compute

recombination distances in centimorgans (cM). All steps performed in this study were summarized in Figure 2.2.

2.2.6. Cytological analysis

Primary leaves were inoculated with a conidial suspension (1.5 x 10^5 spores/ml). Sixty hours after inoculation, they were harvested and fixed by boiling in alcoholic lactophenol (lactic acid/phenol/glycerol/distilled water/ethanol, 1:1:1:1:8, vol/vol/vol/vol/vol) for 2 min. The specimens were observed under bright and fluorescent fields of an Olympus BX51 microscope (Olympus, Tokyo, Japan) with an exciter filter B for cytological analysis. Observation was focused on appressoria and germlings in the process of penetrating epidermal cells. When more than one appressoria were on one epidermal cell, they were excluded from counting. A total of 300 to 400 appressoria on six leaves were counted for each isolate or progeny. Cell death was visualized with trypan blue staining, as described previously (Inoue et al. 2013).

2.3. Results

2.3.1. Molecular mapping of *PWT3*

To confirm the monofactorial segregation conferred by *PWT3*, 73Q2 (an F_1 from Br58 x Br48 carrying *PWT3* alone) was backcrossed with Br48 to produce 39 BC_1F_1 random progenies (Fig. 2.3). Pathogenicity of the parental isolates and the BC_1F_1 progenies was tested on wheat cv. Norin 4 (N4). 73Q2 produced a few brown pinpoint lesions without visible centers, and was regarded as avirulent. In contrast, Br48 produced many water-soaked patches by 3 days after inoculation. They enlarged and coalesced with time and, finally, the whole leaf shriveled with its color remaining green by 5 days after inoculation. When N4 was inoculated with the 39 BC_1F_1 cultures, avirulent (infection type 0-5B) and virulent (infection type 3G-5G) cultures segregated in a 1:1 ratio. Based on

these reactions on N4, genotypes of each culture were determined; the avirulent cultures were considered as *PWT3* carriers while the virulent cultures were considered as *pwt3* carriers.

Using SSR markers reported by Zheng et al. (2008) and some makers designed using the database of *P. oryzae* 70-15 genomic sequence (*http://www.broadinstitute.org/annotation/fungi/magnaporthe/*), *PWT3* locus was mapped on chromosome 6 with two flanking SSR markers MGM130 and MoSSR6-3 (Fig. 2.4). The SSR maker most closely linked to *PWT3* was MoSSR6-1.

2.3.2. Identification of color mutants in BC_3F_1 and BC_4F_1 cultures

For further analysis, Yt3N1, a BC_1F_1 cultures avirulent on N4 (*PWT3* carrier) was backcrossed with Br48 twice (Fig. 2.3). When cultured on PDA media, the BC_3F_1 generation contained some white cultures (Fig.2.5). They were considered to be color mutants because both of their parents (h12-1-6 and Br48) produced black colonies. In some tetrads of BC_3F_1 generation, white and black (normal) cultures segregated in a 1:1 ratio (Fig. 2.5).

Moreover, when a BC_3F_1 culture (h22-4-7) with white mycelia was further backcrossed with Br48, white and black cultures again segregated in a 1:1 ratio (41:40) (Table 2.1), suggesting that the color difference is controlled by a single gene.

Color mutants are known to be deficient in penetration ability. In fact, the four white BC_3F_1 culture were avirulent on N4 while the four black BC_3F_1 cultures were virulent (Fig. 2.5). Furthermore, in the BC_4F_1 population, the segregation of the colony color was perfectly concordant with that of virulence on N4 (Table 2.2). These results indicate that the colony color is perfectly associated with virulence on N4.

The importance of appressorial melanization in penetration was first noted in *Colletotrichum lagenarium* albino mutants (Kubo et al. 1982). Melanin is known to play an essential role in appressorial penetration in *Colletotrichum* and *Pyricularia*. However, our white cultures were considered to be different from the melanin-deficient mutants reported by those researchers that lost the basic capacity of penetration completely, because the white cultures were virulent on a tetraploid accession, Tat14 (Table 2.2).

It should be noted that the white BC_3F_1 and BC_4F_1 cultures carried the Br48 alleles at the MGM130, MoSSR6-1, and MGM134 loci (Fig.2.4**).** This result indicates that these white (avirulent) cultures do not carry *PWT3*, and therefore that the avirulence is not attributable to *PWT3*.

2.3.3. Identification of a hidden resistance gene to color mutants

The three wheat cultivar/accessions, N4, Tat4, and Tat14, were inoculated with Br58, Br48, F_1 (73Q2), BC_1F_1 (Yt3N1), BC_2F_1 (h12-1-6), and BC_3F_1 (h22-4-7). N4 was resistant to Br58 and F_1 through BC_3F_1, although its resistance to 73Q2 was weak (infection type 3BG - green blast lesions surrounded by brown tissues). By contrast, a tetraploid wheat accession, Tat14, was susceptible not only to all black cultures from the parents (Br58 and Br48) through the BC_4F_1 generation but also to all white cultures in the BC_3F_1 and BC_4F_1 generations (Table 2.2).

Interestingly, another tetraploid wheat accession, Tat4, which was susceptible to all cultures from parent (Br58 and Br48) through the BC_2F_1 generation, showed moderately resistant reaction to the white BC_3F_1 cultures (Fig.2.5, Table 2.2). This moderate resistance of Tat4 was also expressed against white BC_4F_1 cultures (Table 2.2).

Against h22-4-7 (BC_3F_1), Tat4 was resistant (infection type 3B) while Tat14 was susceptible (infection type 5G). When F_2 seedlings derived from Tat4 x Tat14 were inoculated with h22-4-7, resistant and susceptible seedlings segregated in a 3:1 ratio (Table 2.3), suggesting that the resistance of Tat4 is controlled by a single gene. To confirm the single gene control, another white culture (A1-3) was chosen from the BC_4F_1 cultures and employed for segregation analysis. Against A1-3, Tat4 was resistant (infection type 2B) while Tat14 was susceptible (infection type 5G). When inoculated with A1-3, the F_2 population from Tat4 x Tat14 showed a clear segregation of resistant and susceptible seedlings (Table 2.3). On the other hand, this gene was not detected with Br58, 73Q2 (F_1), Yt3N1 (BC_1F_1), nor h12-1-6 (BC_2F_1); Tat4 and all F_2 seedlings from Tat4 x Tat14 were susceptible to these cultures (Tables 2, 3). Here, we successfully detected a "hidden" resistance gene in wheat. This gene was tentatively designated as *RmgTd(t).*

2.3.4. Molecular mapping of the *RmgTd(t)* locus

To map the *RmgTd(t)* locus, 86 F_2 plants derived from the cross Tat4 x Tat14 were grown in a field and selfed. Twenty seeds were retrieved from each of 86 F_3 lines, grown for 7 days, and inoculated with h31-2-7 (a white BC_4F_1 culture). Segregation of homozygous resistant, segregating, and homozygous susceptible lines were 20:42:24, fitting 1:2:1 ratio (a $\chi^2 = 0.42$) as expected.

Another set of twenty seeds was retrieved from each of the 86 F_3 lines, grown for 5-6 days, and used for DNA extraction. Selected SSR loci (as described 3.2.3) were amplified from these DNA samples. Among 350 SSR markers on 14 chromosomes screened by BSA, about 30% were polymorphic between Tat4 and Tat14. By combining molecular data with inoculation data, the *RmgTd(t)* gene was mapped on chromosome 7B with 3 markers,

Xbarc1073, Xwmc276 , Xhbg338 (Fig. 2.6). The most closely linked SSR makers to *RmgTd(t)* was Xhbg338 (10.5cM).

2.3.5. Cytological responses associated with *RmgTd(t)*

To determine cytological responses controlled by *RmgTd(t)*, primary leaves of Tat4 and Tat14 were inoculated with the parental isolates (Br58, Br48) and a white culture (h22-4-7). Sixty hours after inoculation, wheat leaves were fixed in alcoholic lactophenol. Not only the wild isolates (Br58 and Br48) but also the white culture produced mature, pigmented appressoria, and attempted penetration (Fig. 2.7). On both accessions, appressorial penetration by Br58 and Br48 was blocked at the papilla stage in low frequencies (1.6% and 3.1%, respectively) (Table 2.4). Most of the germlings that overcame the papilla stage produced infection hyphae and effectively colonized the epidermal cells, leading to susceptible reactions. Similar results were obtained in Tat14 inoculated with h22-4-7 (Table 2.4). On the other hand, in Tat4 inoculated with h22-4-7, more than 90% of germling that penetrated cell walls successfully produced infection hyphae but subsequently induced HR of mesophyll cells, resulting in the failure of causing disease (Table 2.4, Fig. 2.7).

2.4. Discussion

Genetic analysis of the progenies derived from crosses of Br48 (*Triticum* isolate) with GFSI1-7-2 (*Setaria* isolate) (Murakami et al. 2000), Br58 (*Avena* isolate) (Takabayashi et al. 2002) and PO12-7301-2 (*Oryza* isolate) (Tosa et al. 2006) made it possible to identify *Pwt1*, *Pwt2*, *Pwt3*, and *Pwt4*, that conditioned their specific pathogenicity on wheat. The *Oryza* and *Setaria* isolates shared two avirulence genes, *PWT1* and *PWT2*, while the *Aveva* isolates carried the other avirulence genes (*PWT3*, *PWT4*). Interestingly, *PWT4-Rwt4* interaction was temperature-insensitive and effective

even at high temperature whereas the interaction between *PWT3* and its corresponding, hypothetical resistant gene *Rwt3* was temperature-sensitive and ineffective at high temperature (Takabayashi et al. 2002).

In a preliminary experiment, we inoculated many cultivars/accessions of hexaploid wheat, tetraploid wheat, and barley with the two parental isolates (Br48 and Br58) and a BC_1F_1 culture (73Q2). Although no barley cultivars responded to *PWT3*, many hexaploid and some tetraploid cultivars/accessions responded to *PWT3* (data not shown). These results suggest that *PWT3* could be ubiquitously involved in the avirulence of *Avena* isolate on wheat possibly because its corresponding resistance gene, *Rwt3*, is ubiquitously distributed in these cultivars/accessions.

Avirulence genes of *P. oryza*e tend to be located on telomeric or subtelomeric regions (Farman 2007; Orbach et al. 2000). *PWL1*, a cloned avirulence gene involved in species specificity on weeping lovegrass, was suggested to be linked to a telomere on the basis of cosegregation of a *PWL1*-flanking sequence with a telomere signal (Kang et al. 1995). Another cloned avirulence gene on weeping lovegrass, *PWL2*, was closely linked to a telomere and was located on an unstable region (Sweigard et al. 1995). *PWT1*, an avirulence gene of *P. oryza*e on wheat and barley, is tightly linked to a telomere and probably located on a subtelomeric region (Chuma et al. 2010). In this study we showed that *PWT3*, an avirulence gene of an *Avena* isolate, is located on chromosome 6 (Fig. 2.4). Its flanking markers will provide a starting point for cloning and make it possible to recognize differences between these avirulence genes.

For further analysis, Yt3N1, a BC_1F_1 cultures avirulent on N4 (*PWT3* carrier) was backcrossed with Br48 twice. Interestingly, some tetrads in the BC_3F_1 generation contained white cultures, which were considered to be color mutants because both of their parents (h12-1-6 and Br48) produced black colonies. White BC_3F_1 culture h22-4-7 was

further backcrossed with Br48. In the BC_4F_1 generation, white and black cultures segregated in a 1:1 ratio (Table 2.1). These results suggested that this mutation involved a single gene. Furthermore, the segregation of the colony color was perfectly concordant with that of virulence on N4.

Kubo et al. (1982) found that melanin plays an essential role in appressorial penetration in *Colletotrichum*. *Pyricularia* species were found to penetrate cell walls with similar mechanisms (Howard and Valent 1996). The white cultures found in this study were considered to be different from the melanin-deficient mutants reported by those researchers that lost the basic capacity of penetration completely, because the white cultures were virulent on a tetraploid accession, Tat14 (Table 2.2). Besides that, these white cultures produced dark-brown appressoria and infection hyphae.

It is important to identify a resistance gene that interacts with the gene associated with the color and avirulence. In this study, actually, we found a "hidden resistance gene" in a tetraploid wheat accession, Tat14, located on chromosome 7B. This resistance gene was tentatively designated as *RmgTd(t)*. The interaction between this "avirulence" gene and *RmgTd(t)* may also be under the control of gene-for-gene theory.

As mention before, our laboratory has identified several major genes for resistant to the blast fungus in wheat. The first gene was *Rmg1* (*Rwt4*) on chromosome 1D. Subsequently, two resistance gene, *Rmg2* and *Rmg3* were found on chromosome 7A and 6B, respectively (Zhan et al. 2008). Two other wheat genes, *Rmg4* and *Rmg5* were located on chromosomes 4A and 6D, respectively (Nga et al. 2009). Recently, we identified *Rmg6* on chromosome 1D (Vy et al, 2014). *RmgTd(t)* were considered to be different from these resistance genes because it was located on a different chromosome, 7B. Furthermore, Tat4 was highly susceptible to Br58, F_1, BC_1F_1 and BC_2F_1 (Table 2.3), which carried *PWT3*.

Several cytological studies on the process of blast infection were performed to characterize interactions between rice and the blast fungus (Arase et al. 1983; Koga and Kobayashi, 1982; Koga, 1994). To elucidate mechanisms of the resistance conferred by *RmgTd(t)*, we examined cytological responses with a fluorescence microscope. When Tat4 and Tat14 were inoculated with virulent isolates (Br48 and Br58), infection type 5G (highly susceptible) were produced. At the cytological level, this reaction was reflected to low frequencies of the no reaction type, the papilla formation type, and the HR type, and a high frequency of hyphal growth (Table 2.4). A cytological difference between incompatible and conpatible interactions was found in HR of mesophyll cells. On Tat4 appressoria of a color mutant successfully produced infection hyphae, but subsequently induced HR of mesophyll cells. These reactions may explain the intermediate resistance of Tat4.

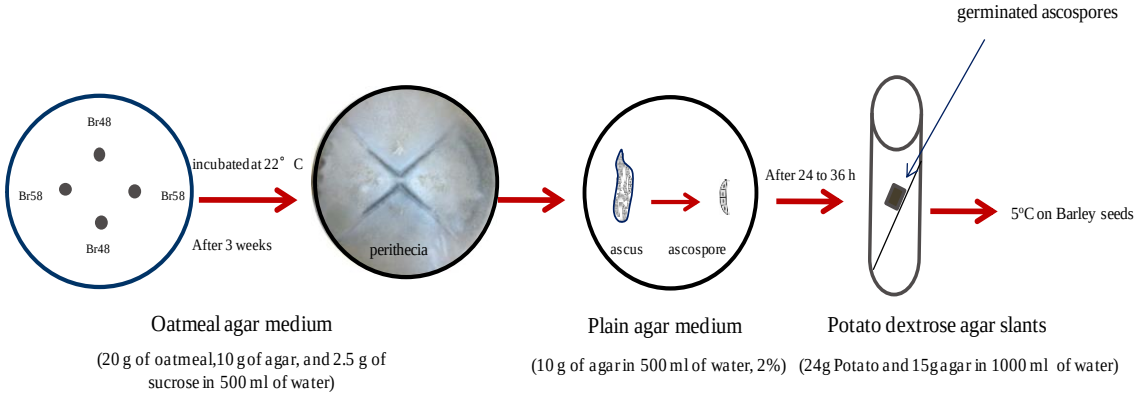

Fig. 2.1. Genetic crosses of fungal strains

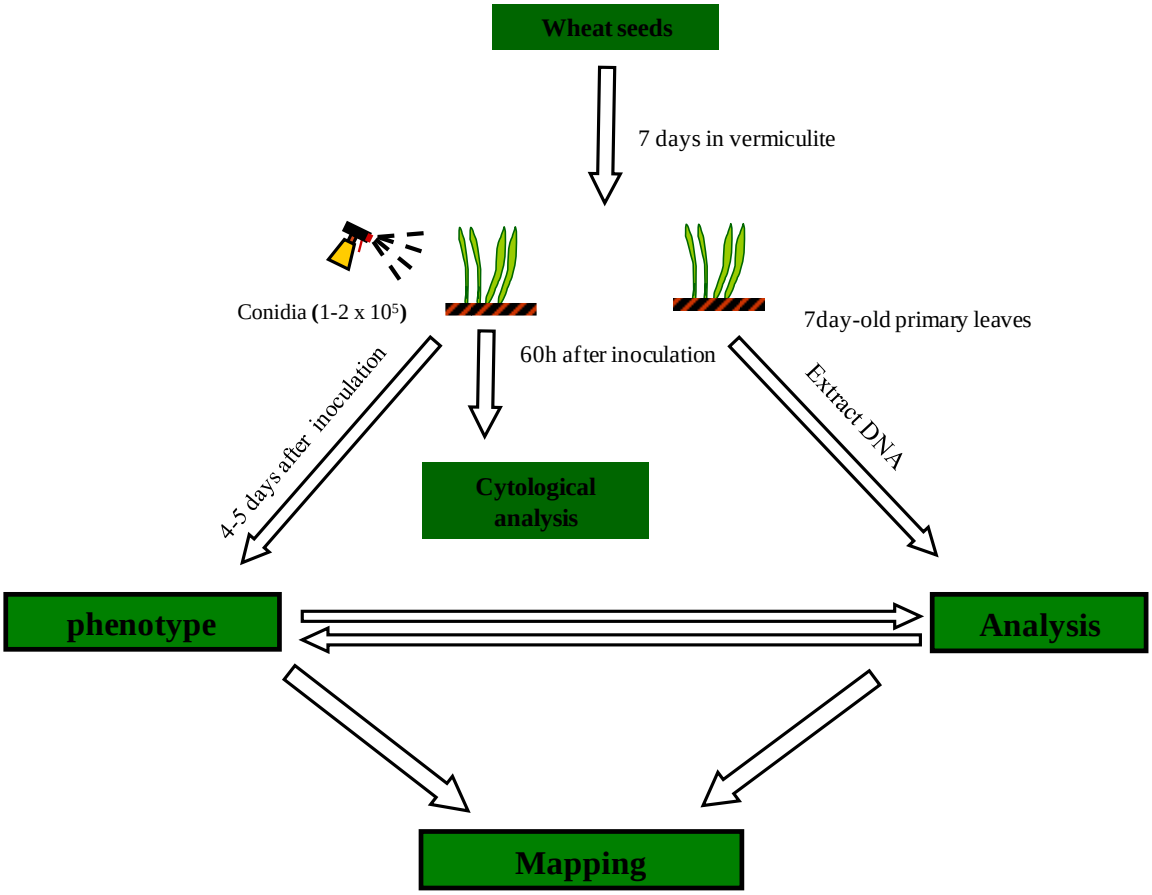

Fig. 2.2. Outline of experiments

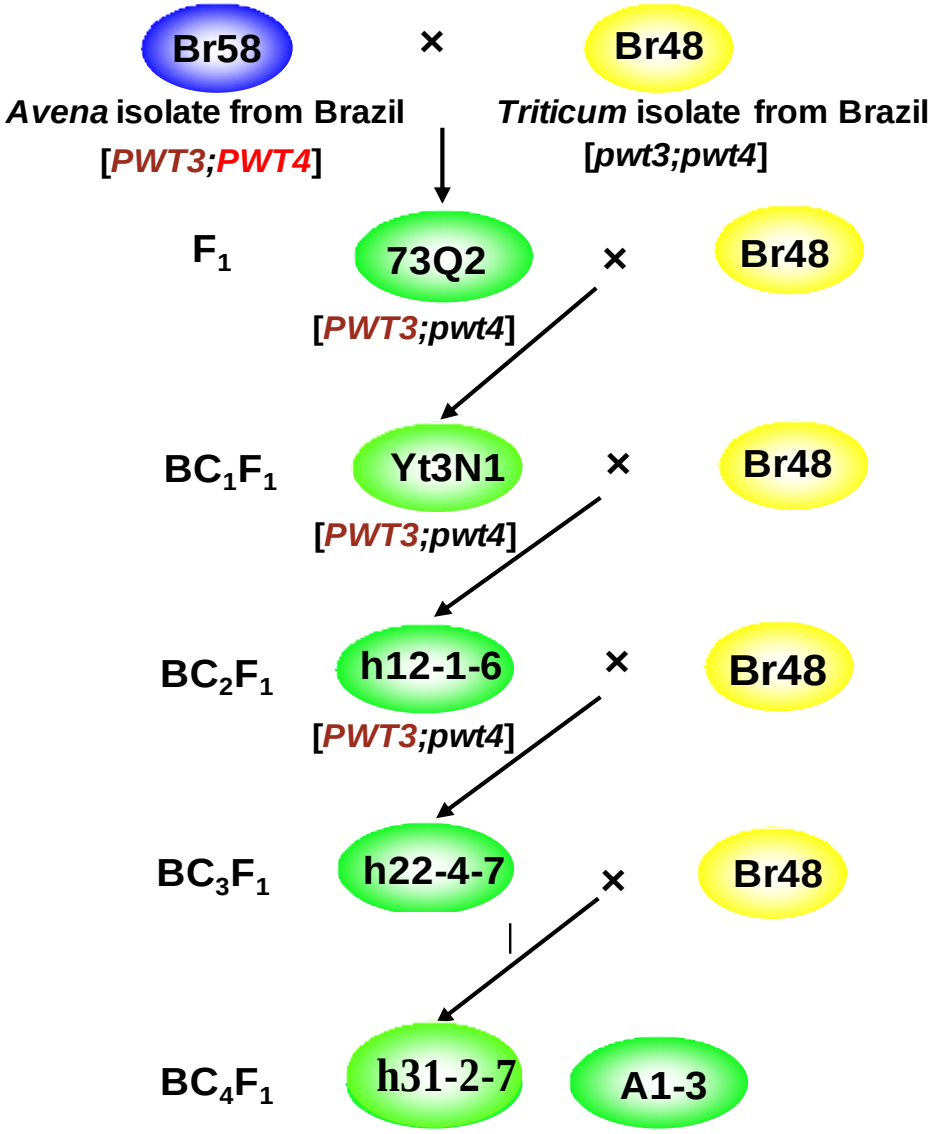

Fig. 2.3. Pedigree of *P. oryzae* cultures used in this study

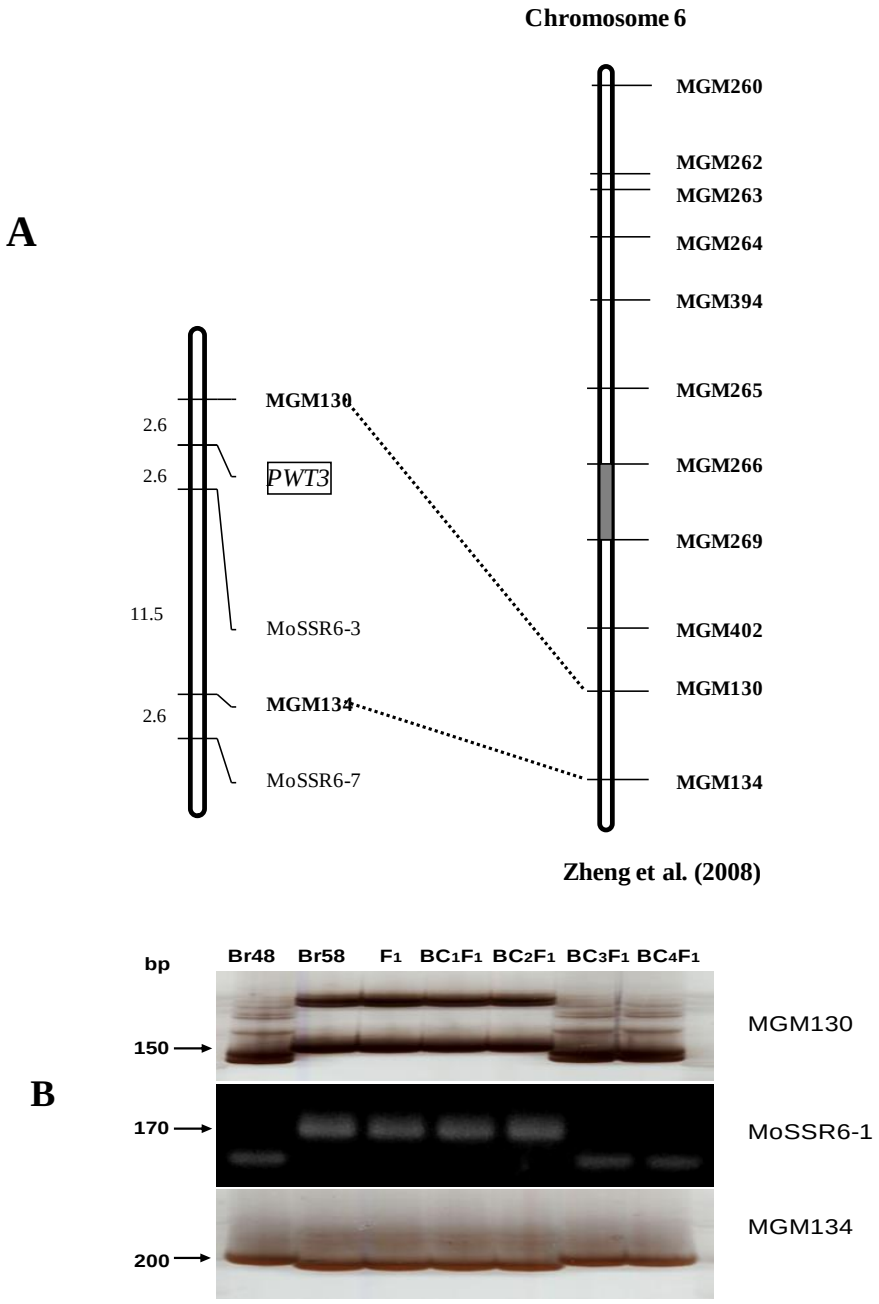

Fig. 2.4**.** Analysis of backcross progenies with molecular markers. A, Geneticmap of the *PWT3* region constructed using a BC_1F_1 population derived from 73Q2 × Br48. Markers prefixed with MGM are from Zheng et al. (2008). Others were designed using the database of the *Pyricularia oryzae* 70-15 genomic sequence. B, Polyacrylamide gel electrophoresis analysis of backcross progenies. Two *PWT3*-flanking simple-sequence repeat loci (MGM130 and MGM134) were amplified from genomic DNA of Br48 (*Triticum* isolate), Br58 (*Avena* isolate), 73Q2 (F_1), Yt_3N_1 (BC_1F_1), h12-1-6 (BC_2F_1), h22-4-7 (BC_3F_1), and h31-2-7 (BC_4F_1), and run on a 6% polyacrylamide gel.

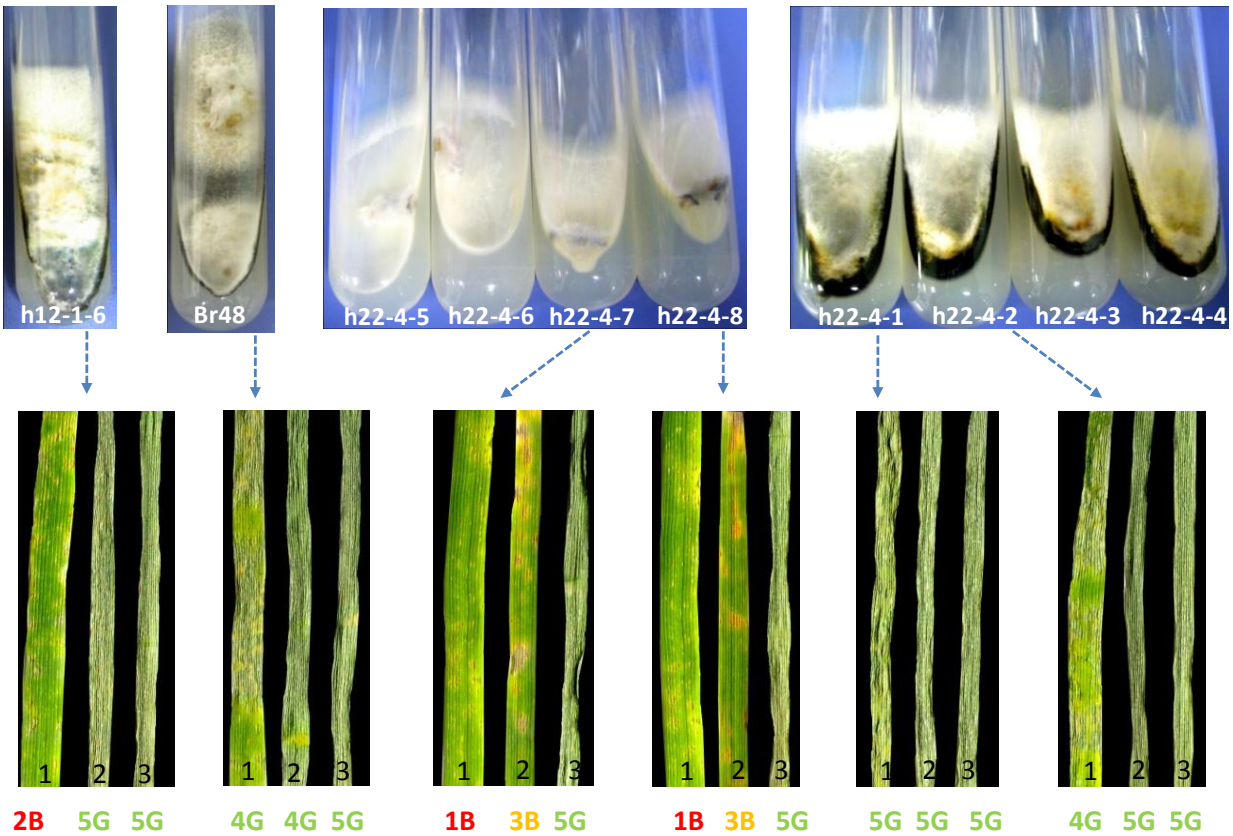

Fig. 2.5**.** Infection of N4 (1), Tat4 (2) and Tat14 (3) cultivars with BC_3F_1 cultures and their parents (h12-1-6 and Br58), 5 days after inoculation.

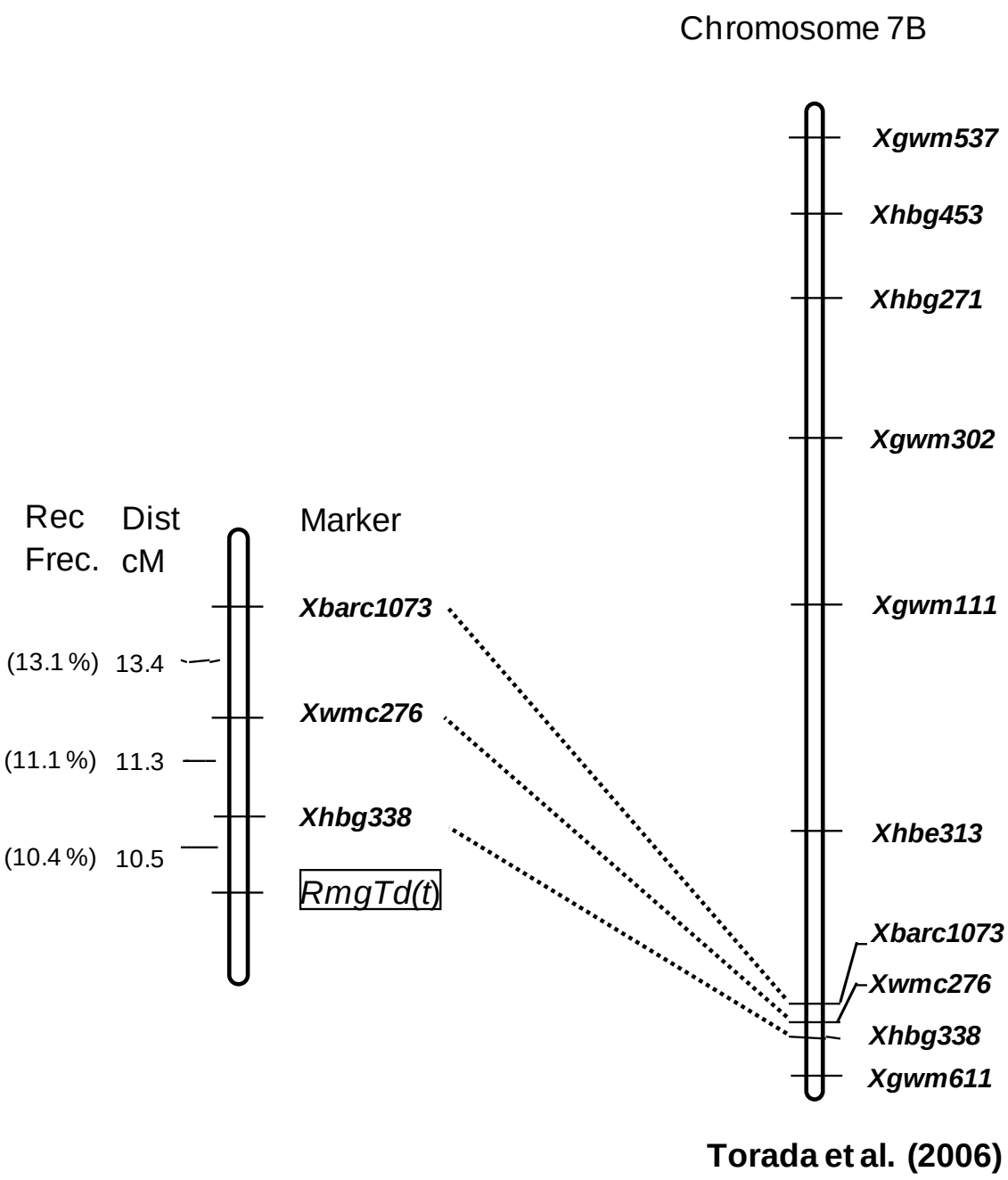

Fig. 2.6. Linkage map around *RmgTd(t)* constructed using F_3 lines derived from Tat4 x Tat14

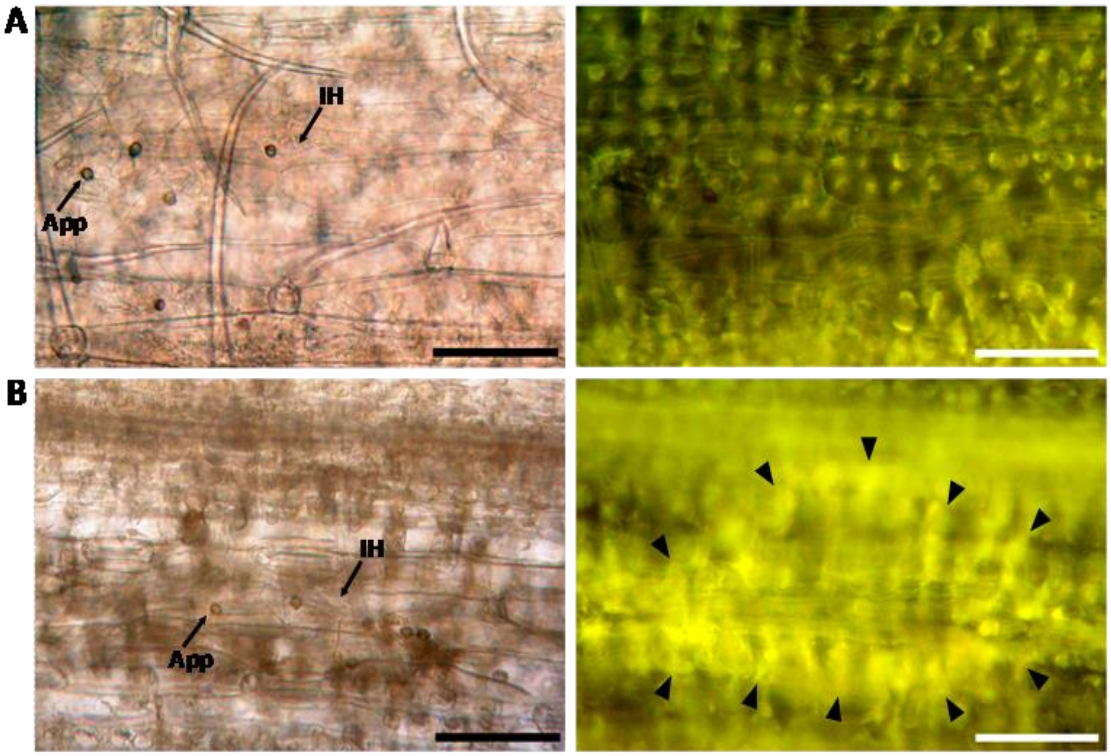

Fig. 2.7. Cytological responses in Tat14 (A) and Tat4 (B), 60 hrs after inoculation with h22-4-7.Left, bright field; right, fluorescent field. App: appressoria; IH: infection hyphae. Arrowhead: fluorescent mesophyll cells. Scale bars; 100 µm

Table 2.1. Segregation of cololy colors in an BC_4F_1 population derived from a backcross, h22-4-7 (BC_3F_1) x Br48

Ascus No.	No. of BC_4F_1 cultures		
	White	Black	Total
1	4	4	8
2	4	3	7
3	4	4	8
4	4	4	8
5	3	4	7
6	3	4	7
7	4	4	8
8	4	3	7
9	4	3	7
10	3	4	7
11	4	3	7
Total	41	40	81

Table 2.2. Responses of wheat cultivar/accessions to *Avena* isolate Br58, *Triticum* isolate Br48, and progenies derived from backcrosses with Br48

Plant species	Cultivar/Accession	Infection type with						No. of BC_4F_1 cultures from h22-4-7 x Br48			
		Br58	73Q2 (F_1)	Yt3N1 (BC_1F_1)	h12-1-6 (BC_2F_1)	h22-4-7 (BC_3F_1)	Br48	White A[a]	White V[a]	Black A	Black V
T. aestivum (6X)	N4 (Norin 4)	0-1B	3BG	2B	2B	1B	4G	41	0	0	40
T.dicoccoides (4X)	Tat4 (KU109)	5G	5G	5G	5G	3B	4G	41	0	0	40
T. paleocolchicum (4X)	Tat14 (KU156)	5G	5G	5G	5G	5G	5G	0	41	0	40

[a]A, avirulent (infection type 0-3BG); V, virulent (infection type 3G-5G)

Table 2.3. Frequency distribution of infection types with *Avena* isolate Br58 and its backcross progenies in F_2 seedlings derived from a cross between tetraploid wheat accessions, Tat4 and Tat14

Culture	No. of F_2 seedlings from Tat4 x Tat14[a]										$\chi 2$ for 3:1	P
	0/1B[b]	2B/2BG	3B	3BG	3G	4G	5G	Total	Resistant	Susceptible		
Br58	0	0	0	0	0	3	37	40	0	40	-	-
73Q2 (F_1)	0	0	0	0	3	2	33	38	0	38	-	-
Yt3N1 (BC_1F_1)	0	0	0	0	1	5	31	37	0	37	-	-
h12-1-6 (BC_2F_1)	0	0	0	0	0	5	32	37	0	37	-	-
h22-4-7 (BC_3F_1)	19	5	2	0	3	2	3	34	26	8	0.04	0.84
A1-3 (BC_4F_1)	39	33	9	0	5	2	15	103	81	22	0.73	0.39

[a] Tat4, *Triticum dicoccoides* accession KU109; Tat14, *Triticum paleocolchicum* accession KU156.

[b] Infection type at 5 days after inoculation. 0-3BG, resistant; 3G-5G, susceptible.

Table 2.4. Cytological responses of wheat accessions against *Avena* isolate Br58, *Triticum* isolate Br48, and BC_3F_1 culture h22-4-7

			% appressoria[a] accompanied by				
Culture	Accession	Infection type	no reaction (A)	cell wall defense (B)[b]	HR of epidermal cell (C)	hyphal growth with HR of mesophyll cells (D)	hyphal growth with no HR (E)
Br58	Tat 4	5G	13.3	1.6	0.5	8.0	77.4
	Tat14	5G	17.1	4.6	7.5	3.4	67.7
h22-4-7	Tat 4	3B	0.0	1.6	0.0	96.7	1.6
	Tat14	5G	10.9	2.2	2.1	0.0	85.1
Br48	Tat 4	4G	5.5	3.1	33.6	0.8	57.5
	Tat14	5G	11.4	3.1	20.0	1.0	65.1

[a] Average of two replications. More than 300 appressoria on three leaves were counted in each replication.

[b] Appressoria that attempted penetration but were stopped at the cell wall stage by papilla formation etc.

CHAPTER III

Identification of *Rmg8*, a new gene for resistance to *Triticum* isolates in hexaploid wheat

3.1. Introduction

Development of resistant cultivars carrying resistance (*R*) genes is the most effective and economical way to control disease. Historically, however, many R genes have been rendered ineffective only a few years after their use in farmers' fields, resulting in a "breakdown of resistance", because of the development of new races (Kiyosawa 1982; Leach et al. 2001). To prevent such breakdown, cultivars with durable R genes are required.

Wheat blast caused by *Triticum* isolates (Urashima et al. 1993) significantly reduces wheat yields and grain quality; yield losses reach to 70 - 100% when favorable climatic conditions occur at the critical developmental stages for infection (Cruz et al. 2012; Kohli et al. 2011). The first epidemic of this disease occurred in Paraná State, southern Brazil in 1985 (Igarashi et al. 1986). Since then, it has become widely distributed across most of the wheat-producing areas in Brazil, Argentina, Paraguay, and Bolivia (Cruz et al. 2012; Kohli et al. 2011) because of a lack of resistant cultivars and effective fungicides (Maciel et al. 2014). Recently, wheat blast was also found in North America. In May 2011, *P. oryzae* was isolated from a single severely blasted wheat head at a research plot at the University of Kentucky Research and Education Center in Princeton. This infection was possibly caused by a host jump from an annual ryegrass pathogen (Pratt 2012). Although no serious outbreaks have been reported outside of

South America, there is an increasing threat of wheat blast epidemics in other parts of the world (Kohli et al. 2011).

To date, seven blast R genes have been identified in wheat. The first R gene designated *Rmg1* (*Rwt4*) was effective against an *Avena* isolate but not against *Triticum* isolates (Takabayashi et al. 2002). *Rmg4* and *Rmg5* conditioned resistance to a *Digitaria* isolate, but not to *Triticum* isolates (Nga et al. 2009). *Rmg6* was effective against a *Lolium* isolate but not against *Triticum* isolates (Vy et al. 2014). These four R genes governed resistance to inappropriate subgroups. On the other hand, the other three genes were effective against isolates of an appropriate subgroup (*Triticum* isolates), including *Rmg2* and *Rmg3* identified in common wheat cultivar 'Thatcher' (Zhan et al. 2008) and *Rmg7* identified in tetraploid accessions, St24, St17, and St25 (Tagle et al. 2015). These genes may be useful for wheat breeding, but have not yet been mapped.

In the present study, we identified a novel gene for resistance to *Triticum* isolate Br48 on chromosome 2B of a hexaploid wheat cultivar, 'S615' (S615), and designated it *Rmg8*. We also identified its corresponding avirulence (AVR) gene by using progeny derived from a cross between Br48 and a laboratory strain 200R29.

3.2. Materials and Methods

3.2.1. Fungal materials

Isolates used for a preliminary screening were 14 *Triticum* isolates collected in Brazil in 1990 - 1992 (Table 3.1). After screening, a representative *Triticum* isolate, Br48, was used for segregation analyses of wheat F_2 and F_3 populations. To identify an AVR gene, Br48 (*MAT1-1*) was crossed with 200R29 (*MAT1-2*), a highly virulent F_1 culture derived from a cross between *Eleusine* isolate MZ5-1-6 and Br48 (Tagle et al. 2015), as described previously (Murakami et al. 2000). A random hybrid population was produced by sampling

one ascospore from each ascus. These cultures were maintained on barley seed media as described previously (Hirata et al. 2007).

3.2.2. Plant materials.

Plant materials used for the preliminary screening were 31 cultivars of common wheat (*Triticum aestivum* L.) provided by Dr. K. Tsunewaki, formerly Kyoto University, and Dr. U. Hiura, formerly Okayama University. After the screening, 3 representative cultivars were selected for further analyses. A common wheat cultivar, S615, was resistant to Br48, whereas two cultivars, 'Shin-chunaga' (Sch) and 'Hope', were susceptible (Table 3.2). Tetraploid accession, *T. dicoccum* KU-120 (St24), was employed for a comparison of R genes because it carried *Rmg7* (Tagle et al. 2015). Wheat seeds were pre-germinated and sown in vermiculite supplied with liquid fertilizer in a seedling case as described in chapter II. Leaves were then fixed onto a hard plastic board for inoculation.

3.2.3. Infection assays

Inocula were prepared following the protocol described by Tagle et al. (2015). The inoculum suspension (1×10^5 conidia/ ml) was sprayed onto adaxial surfaces of the 7-day-old primary leaves using an air compressor. The inoculated plants were incubated for 24 h in darkness in humid trays at 22 or 25°C, after which they were transferred to dry conditions under fluorescent lighting, and further incubated at the same temperature. Disease symptoms were evaluated four or five days after inoculation as described in chapter II.

For infection assays of wheat plants at the heading stage, stems with spikes at the stage of full head emergence were cut from wheat plants that had been sown in an experimental field in the autumn of the previous year, and put in flasks with water. Spikes of the susceptible cultivar (Sch) could not be harvested simultaneously with the resistant cultivar (S615) because of different heading times; Sch was early-heading while S615 was

late-heading. Therefore, a late-heading cultivar, Hope, was used as a susceptible control for assays at the heading stage. Harvested spikes were inoculated with conidial suspensions (1×10^5 spores/ml), covered with a plastic bag, and placed in darkness at 25°C for 24 h. The plastic bag was removed and the set-up was incubated further at the same temperature with 12 h alternate light and dark conditions. Five days after inoculation, spike infections were rated with six progressive grades from 0 to 5: 0 = no visible evidence of infection; 1 = pinhead spots; 2 = small lesions (<1.5 mm); 3 = scattered lesions of intermediate size (<3 mm); 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. Three spikes were used for each wheat cultivar per replication. All infection assays at the heading stage were repeated twice.

3.2.4. Molecular mapping

To map *Rmg8* and *Rmg7*, 93 F_2 plants derived from a cross between S615 and Sch and 94 F_2 plant derived from a cross between St24 and Tat14 (*T. paleocolchicum* KU-156, highly susceptible to Br48) (Tagle et al. 2015) were grown in a field and selfed. From each of the resulting F_3 lines, 20 seeds were used for disease assays with Br48. Another 20 seeds were grown at 22ºC for 5-7 days and pooled leaf tissue samples were used for DNA extraction.

Based on phenotypic data, five homozygous resistant (*RR*) and five homozygous susceptible (*rr*) F_3 lines were selected for bulked segregant analysis (Michelmore et al. 1991). Resistant and susceptible bulks (10 ng/µl) were constructed by mixing equal amounts of DNA of the five *RR* and five *rr* F_3 lines, respectively. Simple-sequence repeat (SSR) loci reported by Somers et al. (2004) and Torada et al. (2006) were amplified from DNA of the parental cultivars and the two bulks. PCR products were separated in 2% agarose or in 13% nondenaturing polyacrylamide gels using a high efficiency genome

scanning system (Nippon Eido, Tokyo) (Hori et al. 2003). Markers showing a possibility of linkage were then subjected to analyses on the F_3 lines to construct a linkage map. A genetic map was constructed using software MAPMAKER (Lander et al. 1987). Thresholds of linkage for map construction were minimum log of likelihood ratio of 4.0 and a maximum recombination fraction of 0.4. The Kosambi mapping function was employed to compute map distances in centimorgan (cM).

3.3. Results

3.3.1. Infection assays at the seedling stage

Screening of the common wheat cultivars revealed that S615 was resistant to all *Triticum* isolates at both 22ºC and 25ºC while Sch and Hope were susceptible (Table 3.1 and 3.2, Fig. 3.1A). Consequently, we selected these three cultivars for further analyses. When an F_2 population derived from a cross between S615 and Sch were inoculated with Br48, resistant and susceptible seedlings segregated clearly (Fig. 3.2) in a 3:1 ratio (164:42, $\chi 2$ = 2.34). In F_3 populations non-segregating resistant, segregating, and non-segregating susceptible lines were distributed in a 1:2:1 ratio (21:52:20, $\chi 2$ = 1.32), indicating that the resistance of S615 to Br48 was controlled by a single dominant gene. This gene was designated *Rmg8* (Resistance to *Magnaporthe grisea 8*).

3.3.2. Infection assays at the heading stage

To check whether the resistance of S615 is expressed at the heading stage, its spikes were inoculated with Br48 together with those of the susceptible control Hope, and incubated at 25ºC. The spikes of S615 showed infection type 2 with small brown lesions caused by a hypersensitive reaction (Fig. 3.1B) as expected. By contrast, the spikes of Hope were completely blighted with infection type 5 (Fig. 3.1B).

3.3.3. Identification of an AVR gene in a *Triticum* isolate

In order to identify an AVR gene corresponding to *Rmg8*, an isolate virulent on the *Rmg8*-carrier is required as a mating partner of Br48. However, S615 was resistant to all *Triticum* isolates tested (Table 3.1). To find a mating partner, S615 was inoculated with isolates from other host-specific subgroups (e.g. *Eleusine* isolates, *Setaria* isolates) and their descendants, and incubated at 22 and 25°C. Several virulent cultures were found in an F_1 population derived from MZ5-1-6 (*Eleusine* isolate) x Br48. Their mating types were checked using *MAT1-1* and *MAT1-2*-specific primers (Zheng et al. 2008). Finally, 200R29 was selected as the mating partner because it carried the *MAT1-2* allele and was highly virulent on S615 at 25°C (Table 3.2). Br48 (*MAT1-1*) was crossed with 200R29, resulting in 73 random hybrid cultures. When they were sprayed on S615 and incubated at 25°C, 31 and 40 cultures were stably avirulent and virulent, respectively (Table 3.3). There were two exceptional cultures whose infection types were unstable (i.e., fluctuated among replications). Regardless of their categorization, however, segregation of avirulent and virulent cultures fitted a 1:1 ratio (Table 3.3), suggesting that a single gene is involved in the avirulence of Br48 on S615. This AVR gene was designated *AVR-Rmg8*.

When the 73 cultures were sprayed onto St24 carrying *Rmg7*, avirulent and virulent cultures clearly segregated in a 1:1 ratio (Table 3.3). This gene was considered to be *AVR-Rmg7* corresponding to *Rmg7* (Tagle et al. 2015). To check if *AVR-Rmg8* and *AVR-Rmg7* are located at the same locus, the segregation patterns on S615 and St24 were compared. When the unstable two cultures were classified as “virulent”, the segregation patterns on S615 and St24 were perfectly concordant (Table 3.4). When these two cultures were classified as “avirulent”, however, they were regarded as recombinants (Table 3.4).

3.3.4. Molecular mapping of *Rmg8*

To determine the chromosomal position of *Rmg8*, bulked-segregant analysis was performed with the F_3 lines derived from a cross between S615 and Sch. Of 725 SSR markers tested, 293 were polymorphic between the parental accessions (Table 3.5). Twelve polymorphic markers located on chromosome 2B appeared to be associated with *Rmg8* in bulked segregant analysis. Genotyping of all F_3 lines indicated that *Rmg8* was flanked by SSR markers, *Xwmc317* and *Xbarc159* (Fig. 3.3), assigned to the distal region in the long arm of chromosome 2B (Somers et al. 2004).

To determine the chromosomal position of *Rmg7*, 94 F_3 lines derived from a cross between St24 (*Rmg7*) and Tat14 (*rmg7*) were employed. When they were inoculated with Br48, non-segregating resistant, segregating, and non-segregating susceptible lines were distributed in a 1:2:1 ratio (26:45:23, $\chi 2 = 0.36$). Molecular mapping with these F_3 lines indicated that *Rmg7* was flanked by SSR markers, *Xcfd50* and *Xhbg327* (Fig. 3.3), assigned to the distal region in the long arm of chromosome 2A (Somers et al. 2004).

3.4. Discussion

In the present study, we identified *Rmg8*, a dominant gene conferring strong resistance to *Triticum* isolate Br48 in common wheat cultivar S615. This gene was located on the long arm of chromosome 2B (Fig. 3.3). *Rmg8* should be different from *Rmg2* and *Rmg3*, two of the three R genes previously identified as effective against *Triticum* isolates, because *Rmg2* and *Rmg3* are located on chromosomes 7A and 6B, respectively (Zhan et al. 2008). *Rmg8* should be also different from the other (third) R gene, *Rmg7*, which was identified in a tetraploid wheat accession (St24) by Tagle et al. (2015), because *Rmg7* is located on chromosome 2A (Fig. 3.3).

Segregation analysis of a fungal hybrid population suggested that their corresponding AVR genes, *AVR-Rmg7* and *AVR-Rmg8*, are closely linked or located at

the same locus (Table 3.4). If these AVR genes are the same, this means that a single AVR gene matches to *Rmg7* in tetraploid wheat and *Rmg8* in hexaploid wheat. It should be noted that *Rmg8* and *Rmg7* are located in the distal regions of long arms of homoeologous chromosomes 2B and 2A, respectively (Fig. 3.3). There is a possibility that *Rmg8* in hexaploid wheat and *Rmg7* in tetraploid wheat are homoeologous genes. To verify the homoeologous relationship between *Rmg8* and *Rmg7*, fine mapping of these genes should be performed in further studies.

R genes to be used in breeding for resistance to wheat blast must fulfill some requirements. First, they must be expressed at the heading stage because wheat blast in the field is primarily a spike disease. Variation in blast response among wheat cultivars has been recognized at both the seedling and adult plant stages (Cruz et al. 2012; Prestes et al. 2007; Urashima et al. 2004). Cruz et al. (2012) suggested that seedling infection assays were not reliable in predicting the severity of spike infection. Maciel et al. (2014) showed that there was not a strong correlation between seedling and spike reactions to the wheat blast pathogen. On the other hand, Tagle et al. (2015) showed that tetraploid accessions carrying *Rmg7* (St17, St24, and St25) were highly resistant even at the heading stage. In the present study, we found that S615 carrying *Rmg8* conferred resistance not only at the seedling stage but also at the heading stage. If resistance at the heading stage is conferred by *Rmg8,* then it is considered to fulfill the first requirement.

Secondly, R genes must be durable. The durability of R genes may be predicted by evaluating a fitness cost imposed by mutation of, or constraints imposed on, corresponding AVR genes (Janzac et al. 2009; Leach et al. 2001; Vera Cruz et al. 2000) or by estimating stability of corresponding AVR genes based on their chromosomal location and molecular structure around them (Chuma et al. 2011; Tosa and Chuma 2014). In any case identification of AVR genes is a prerequisite to evaluate durability of new R

genes. To identify an AVR gene corresponding to an R gene (*R1*), we need an isolate virulent on *R1*. However, this requirement includes an intrinsic antinomy; durable R genes should be resistant to all prevailing *Triticum* isolates. In this respect, *Rmg7* and *Rmg8* were effective to all *Triticum* isolates tested. Tagle et al. (2015) solved this problem by using temperature-sensitivity of avirulence of an *Eleusine* isolate on the *Rmg7* carriers. In the present study, we overcame this problem by producing a culture virulent on *Rmg8* (200R29) through crossing a *Triticum* isolate (Br48) with an *Eleusine* isolate (MZ5-1-6). By backcrossing this virulent isolate with the *Triticum* isolate, we successfully identified *AVR-Rmg8*. Cloning of *AVR-Rmg8* will be reported in the next chapter.

Figure 3.1. Symptoms on seedlings (A) and spikes (B) of resistant cultivar S615 and susceptible cultivars Shin-chunaga (Sch) or Hope, 5 days after inoculation with *Triticum* isolate Br48 at 25ºC.

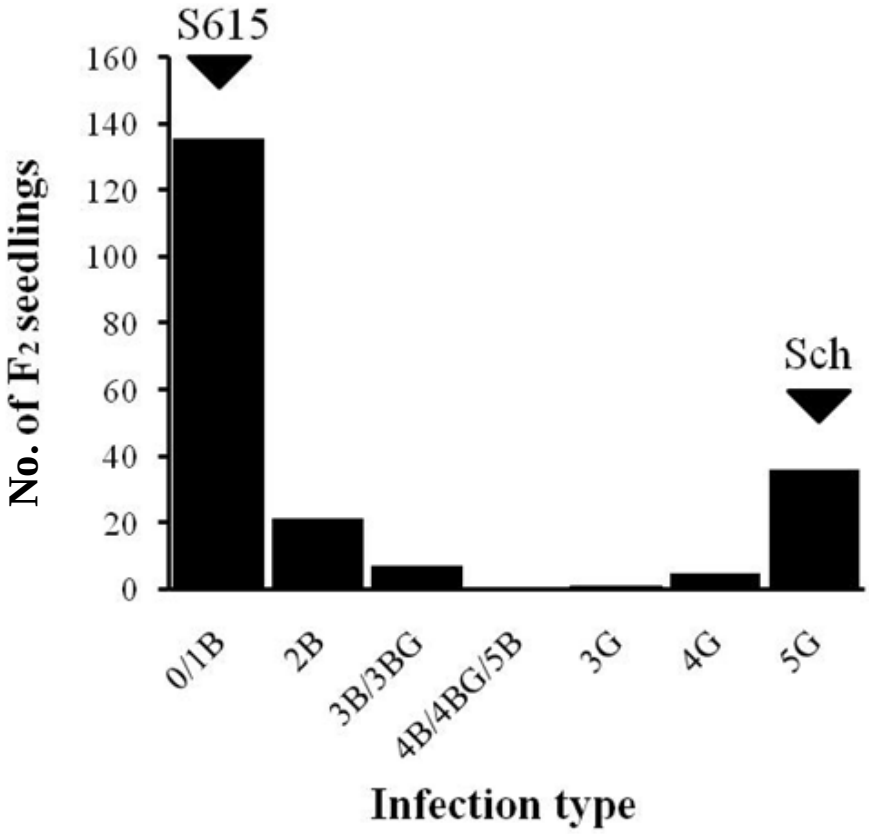

Fig.3.2. Frequency distribution of infection types with *Triticum* isolate Br48 at 22 ^{o}C in F_2 seedlings derived from the cross between S615 and Sch.

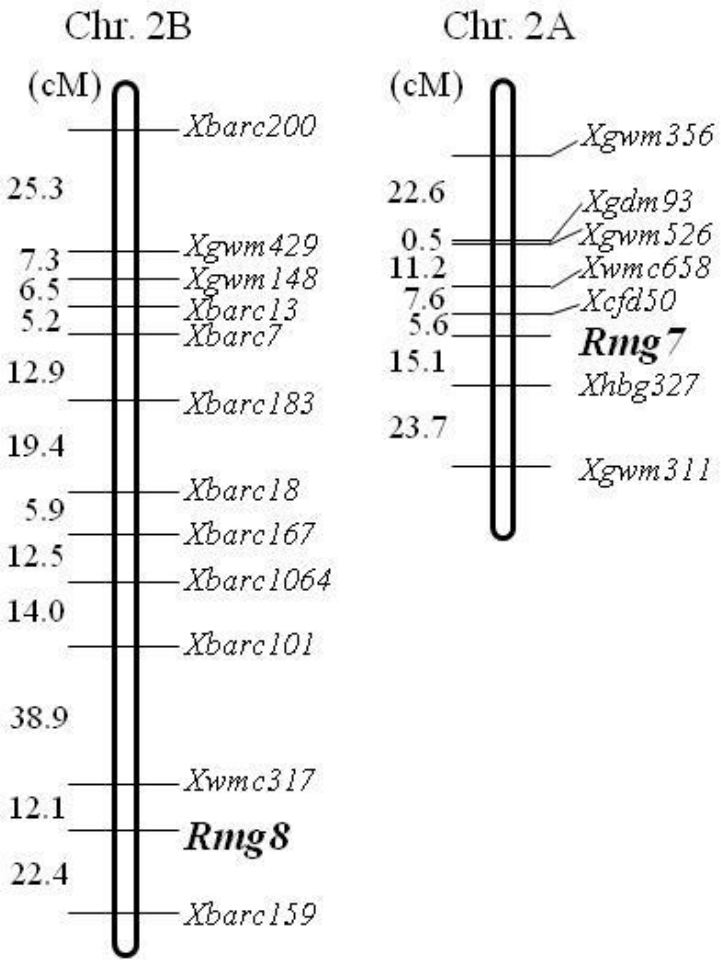

Fig. 3.3. Genetic map of the *Rmg8* (left) and *Rmg7* (right) regions constructed using F_3 lines derived from crosses between two common wheat cultivars (S615 and Sch) and two tetraploid wheat accessions (St24 and Tat14), respectively.

Table 3. 1. Reactions of wheat lines to *Triticum* isolates of *P. oryzae*

Isolate	Locality	Year of collection	Infection type[a]					
			S615[b]		Sch		Hope	
			22ºC	25ºC	22ºC	25ºC	22ºC	25ºC
Br2	Londrina, Parana, Brazil	1990	1-2B	2B	5G	5G	5G	5G
Br3	Londrina, Parana, Brazil	1990	1B	3B	5G	5G	5G	5G
Br5	Jagupita, Parana, Brazil	1990	0-1B	2B	5G	5G	5G	5G
Br8	B.V.Praiso, Parama, Brazil	1990	2B	3B	5G	5G	5G	5G
Br46	Rio Brilhante, Mato Grosso do Sul	1990	1-2B	1B	3-4G	5G	3B	5G
Br48	Itapora, Mato Grosso do Sul, Brazil	1992	0-1B	1-2B	5G	5G	5G	5G
Br49	Dourados , Mato Grosso do Sul, Brazil	1992	1-2B	2B	5G	5G	5G	5G
Br50	Rio Brilhante , Mato Grosso do Sul, Brazil	1992	1-2B	1-2B	5G	5G	5G	5G
Br108.1	Assai ,Parana, Brazil	1992	2B	1B	5G	5G	5G	5G
Br116.5	Santa Mariana , Parana, Brazil	1992	1B	2B	5G	5G	5G	5G
Br117.1	Santa Mariana , Parana, Brazil	1992	1B	1-2B	4G	5G	5G	5G
Br127.11	Sertanopolis, Parana , Brazil	1992	1B	1B	5G	5G	4G	5G
Br128.1	Sertanopolis, Parana , Brazil	1992	2B	1B	5G	5G	5G	5G
Br130.8	Rolandia , Parana , Brazil	1992	1-2B	2-3B	5G	5G	5G	5G

[a] Evaluated 5 days after inoculation. 0 = no visible evidence of 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] Wheat lines. *Triticum aestivum* 'S615', 'Shin-chunaga' (Sch), and 'Hope'

Table 3.2. Infection types of wheat seedlings with *Triticum* isolate Br48, *Eleusine* isolate MZ5-1-6, and F_1 culture 200R29 derived from the cross, MZ5-1-6 x Br48.

		Infection type[a] with					
		Br48		200R29		MZ5-1-6	
Wheat line	Lab code	22°C	25°C	22°C	25°C	22°C	25°C
Triticum aestivum 'S615'	S615	0-1B*	1-2B*	3BG*	5G	1B*	2B*
Triticum aestivum 'Shin-chunaga'	Sch	5G	5G	3-4G	5G	1B*	1B*
Triticum aestivum 'Hope'	Hope	5G	5G	5G	5G	0-1B*	0*
Triticum dicoccum KU120	St24	0*	1-2B*	1-2B*	5G	2-3B*	5G

[a] Evaluated 5 days after inoculation. 0 = no visible evidence of 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. Asterisks indicate resistant reactions.

Table 3.3. Responses of wheat seedlings to a population derived from the cross, Br48 x 200R29

Wheat line	No. of cultures												$\chi 2$ (1:1)
	0/1B[a]	2B	3B/3BG	4B/4BG/5B	3G	4G	5G	Subtotal	A[b]	U[c]	V[d]	Total	
S615	9	16	6	0	0	0	40	71	31	2	40	73	0.67[e] [(A+U):V]
													1.66[e] [A: (U+V)]
St24	14	11	6	0	0	0	42	73	31	0	42	73	1.66[e] [A:V]
Sch	0	0	0	0	0	5	68	73	0	0	73	73	-

[a] Infection type, 5 days after inoculation at 25°C. 0 = no visible evidence of 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] Avirulent (infection type 0 to 3BG)

[c] Unstable (fluctuate from 4B/4BG to 4G)

[d] Virulent (infection type 4G to 5G)

[e] Not significant at P=0.05

Table 3.4. The combined segregation of virulence on two wheat lines in a population derived from the backcross, Br48 x T17

Categorization of unstable cultures	No. of cultures				
	$A_{S615}A_{St24}$ [a]	$A_{S615}V_{St24}$	$V_{S615}A_{St24}$	$V_{S615}V_{St24}$	Total
Avirulent	31	2	0	40	73
Virulent	31	0	0	42	73

[a] A, avirulent (infection type 0 to 3BG); V, virulent (infection type 4G to 5G)

For example, $A_{S615}V_{St24}$ represents cultures that are avirulent on S615 and virulent on St24.

Table 3.5. Screening the SSR markers for mapping of *Rmg8* against Br48 in cv. S615

Chromosome	Checked markers	Amplified markers	Polymorphic markers
1A	29	28	17
1B	48	42	26
1D	24	23	8
2A	25	22	16
2B	31	30	19
2D	48	43	19
3A	41	35	15
3B	20	13	11
3D	48	43	15
4A	41	26	8
4B	36	26	14
4D	48	43	18
5A	37	30	12
5B	16	15	7
5D	32	19	14
6A	23	20	10
6B	22	20	9
6D	48	45	15
7A	37	31	12
7B	20	16	11
7D	51	43	17
Total	725	613	293

CHAPTER IV

Molecular cloning of an avirulence gene, *AVR-Rmg8*, corresponding to *Rmg8*

4.1. Introduction

Many strategies have been developed to breed resistant varieties, but often such attempts have been negated by the pathogen that overcame the resistance. Such a breakdown of resistance was caused by mutations of AVR genes leading to loss of recognition by the corresponding R gene. For instance, breakdown of rice R genes against *P. oryzae* has been caused by the insertion of transposons into AVR genes or their promoter sequences, point mutations, deletion, or translocation of AVR genes (Chuma et al. 2011). Therefore, cloning of AVR genes and evaluation of their stability may lead to predict durability of corresponding R genes. Furthermore, cloning of *AVR* genes is necessary to understand mechanisms of pathogenesis and mechanisms involved in co-evolution of fungal effectors and their host targets. In the previous chapter, I identified *Rmg8*, a dominant gene conferring strong resistance to *Triticum* isolate Br48 in common wheat cultivar S615. This gene was located on the long arm of chromosome 2B. In this chapter, I tried to clone its corresponding avirulence gene, *AVR-Rmg8*.

4.2. Materials and Methods

4.2.1. Fungal materials

A hybrid population derived from the cross between Br48 (avirulent on S615) and 200R29 (virulent on S615) were used for mapping. 200R29 was also used as a recipient for *P. oryzae* transformation assay.

4.2.2. Plant materials

Common wheat cultivars employed were S615 and Shin-chunaga (Sch), which are resistant and susceptible to Br48, respectively. *T. dicoccum* KU120 (St24), a tetraploid accession carrying *Rmg7*, was employed for a comparison of R genes (Tagle et al. 2015). Wheat seeds were pre-germinated and grown for 1 week as described above. Primary leaves were then fixed onto a hard plastic board for inoculation.

4.2.3. Infection assay

The 7-day-old wheat seedlings were inoculated with conidia, incubated at 25°C, and evaluated 5 days after inoculation as described in Chapter III. Pathogenicity tests for parents, progeny, and transformants were repeated three times.

4.2.4. Outline of mapping

We adopted two rounds of genetic analyses to delimit the location of the *AVR-Rmg8* gene. In the first round, bulked segregant analysis (BSA, Michelmore et al. 1991) was performed to screen Simple-Sequencing Repeat (SSR) markers produced by Zheng et al. (2008). Promising markers were then subjected to segregation analysis with 73 F_1 random cultures derived from Br48 x 200R29. In the second round, a larger population consisting of 449 random cultures (derived from the same cross) were screened with two SSR markers flanking *AVR-Rmg8*. Out of the 449 progenies, 58 recombinants were selected with the two flanking markers. For fine-mapping whole genome sequences of *Triticum* isolate Br48 and *Eleusine* isolate Z2-1 were screened for Single-Nucleotide Polymorphism Sites (SNPs), which were then converted to CAPs markers using SGN CAPS designer (http://solgenomics.net/)

4.2.5. PCR and electrophoresis

Total genomic DNA was isolated as described by Nakayashiki et al. (1999). Each PCR amplification was performed in a total volume of 20 μl containing 20 ng of genomic DNA, forward and reverse primers each at 0.5 μM, 10× PCR buffer, 1.5 mM $MgCl_2$, 200

µM dNTPs and 1 U of Taq DNA polymerase. The PCR consisted of a first step at 94°C for 2 min followed by 30 cycles of 30 s denaturation at 94°C; 30 s annealing at 50-60°C (based on T_m of primers); 1 min extension at 72°C and 5 min at 72°C for final extension. Amplified products were electrophoresed in 4.0% polyacrylamide gels (NacalaiTesque, Kyoto, Japan) and the banding patterns were visualized using SILVER SEQUENCE DNA Staining Regents (DNA Silver Staining System; Promega Crop., Madison, WI). For CAPs markers, a 10 µl sample of each reaction was loaded onto a 0.7 % agarose gel to ascertain whether PCR amplification was successful. The remaining reaction (10 µl) was digested with relevant restriction enzymes following manufacturer's instructions. Digested samples were separated on a 0.7 % agarose gel containing TAE buffer at 110 V for 20 min and visualized under UV light after staining with ethidium bromide.

4.2.6. Complementation test

The candidate genes were amplified by PCR using primers listed in Table 4.1 and ligated into pBluescript II SK (+) digested with *Eco*RV. Ligation products were transferred into *E.coli* DH5α. Cloned fragments were transformed into protoplast of 200R29 through PEG-mediated cotransformation with pSH75 carrying a hygromycin B phosphotransferase gene as described by Tosa et al. (2005). Ten stable transformants were obtained for each clone. They were grown on potato-dextrose agar medium for complementation test.

4.3. Results and Discussion

4.3.1. Rough mapping of *AVR-Rmg8*

As described in Chapter III, avirulent and virulent cultures segregated in a 1:1 ratio in the 73 cultures derived from Br48 x 200R29. They were considered to be carriers and non-carriers, respectively, of *AVR-Rmg8*. Five cultures were arbitrary chosen from

each of the carriers and non-carriers, i.e., S5, S7, S12, S15, and S24 from the avirulent cultures and S26, S33, S37, S44, and S64 from the virulent cultures. Equal amounts of their genomic DNA were mixed, resulting in R bulk and S bulk.

To determine chromosomal location of *AVR-Rmg8*, a total of 200 SSR markers (Zheng at al. 2008) covering the majority of the genome of *P. oryzae* were screened by BSA. Markers polymorphic between the two bulks were MGM282, MGM147, MGM292, MGM294, MGM296, MGM301, and MGM302 together with *MAT1-2* and pyrm07.08, all of which were located on chromosome 7. These markers were subjected to a linkage analysis with avirulence/virulence using 67 cultures (excluding 6 unstable or attenuated cultures) derived from Br48 x 200R29. The analysis showed that *AVR-Rmg8* was flanked by *MAT1-2* and MGM301 with one recombinant (Fig. 4.1).

4.3.2. Fine mapping of *AVR-Rmg8*

For further narrowing down the genetic region of *AVR-Rmg8* we increased the size of mapping population from 73 to 449 and screened for recombinants with two flanking markers, *MAT1-2* and MGM301. Out of 449 cultures, 58 recombinants were selected.

When the whole genome sequence of Br48 was analyzed, 10 candidate genes were found between the two flanking markers. We developed CAPs markers by comparing the whole genome sequence of Br48 with that of *Eleusine* isolate Z2-1. Typing of the recombinants with these new markers revealed that *AVR-Rmg8* is located on a 48kb fragment flanked by 1338_1_2 and 1106_3_1 (Fig. 4.2). This fragment contained 5 genes (755-4; 755-2; 2570-1; 4315-4; 1106-3), all of which co-segregated perfectly with *AVR-Rmg8*.

4.3.3. Complementation test

The five clones 755-4, 755-2, 2570-1, 4315-4, and 1106-3 were tentatively designated as I, II, III, IV, and V, respectively. Ten transformants for each fragment (I-V) were sprayed on S615 and Sch. All transformants containing fragments I, II, III or V were virulent on S615 whereas six of the ten transformants containing fragment IV showed avirulence on S615 (Fig. 4.2). The virulence of the four other transformants with fragment IV was not determined because they did not produce lesions on susceptible cultivar Sch lacking *Rmg8*. These cultures might have lost their basic pathogenicity during transformation. From these results, the 2.3-kb fragment IV (4315_4) was presumed to contain *AVR-Rmg8*.

To localize *AVR-Rmg8* further, 5 subclones of fragment IV (4315_4) were produced by PCR amplification using the primer sets listed in Table 4.1 followed by their ligation into pBluescript II SK (+). Three clones (Vb, Vc, and Vd) were lacking the 3′ end of fragment IV while two fragments (Ve and Vf) were lacking its 5′ end (Fig. 4.3). They were introduced into 200R29. Ten transformants for each of the 5 clones were sprayed on S615. Transformants containing Vb, Ve and Vf were avirulent on S615 whereas those with Vc and Vd were virulent (Fig. 4.3). Consequently, the region harboing *AVR-Rmg8* was narrowed down to the 829-bp region shared by all the four fragments.

The 829-bp region designated as fragment Vg was amplified by PCR and resequenced. The DNA sequence of Vg contained two open reading frames (ORFs) including a longer ORF (330 bp) encoding a polypeptide of 110 amino acids and a short ORF (240 bp) encoding a polypeptide of 80 amino acids, both of which started with ATG (Fig. 4.3). However, we have yet to identify which ORF encodes *AVR-Rmg8*. In addition, there remains a possibility that other gene candidates exist because the prediction of intron–exon structure has not been performed as yet.

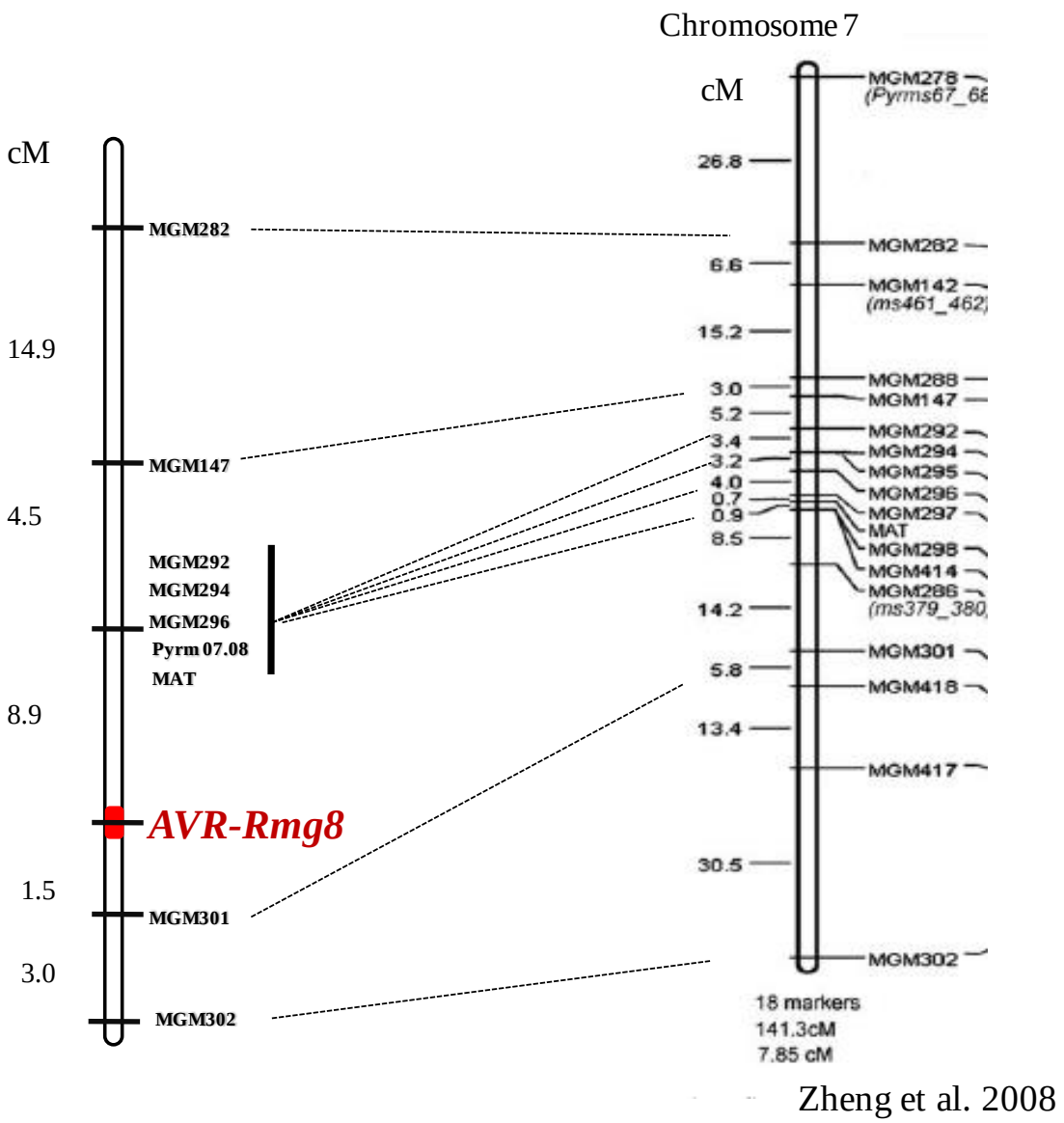

Fig. 4.1. Genetic map around *AVR-Rmg8* constructed using 67 BC_1F_1 cultures derived from the cross, Br48 x 200R29

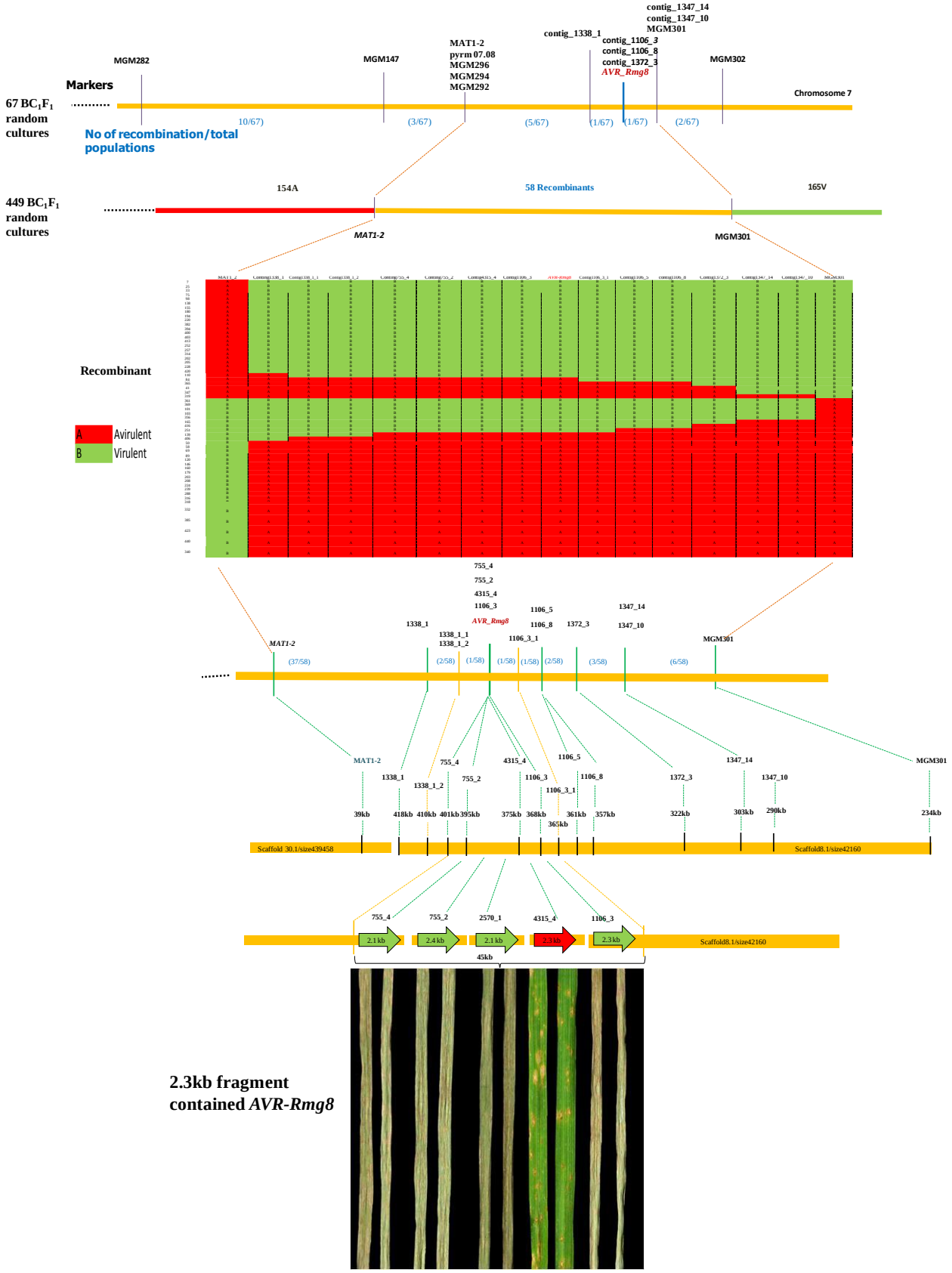

Fig. 4.2 Fine map of *AVR-Rmg8* constructed using 449 BC_1F_1 cultures derived from the cross, Br48x200R29

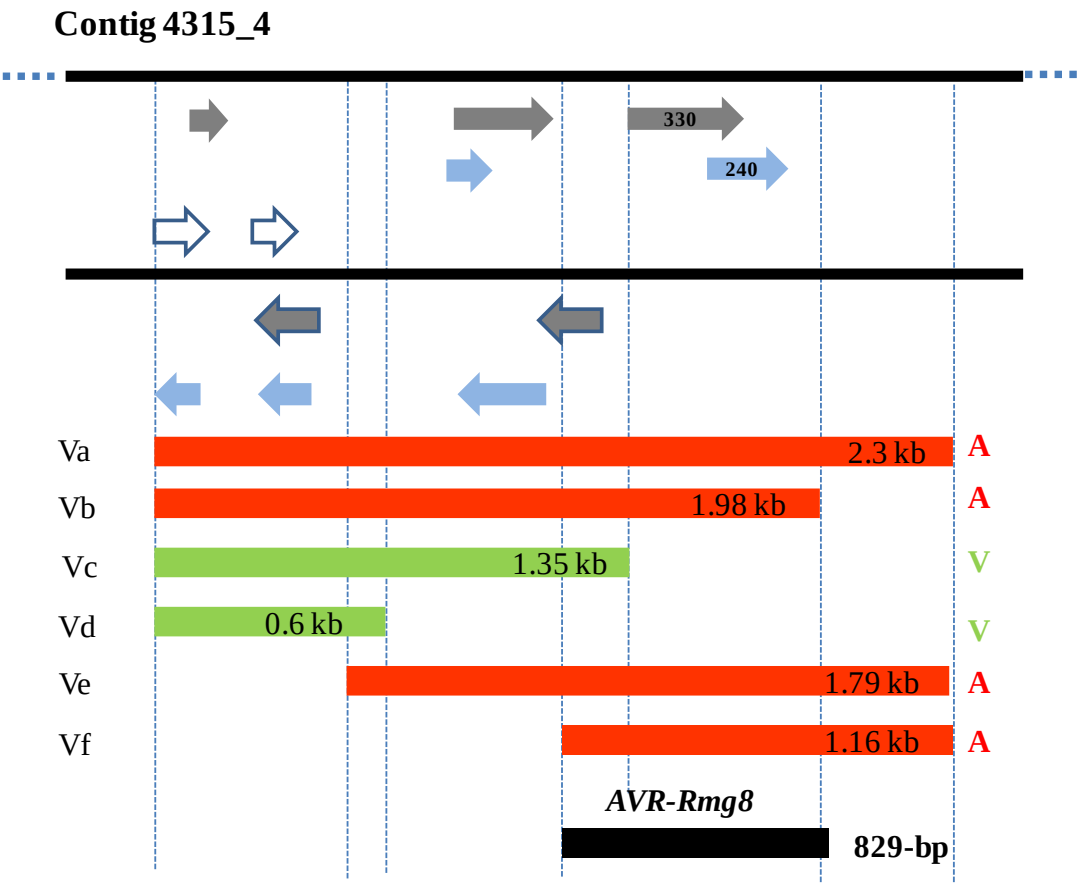

Fig.4.3. Complementation tests with subclones of the 2.3kb fragment of contig 4315_4 .

Arrows indicate ORFs. A: Avirulent; V: Virulent

Table 4.1. List of primers used for sub-cloning

Target fragment	Primer pair (F/R)
contig_755_4_F	AACCAAAGGCCCAACTACTG
contig_755_4_R	GCATTGTTGACTCGAAGTGC
contig_755_2_F	GTCCTGGCCTTTTCATTGCG
contig_755_2_R	CAGAGCTGATAGGAAGGCGG
contig_2570_1_F	GCGCCTACCAGAGCATGTAT
contig_2570_1_R	CGCACCGTACAAGCAAAAGG
contig_4315_4_F	TGCGGCTGTGTATTGTACCT
contig_4315_4_R	TTGCACCATTTATCGCCTGC
contig_1106_3_F	CGGCCTCATATCTTACCACG
contig_1106_3_R	ATGGAGCTGGACGATTTCAC
4315_4_Va_F	TGCGGCTGTGTATTGTACCT
4315_4_Va_R	TTGCACCATTTATCGCCTGC
4315_4_Vb_R	ACCACGCCCTTCAAATAGGA
4315_4_Vc_R	GCATGGTGGGAAATCAAAGCA
4315_4_Vd_R	AGGTGCGATTTGCTATCCAGTG
4315_4_Ve_F	CAAAAGGTTGCACCCAGTGT
4315_4_Vf_F	AAGCAGCCTAGCCACTGAAG
4315_4_Vg_F	CGACTGAACACTCGGAGCTT

CHAPTER V

General Discussion

In this study we identified a new resistance gene *Rmg8* in S615, which is a fully-awned, brown-glumed Portuguese variety obtained by the Experimental Farm at Swift Current from Lincoln College, Christchurch, New Zealand (Larson 1959). This *R* gene is expressed at both 22°C and 25°C in the seedling stage. Moreover, this R gene also seems to confer resistance at the heading stage. There are several reports showing the change of resistance level during plant development. For example, young plants of *indica* rice cultivar 'Modan' carrying *Panicle blast 1* (*Pb1*) is susceptible to blast disease, while its mature plants are resistant, and the resistance is durable even after heading (Fujii and Hayano-Saito 2007). However 'S615' showed high resistance at the seedling and heading stage. These finding suggest that this *R* gene may be useful for breeding new cultivars resistant to *P. oryzae.*

Aside from that, a hidden *R* gene *RmgTd*(*t)* against a laboratory strain of *P.oryzae* was identified using a mutant strain. This gene was located on chromosome 7B in tetraploid accession Tat4. The incompatibility associated with *RmgTd*(*t*) occurs only when it encounters cultures with the color mutation. There may be other hidden resistance genes in the wheat genome.

Besides the identification of resistance genes, more than 40 *AVR* genes have been identified in *P. oryzae* but only a few avirulence genes have been characterized such as *AVR-pita* (Orbach et al. 2000), *AVR-CO39* (Farman and Leong 1998), *PWL1* (Kang et al. 1995), *PWL2* (Sweigard et al. 1995) and *ACE1* (Bohnert et al. 2004). The first pair of

cloned resistance gene and avirulence gene was *Pi-ta* and *AVR-pita* (Bryan et al. 2000; Orbach et al. 2000). *Pi-ta* has been used as an effective resistance gene to control rice blast in southeast production areas of US, and is now commonly found in rice germplasm throughout the world (Wang et al. 2007).

In this study, we identified a pair of genes, *Rmg8* and *AVR-Rmg8*, interacting in a gene-for-gene manner. *AVR-Rmg8* was narrowed down to a 829-bp region. This region contained two open reading frames (ORFs) starting with ATG; a long ORF (330 bp) encoding a polypeptide with 110 amino acids and a short ORF (240 bp) encoding a polypeptide with 80 amino acids (Fig. 4.3). Their homologous sequence could not be detected in the genomic database of *P. oryzae.* It should be noted that *AVR-Rmg8* could be cloned through a simple chromosome walking. This suggest that the genomic structure around *AVR-Rmg8* is very simple. Thus, *AVR-Rmg8* may be located on a stable genomic region. If an AVR is stable, its corresponding R gene should be durable. *Rmg8* may be a durable resistance gene.

More interestingly, *Rmg8* and *Rmg7* are located in the distal regions of long arms of homoeologous chromosomes 2B and 2A, respectively (Fig. 3.3). There is a possibility that *Rmg8* in hexaploid wheat and *Rmg7* in tetraploid wheat are homoeologous genes. To verify the homoeologous relationship between *Rmg8* and *Rmg7*, fine mapping of these genes are needed.

Summary

In this study, I identified two resistance genes to *P. oryzae* and cloned an AVR gene. First, during the process of backcrossing, I found a new fungal gene associated with mycelial color (black/white). This color mutation was perfectly associated with virulence/avirulence on a specific cultivar. By using the color mutants a hidden resistance gene, *RmgTd(t),* was identified in a tetraploid accession. Molecular mapping showed that *RmgTd(t)* was located on chromosome 7B. Cytological analysis revealed that the moderate resistance controlled by *RmgTd(t)* was associated with hypersensitive reaction of mesophyll cells.

Second, a new gene for resistance to *Triticum* isolates of *P. oryzae* was identified in common wheat cultivar S615 and designated as *Rmg8* (Resistance to *Magnaporthe grisea 8*). This gene was located on the long arm of chromosome 2B (Fig. 3.3).

Finally, an avirulence gene corresponding to *Rmg8* was identified and designated as *AVR-Rmg8*. *AVR-Rmg8* was mapped between two markers, *MAT1-2* and *MGM301,* located on chromosome 7. Based on fine mapping and complementation tests, *AVR-Rmg8* was delimited to a 829-bp fragment. This is a key step toward performing functional and structural analyses of *AVR-Rmg8* for evaluating the durability of *Rmg8.*

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