



Genetic and molecular mechanisms of the evolution of wheat blast fungus from its relatives

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Doctoral dissertation

**Genetic and molecular mechanisms of the
evolution of wheat blast fungus from its relatives**

コムギいもち病菌のその近縁系統からの
進化メカニズムの遺伝的・分子生物学的解析

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Chapter 1

General introduction

Plant defenses against pathogens are composed of by two lines. The first line provides defense against all potential pathogens through recognizing the presence of pathogens via pathogen-associated molecular patterns (PAMP), which results in PAMP-triggered immunity (PTI) and prevents infection and/or limit reproduction after infection of pathogens (Rouxel et al. 2010). The second one follows gene-for-gene interactions in which one resistance gene product recognizes one matching avirulence gene product (Flor 1956). Plant – pathogen co-evolution in nature according to any change in the virulence of pathogens appears to continually balance by changes in the resistance of their host, and vice versa.

Host species specificity and cultivar specificity are two typical types of host – parasite specificity between plants and pathogen. Some of host species specificity were demonstrated to be conditioned by gene-for-gene interactions. The forma specialis - genus specificity in *Blumeria graminis* is an example. *B. graminis*, a causal agent of powdery mildew disease on gramineous plants, is composed of formae speciales, each of which infects just one plant genus. Tosa and his coworkers (Tosa 1989a, b) identified four avirulence genes in *B. graminis* f. sp. *agropyri* (virulent on *Agropyron*, but avirulent on wheat) designated them as *Ppm10*, *Ppm11*, *Ppm14*, and *Ppm15*. They corresponded to four resistance genes of wheat, *Pm10*, *Pm11*, *Pm14*, and *Pm15*, respectively. They concluded that f.sp. *agropyri* is avirulent on wheat because of carrying avirulence genes recognized by resistance genes in wheat. These results suggested that the resistant of wheat to inappropriate formae speciales is conditioned by gene-for-gene interactions, and

intrinsically considered as cultivar resistance (Tosa, 2009). In addition, the subgroup-genus specificity in the *Pyricularia oryzae*-gramineous plant system is also under the control of gene-for-gene interactions. *Pyricularia oryzae* (*Magnaporthe oryzae*) (Couch and Kohn 2002) is a causal agent of blast disease on 50 gramineous species (e.g., rice, foxtail millet, finger millet) but is classified into several genus-specific subgroups such as those pathogenic on *Oryza* spp., *Setaria* spp., *Eleusine* spp. (Kato et al. 2000). Isolates belonging to these subgroups have been designated using their host genera, e.g., *Oryza* isolates, *Setaria* isolates, *Eleusine* isolates and so on. Valent and her coworkers (Valent and Chumley 1994; Valent et al. 1986) identified genes *PWL1* and *PWL2* conditioning the pathogenicity toward weeping lovegrass. Cloning of these genes revealed that *PWL1* and *PWL2* conditioned avirulence of *Eleusine* and *Oryza* isolates, respectively, toward weeping lovegrass, just like avirulence genes conditioning cultivar specificity (Kang et al. 1995; Sweigard et al. 1995). Two fungal avirulence loci of *Avena* isolate Br58, *Pwt3* and *Pwt4*, were found by crossing a *Triticum* isolate Br48 with the *Avena* isolate (Takabayashi et al. 2002). *Rmg1* was identified as a resistance gene in common wheat corresponding to the avirulence gene *PWT4* (Takabayashi et al. 2002). Recently, Nga et al. (2009) identified two genes, *Rmg4* and *Rmg5*, which conditioned the resistance of common wheat to a *Digitaria* isolate (*P. grisea*), suggesting that the resistance to the inappropriate species is also conditioned by gene-for-gene interactions.

The most cloned resistance gene encodes proteins of a conserved class, whereas pathogen avirulence genes are highly diverse. To overcome host resistance, many avirulence genes evolved through loss of gene function via deletions, gene disruption or point mutation (Farman et al. 2002). The loss of specific avirulence gene might play a key role in the evolution of subgroups of *P.*

oryzae (Tosa, 2009). *AVR1-CO39* is an avirulence gene derived from an weeping lovegrass isolate corresponding to resistance gene *Pi-CO39(t)* in rice cultivar CO39. The loss of *AVR1-CO39* during the early stage of evolution of the *Oryza* subgroup was verified (Tosa et al. 2005). *AVR1-CO39* homologs are present in *P. oryzae*. Among *P. oryzae* isolates, *Oryza* isolates are noncarriers of an intact *AVR1-CO39*, while *AVR1-CO39* homologs in other subgroups including *Seteria*, *Panicum*, *Eleusine*, *Triticum*, *Avena*, and *Lolium* carry a functional avirulence gene (Tosa et al. 2005). Under selection pressure *Oryza* isolates lost the function of *AVR1-CO39* through deleting one part or the entire gene to overcome the resistance *Pi-CO39* in rice (Farman et al. 2002, Tosa et al. 2005). Besides, repetitive DNA elements have been a critical factor in genetic evolution. *AVR-Pita*, an avirulence gene identified in rice isolate, matches the resistance gene *Pi-ta* in rice cultivars. A variety of mutation types in *AVR-Pita* was found, including deletion, point mutations, and insertion of a transposon (Orbach et al. 2000, Kang et al. 2001). For example, the fungus became virulent on a cultivar with *Pi-ta* because of insertion of a DNA-type transposon *Pot3* into the promoter region of the gene (Kang et al. 2001).

Wheat blast disease is new subgroups of *P. oryzae* first reported in 1985 in the State of Parana in Brazil. The wheat blast disease spread to other areas and became a serious problem in wheat production just in a short time after the first report. Furthermore, the wheat blast disease appeared in Kentucky, USA, in 2011. Its causal agent was suggested to have arisen through the evolution of a new strain of *P. oryzae* from the annual ryegrass pathogen in favorite environment for disease development (<http://news.ca.uky.edu/article/uk-researchers-find-important-newdisease>). This hypothesis is supported by the close relationship between the wheat pathogen and other grass pathogens causing severe disease on perennial

(*Lolium perenne*), annual ryegrasses (*Lolium multiflorum*), finger millet (*Eleusine coracana*), and oat (*Avena sativa*) (Oh et al. 2002, Tosa et al. 2004, Kusaba et al. 2006). However, there are many gaps in understanding the evolution of the wheat blast pathogen (*Triticum* isolates). 1) The origin of *Triticum* isolates has not been elucidated. 2) *Triticum* pathogen may be able to hide within other plant species which have not yet been determined. Host plant resistance is one significant management plan to prevent yield loss by wheat blast. To date, however, durable resistance has been lacking.

In the 1990s a new genus-specific subgroup of *P. oryzae* evolved in the U.S.A., that is, *Lolium* isolates causing gray leaf spot on perennial ryegrass (*Lolium perenne*) (Tosa et al. 2004, 2007). Gray leaf spot on perennial ryegrass was first reported in southeastern Pennsylvania in 1991 (Landschoot and Hoyland 1992). This disease spread rapidly through the Mid-Atlantic region in 1995 and then expanded to other regions in the U.S.A. and Japan (Tosa et al. 2004, 2007). Interestingly, *Lolium* isolates were genetically close to isolates from wheat and triticale (Farman 2002). *Triticum* isolates and *Lolium* isolates are new groups that appeared in the 1980s and 1990s, respectively. It is reasonable to hypothesize that both groups are derived from the same “mother population” of *P. oryzae*.

The objective of this study is to identify genes in common wheat for resistance to a *Lolium* isolate of *P. oryzae* and their corresponding avirulence genes. Once the avirulence gene of *Lolium* isolate is cloned, their corresponding loci in *Triticum* isolates will be examined. A close relationship between *Triticum* isolates and *Lolium* isolates has been demonstrated. Therefore, we will be able to understand how *Triticum*, and *Lolium* isolates have evolved from their common ancestor based on the comparison of the avirulence loci between the two groups.

Chapter 2

Genetic analysis of host-pathogen incompatibility between *Lolium* isolates of *Pyricularia oryzae* and wheat

2.1 INTRODUCTION

The incompatibility between one host genus and pathogens of other host genera (inappropriate subgroups) is considered stable, and has been analyzed by several workers with an expectation that clues to breeding for durable resistance may be found in mechanisms of this type of incompatibility. Valent and her coworkers (Valent and Chumley 1994; Valent et al. 1986) identified genes *PWL1* and *PWL2* conditioning the pathogenicity toward weeping lovegrass, in *P. oryzae*. Cloning of these genes revealed that *PWL1* and *PWL2* conditioned avirulence of *Eleusine* and *Oryza* isolates, respectively, toward weeping lovegrass, just like avirulence genes conditioning cultivar specificity (Kang et al. 1995; Sweigard et al. 1995).

Tosa and his colleagues identified several genes for resistance to *P. oryzae* in common wheat, *Triticum aestivum*. ‘Thatcher’ was found to be resistant to all *Triticum* isolates of *P. oryzae* tested. This resistance was controlled by two genes located on chromosomes 7A and 6B, and designated *Rmg2* and *Rmg3*, respectively (Zhan et al. 2008). These genes governed resistance to isolates of an appropriate subgroup. They also identified three genes that governed the resistance of common wheat against inappropriate subgroups. The first, identified in ‘Norin 4’, was designated as *Rmg1* (synonymous to *Rwt4*). *Rmg1* corresponded to *PWT4*, an avirulence gene carried by an *Avena* isolate (Takabayashi et al. 2002), and conditioned the resistance of common wheat to this inappropriate subgroup.

Recently, Nga et al. (2009) identified two genes, *Rmg4* and *Rmg5*, which conditioned the resistance of common wheat to a *Digitaria* isolate (*P. grisea*) and suggested that the resistance to the inappropriate species was also under simple genetic control.

In this chapter, we focused on genetic mechanisms of interactions between common wheat and a *Lolium* isolate of *P. oryzae*. We successfully demonstrated that incompatibility between the *Lolium* isolate and common wheat cultivars was conditioned by two gene pairs, *R1-A1* resulting in strong resistance and *R2-A2* resulting in moderate resistance, that interacted in a gene-for-gene manner.

2.2 MATERIALS AND METHODS

2.2.1 Fungal strains and genetic cross

Parental field isolates were *P. oryzae* TP2 (*MAT1-2*) isolated from perennial ryegrass (*L. perenne*) in Japan in 1997 and Br48 (*MAT1-1*) isolated from wheat (*T. aestivum*) in Brazil in 1990. TP2 was virulent on perennial ryegrass and avirulent on wheat whereas Br48 was virulent on wheat and moderately avirulent on perennial ryegrass (Tosa et al. 2004). To reveal genetic mechanisms of this specific parasitism, TP2 was crossed with Br48 on an oatmeal agar medium (20 g of oatmeal, 10 g of agar, and 2.5 g of sucrose in 500 ml distilled water) as described by Murakami et al. (2000).

For long term storage, these cultures were transferred to sterilized barley seeds in vials, grown at 25°C until mycelia covered the barley seeds (~1 month), and kept in a case with silica gel at 4°C or room temperature at the Laboratory of Plant Pathology, Kobe University. Just before use for experiments, a grain was

transferred to potato dextrose agar (PDA, 24 g of potato dextrose broth powder (Difco, Maryland) and 15 g of agar powder (Nacalai Tesque, Kyoto) in 1 L of distilled water) in a test tube and incubated at 25°C.

2.2.2 Plant materials

Plant materials used for the preliminary screening were 30 cultivars of common wheat ($2n=6x=42$, genome AABBDD) provided by Dr. K. Tsunewaki, Kyoto University, and Dr. U. Hiura, Okayama University (Table 1). Based on results from the screening, five representative cultivars, Chinese Spring (CS), Shinchunaga (Sch), Norin 4 (N4), Selkirk (Sk), and Hope, were chosen for identification of resistance genes (Table 2). Wheat lines and their F₂ seeds were stored at 22°C or 4°C at the Laboratory of Plant Pathology, Kobe University. Seeds were pre-germinated on moistened filter paper in plastic Petri dishes for 24 h at 22°C, sown in a seedling case (5.5 x 15 x 10 cm) filled with vermiculite (Asahi-Kyoto, Okayama), and grown at 22°C in a growth chamber with a 12 h photoperiod for 7 days.

2.2.3 Infection assay

Agar plugs of *P. oryzae* isolates to be tested were transferred to oatmeal agar medium (20 g of oatmeal, 10 g of agar, and 2.5 g of sucrose in 500 mL of distilled water) in Petri dishes and incubated in darkness at 25°C for 6 days. Aerial mycelia on the 6-day-old cultures were washed off by rubbing mycelial surfaces with cotton balls. The colonies were exposed to near-ultraviolet light (black light, 360 nm, 40 W) at 25°C for 4 days to induce sporulation (Murakami et al. 2000, Zhan et al. 2008). The conidia produced were suspended in water and adjusted to $1\sim1.5 \times 10^5$ conidia/mL. Ten mL of the conidial suspension supplied with 0.01 % Tween 20 (Nacalai Tesque, Kyoto) were sprayed on the adaxial surface of 7-day-old

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 at 22°C. The inoculated seedlings were then transferred to growth chamber at 22°C with a 12h photoperiod. Three to five days after inoculation, symptoms were evaluated based on the color and the size of lesions (Takabayashi et al. 2002, Zhan et al. 2008). The size was rated using 6 progressive grades from 0 to 5: 0, no visible evidence of infection; 1, pinpoint spots; 2, small lesions (< 1.5 mm); 3, lesions with an intermediate size (< 3 mm); 4, large typical lesions; and 5, complete blighting of leaf blades. These lesions were classified into two categories on the basis of their color, i.e., brown (B) and green (G). Infection types were represented by the combination of the size (intensity) score and the color code (Fig. 1). For example, infection type 1B represents brown lesions with size 1 while infection type 4G represents green lesions with size 4. Infection type 0 and those with brown lesions were considered resistant or avirulent while those with green lesions were considered susceptible or virulent. Sometimes symptoms were accompanied by both brown and green lesions. These types, designated with GB (e.g. 3GB) were considered moderately resistant or moderately avirulent.

Each infection assay was repeated at least three times. For screening and pathogenicity test of F₁ cultures, six seedlings of each wheat lines were evaluated for each isolate per replication. To identify the resistance genes of common wheat against a *Lolium* isolate, at least 40 F₂ seedlings of each F₂ population were used per one trial.

2.3 RESULTS

2.3.1 Identification of resistance genes *R1* and *R2* in wheat cultivars

To identify genes conditioning the resistance of wheat to *Lolium* isolates, exceptional wheat lines susceptible to *Lolium* isolates are needed. As a result of screening of the 30 hexaploid wheat lines with a *Lolium* isolate, TP2, we found two such exceptions, Sk and Hope (Table 1). Consequently, we chose five cultivars for further genetic analysis; Sk and Hope as susceptible cultivars and CS, Sch, and N4 as resistant cultivars (Table 2).

When F₂ seedlings derived from crosses between the three resistant cultivars (CS, Sch, and N4) and a susceptible cultivar (Hope or Sk) were inoculated with TP2, highly resistant, moderately resistant, and susceptible seedlings segregated in 12:3:1 ratios (Table 3, Fig. 2). Crosses among the resistant cultivars yielded no susceptible F₂ seedlings (Table 3). These results suggest that the resistance of these three cultivars is controlled by the same two genes, one strong gene and one weak gene. They were tentatively designated as *R1* and *R2*, respectively.

2.3.2 Identification of avirulence genes *A1* and *A2* in a *Lolium* isolate

Triticum isolates are virulent on wheat, but *Lolium* isolates are avirulent. To reveal genetic mechanisms of this specific parasitism, *Lolium* isolate TP2 was crossed with *Triticum* isolate Br48. A total of 102 F₁ cultures were obtained from 4 mature asci with 8 ascospores and 10 mature asci with 7 ascospores derived from this cross. Based on fingerprints with MAGGY (Farman et al. 1996), MGR586 (Hamer et al. 1989), and MGR583 (Hamer et al. 1989; Valent and Chumley 1991), pairs of sister progenies were identified. F₁ cultures were designated as 301-K1, 301-K2, 301-L1, 301-L2, 301-M1, 301-M2, and so on. Here, the number just before the hyphen (underlined) indicates the ascus (set) number, and progenies with the same letter (K, L, M, and N) are sister progenies of a pair.

One sister progeny was arbitrarily chosen from each pair, and the resulting 56 cultures (14 tetrads) were employed for segregation analysis. When CS, N4, and Sch were inoculated with the 56 F₁ cultures, avirulent, moderately avirulent, and virulent cultures segregated in a 2:1:1 ratio (Table 4, Fig. 3). The segregation patterns on CS, N4 and Sch were completely concordant; the 28 cultures avirulent on CS were also avirulent on Sch/N4 while the 15 cultures virulent on CS were also virulent on Sch/N4. The 13 cultures moderately avirulent on CS were also moderately avirulent on Sch/N4. All cultures were virulent on the susceptible control, Sk, and Hope (Table 4). These results suggest that the avirulence of TP2 on CS, N4, and Sch is conditioned by the same, two unlinked genes, one highly effective and the other less effective. They were tentatively designated as *A1* and *A2*, respectively.

2.3.3 Demonstration of gene-for-gene interactions

The results mentioned above led us to a hypothesis that *R1* and *R2* correspond to *A1* and *A2*, respectively, in a gene-for-gene manner. If this is the case, a monofactorial segregation conditioned by *R1*, the strong gene playing the primary role in the resistance, should be detected by using an F₁ culture carrying *A1* alone. To demonstrate this hypothesis, a tetra type tetrad (set 305) was chosen from the 14 tetrads derived from the cross, TP2 x Br48. This tetrad was composed of two avirulent cultures (305K1, 305M2), one moderately avirulent culture (305L2), and one virulent culture (305N1) (Fig. 3). One of the two avirulent cultures should carry both *A1* and *A2* whereas the other should carry *A1* alone.

When F₂ seedlings derived from crosses between the resistant cultivars (CS, Sch, N4) and Hope were inoculated with 305M2, resistant (including moderately resistant) and susceptible seedlings segregated in a 15:1 ratio (Table 5), suggesting that 305M2 carries both *A1* and *A2*. On the other hand, resistant and susceptible

seedlings segregated in a 3:1 ratio against 305K1 (Table 5), suggesting that 305K1 carries *Al* alone. Crosses among CS, Sch, and N4 yielded no susceptible F₂ seedlings against 305K1 (Table 5), confirming that these cultivars probably carry the same gene, *R1*. The strong resistance gene *R1* was designated as *Rmg6*.

2.4 DISCUSSION

In the present study we successfully identified two resistance genes *R1* and *R2* as a common wheat gene conferring resistance to a *Lolium* isolate of *P. oryzae*. In our knowledge they are first resistant genes of common wheat against an inappropriate subgroup of *P. oryzae*, *Lolium* isolates.

In gene-for-gene systems found in race-cultivar interactions, a dominant resistance gene corresponds to an avirulence gene in the pathogen (Flor 1956). On the other hand, a genes-for-gene relationship was found in *Pyricularia* – barley interactions (Nga et al. 2012). In this system one resistance gene corresponds to two or more avirulence genes. A question is whether some of the previously identified resistance/avirulence genes are involved in the incompatible interaction between wheat and a *Lolium* isolate. For instance, Takabayashi et al. (2002) identified *Rmg1* (*Rwt4*) controlling resistance against *Avena* isolates. This gene was present in N4 but absent in CS. *R1* identified in the present study was present in both N4 and CS, indicating that *R1* is different from *Rmg1*. In N4, *R1* functions against *Lolium* isolates while *Rmg1* functions against *Avena* isolates. Zhan et al. (2008) found two resistance genes, *Rmg2* (7A) and *Rmg3* (6B), in ‘Thatcher’. However, they are genes for resistance to appropriate pathotype, *Triticum* isolates. N4 and CS are susceptible to all *Triticum* isolates that have been tested, and therefore, should not carry these resistance genes. Furthermore, *Rmg4* (4A) and

Rmg5 (6D), genes for resistance to *Digitaria* isolates identified by Nga et al. (2009), are also considered to be different from *R1* because they were identified by using CS as a susceptible cultivar. Similarly, *R2* should be different from *Rmg1*, *Rmg2*, *Rmg3*, *Rmg4*, and *Rmg5*, which are all absent in CS. Consequently, we conclude that the resistance of CS, N4, and Sch against *Lolium* isolate TP2 is conditioned by two new genes, *R1* and *R2*, which are different from previously reported resistant genes.

Crosses between the resistant cultivars (CS, N4, and Sch) and the susceptible cultivars (Hope and Sk) produced resistant, moderately resistant, and susceptible seedlings in a 12:3:1 ratio, indicating that *R1* is epistatic to *R2*. Apparently, *R1* plays the primary role in the resistance. We also identified two avirulence genes, *A1* involved in strong avirulence and *A2* involved in weak avirulence. The segregation of avirulent and virulent F_1 progenies on CS, N4 and Sch fitted a 2:1:1 ratio, indicating that *A1* is epistatic to *A2*. It is reasonable to assume that *A1* and *A2* correspond to *R1* and *R2*, respectively. Taken together, we conclude that the incompatibility between the *Lolium* isolate of *P. oryzae* and common wheat is conditioned by two gene pairs, *R1* – *A1* and *R2* – *A2*, which interact in a gene-for-gene manner. Takabayashi et al. (2002) suggested that the incompatibility between an *Avena* isolate and common wheat was conditioned by two gene pairs interacting in a gene-for-gene manner. Gene-for-gene relationships may generally underlie genus-specific parasitism in the *P. oryzae* – cereal/grass system.

Triticum isolates and *Lolium* isolates are new groups that appeared in the 1980s and 1990s, respectively. Wheat blast was first reported in the northern region of Parana, Brazil, in 1985, then rapidly spread to other wheat growing regions of the country, and became one of the major diseases of wheat in Brazil in 1990s (Urashima et al. 1993, 2004). Gray leaf spot on perennial ryegrass was first

reported in Pennsylvania in 1991 (Landschoot and Hoyland 1992), then spread to other states in U.S.A. and Japan (Tosa et al. 2004, 2007). Comparative analyses with repetitive elements and internal transcribed spacer (ITS) sequences of rDNA showed a close relationship between isolates from perennial ryegrass and those from wheat (Viji et al. 2001). Fingerprinting with the Pot2 and MGR583 transposons revealed that the perennial ryegrass pathogen was similar to the wheat pathogen (Farman 2002). *Lolium* isolates collected in Japan (including TP2) shared a 100% identical ITS2 sequence with *Triticum* and *Eleusine* isolates (Tosa et al. 2004). These results suggest that *Triticum* and *Lolium* isolates share the same genetic background. Furthermore, the present study showed that the incompatibility between a *Lolium* isolate and wheat is under simple genetic control. Therefore, there seems a potential risk that *Lolium* isolates jump hosts and become a threat to wheat production. Once the avirulence gene *Al* is cloned, we will be able to understand how *Triticum*, *Avena*, and *Lolium* isolates have evolved from their common ancestor.

Actually, wheat blast disease appeared in Kentucky, USA, in 2011. Its causal agent was suggested to have arisen through a host jump by the annual ryegrass pathogen, a close relative of the perennial ryegrass pathogen (<http://news.ca.uky.edu/article/uk-researchers-find-important-new-disease>). A comparison of the avirulence loci between the two groups will reveal how the host jump of the annual ryegrass pathogen occurred.

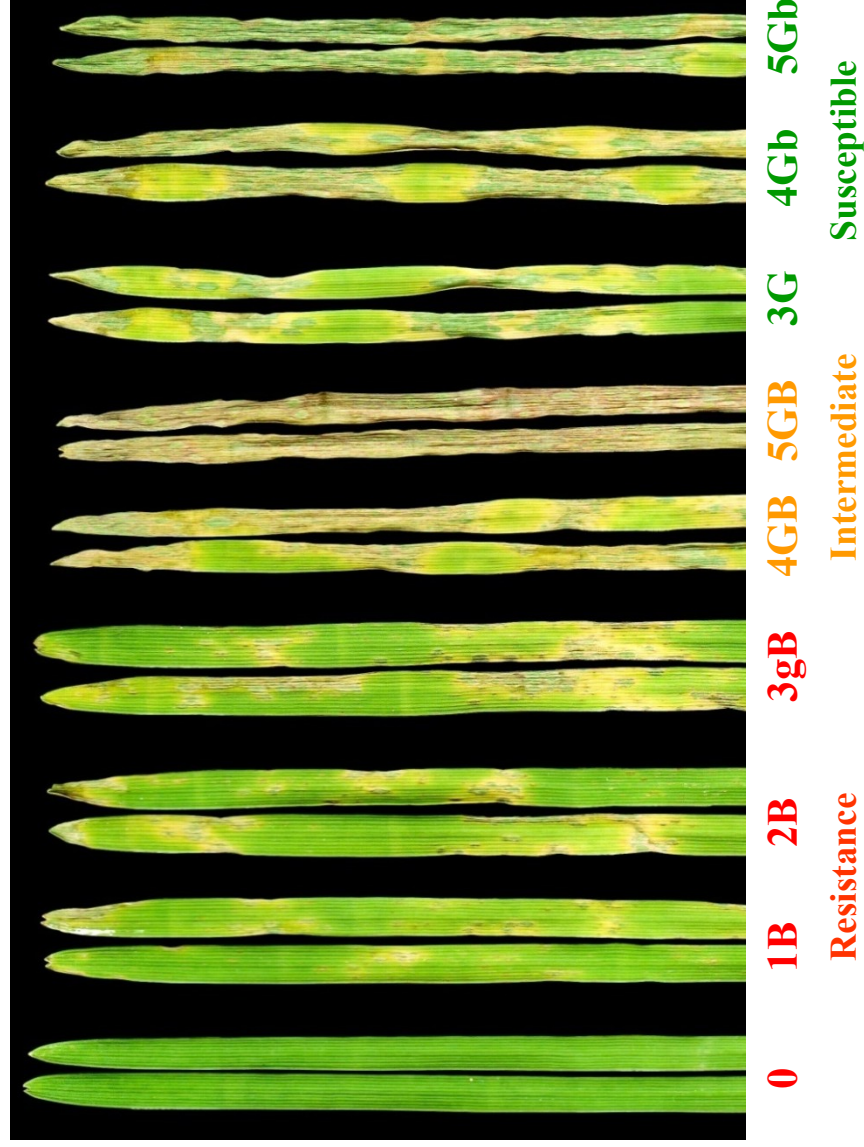


FIGURE 1. Infection types on common wheat leaves of N4 leaf based on the color and the size of lesions, five days after inoculation. **0**, no visible evidence of infection; **1**, pinpoint spots; **2**, small lesions (<1.5mm); **3**, lesions with an intermediate size (<3mm); **4**, large, typical lesions, and **5**, complete blighting of leaf blades. These lesions were classified into two categories on the basis of their color, i.e., brown (B) and green (G). Infection types were represented by the combination of the size (intensity) score and the color code.

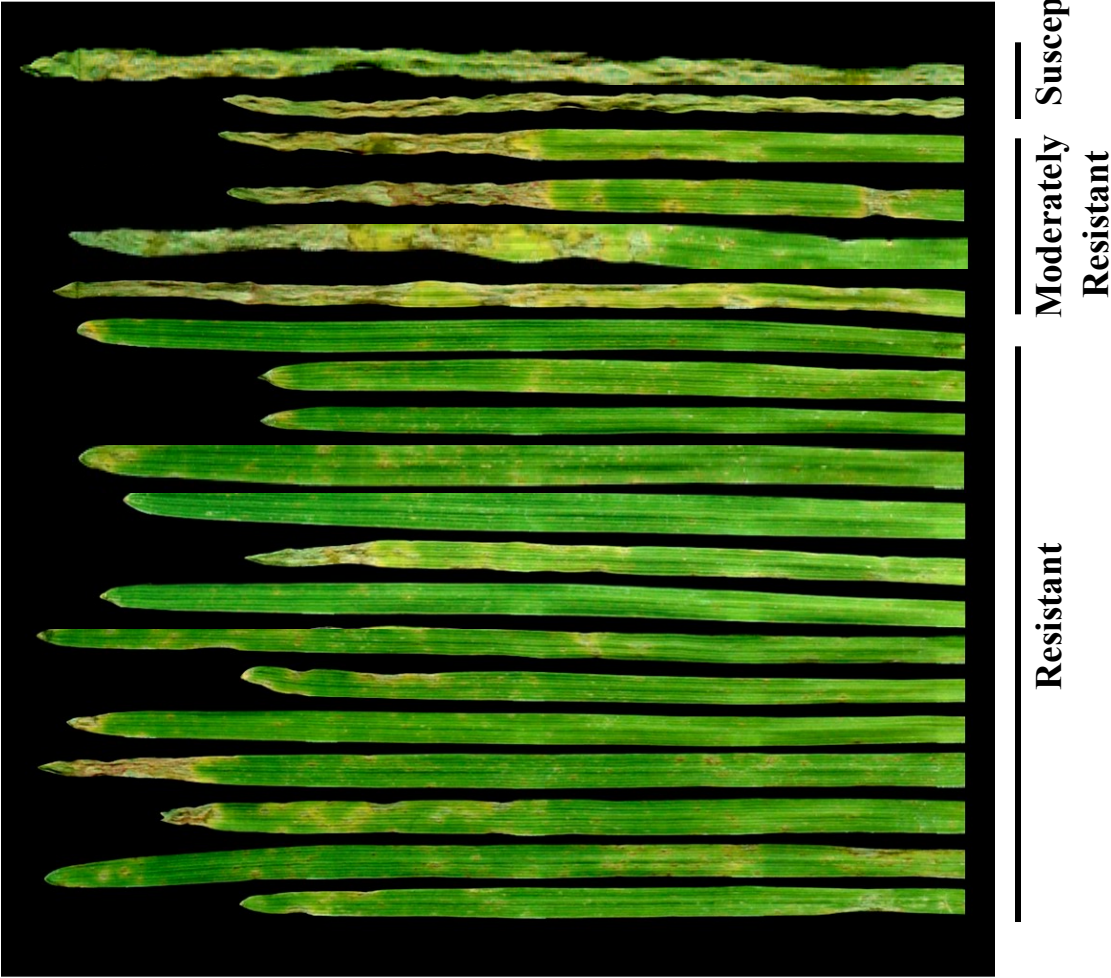


FIGURE 2. Segregation of reactions to *Lolium* isolate TP2 in F₂ seedlings derived from the cross, N4 x Hope, 5 days after inoculation.

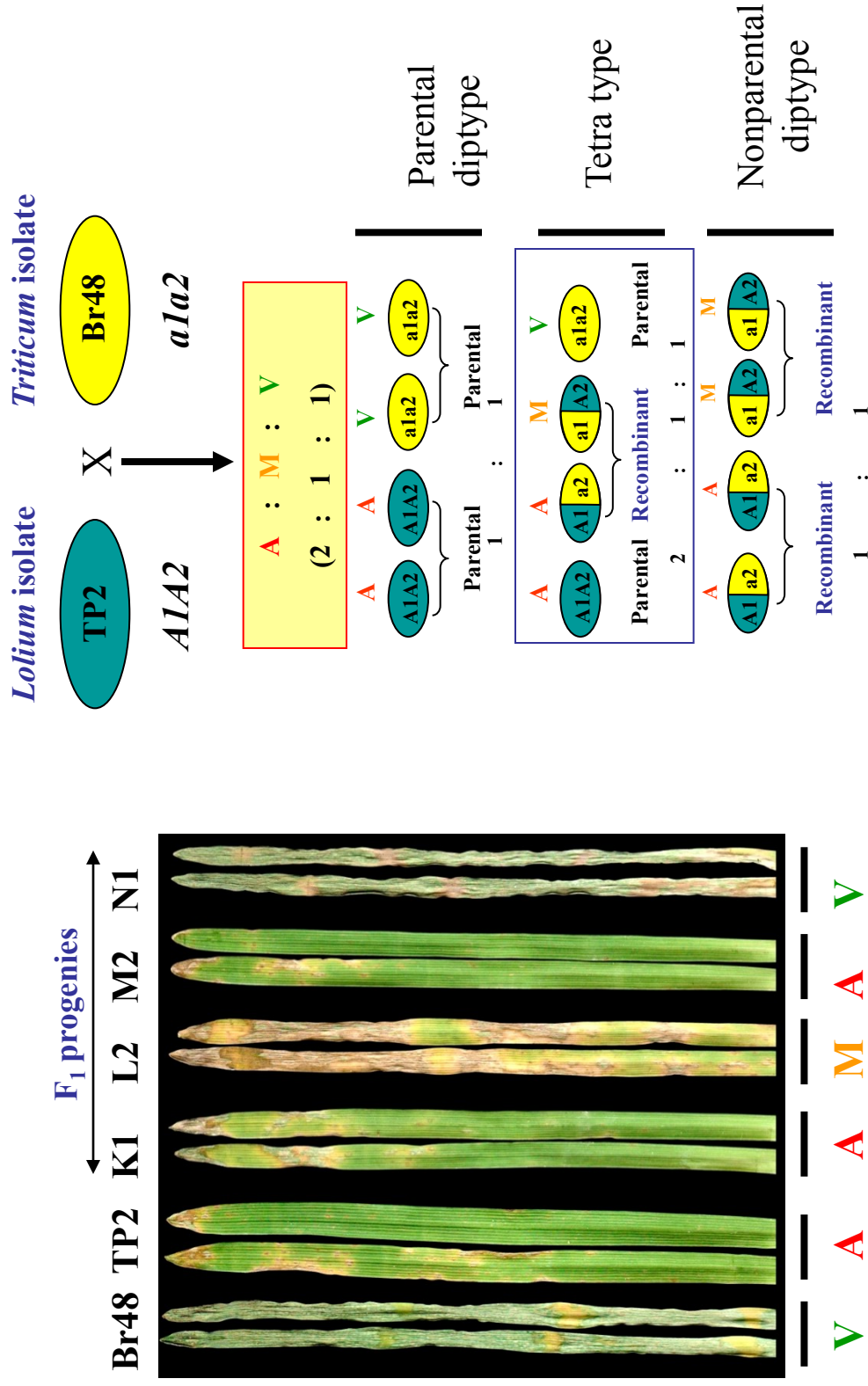


FIGURE 3. Symptoms on wheat cultivar N4, 5 days after inoculation with *Triticum* isolate Br48, *Lolium* isolate TP2, and a tetrad (305K1, 305L2, 305M2, and 305N1) derived from TP2 x Br48. A, avirulent; M, moderately avirulent; V, virulent.

TABLE 1. Common wheat cultivars used for screening and their infection types with a *Lolium* isolate TP2 at 22°C

Lab code	Species	Cultivar	Genome	Infection type at 22°C	
				1 st replication	2 nd replication
1502	<i>T. aestivum</i>	strain P168	ABD	5B	3B
1503	<i>T. aestivum</i>	cv. Chinese Spring (CS)	ABD	0	0
1504	<i>T. aestivum</i>	cv. Norin 26	ABD	1B	1B
1505	<i>T. aestivum</i>	strain Salmon	ABD	1B	1B
1506	<i>T. aestivum</i>	cv. Jones File	ABD	1B	1B
1507	<i>T. aestivum</i>	cv. Selkirk	ABD	5G	5G
1508	<i>T. aestivum</i>	cv. S615	ABD	0	0
1509	<i>T. sphaerococcum</i>	KU161	ABD	0	0
1510	<i>T. compactum</i>	KU151	ABD	1B	1B
1515	<i>T. aestivum</i>	cv. Shin-chunaga (Sch)	ABD	1B	1B
1520	<i>T. aestivum</i>	cv. Norin 10	ABD	1B	1B
1521	<i>T. aestivum</i>	cv. Norin 4 (N4)	ABD	1B	1B
1601	<i>T. aestivum</i>	cv. Hope	ABD	5Gb	5Gb
1602	<i>T. aestivum</i>	cv. Timstein	ABD	0	0
1603	<i>T. aestivum</i>	cv. Cheyenne	ABD	1B	1B
1835	<i>T. aestivum</i>	cv. Red Egyptian	ABD	0	1B
1857	<i>T. aestivum</i>	cv. Thatcher	ABD	2B	2B
H - 1	<i>T. aestivum</i>	cv. Chancellor	ABD	0	1B
H - 11	<i>T. aestivum</i>	cv. Renown	ABD	1B	1B
H - 13	<i>T. aestivum</i>	cv. Spica	ABD	0	1B
H - 14	<i>T. aestivum</i>	cv. Mendos	ABD	0	0
H - 15	<i>T. aestivum</i>	cv. Timvera	ABD	0	0
H - 16	<i>T. aestivum</i>	cv. Transec	ABD	0	0
H - 17	<i>T. aestivum</i>	cv. Transfed	ABD	1B	1B
H - 18	<i>T. aestivum</i>	cv. Kavkaz	ABD	4GB	2GB
H - 20	<i>T. aestivum</i>	cv. Normandie	ABD	1B	1B
H - 21	<i>T. aestivum</i>	cv. Little Club	ABD	1B	1B
H - 22	<i>T. aestivum</i>	cv. Hard Federation	ABD	1B	1B
H - 23	<i>T. aestivum</i>	cv. Ramona	ABD	0	0
H - 24	<i>T. aestivum</i>	cv. Mayo 64	ABD	1B	1B

TABLE 2. Representative common wheat cultivars and their infection types with *Triticum* isolate Br48 and *Lolium* isolate TP2

Cultivar	Infection type ^a with	
	Br48	TP2
Chinese Spring (CS)	4G	0
Shin-chunaga (Sch)	5G	1B
Norin 4 (N4)	5G	1B
Silkirk (Sk)	5G	5G
Hope	5G	5G

^a Refer to the text.

TABLE 3. Segregation of reactions to *Lolium* isolate TP2 in F₂ populations derived from crosses between wheat cultivars

Cross	No. of F ₂ seedlings			$\chi^2(12:3:1)$	P	
	R ^a	M ^b	S ^c			
CS x Hope	92	15	10	117	3.39	0.18
Sch x Hope	93	18	9	120	1.30	0.52
N4 x Hope	93	24	3	120	2.90	0.24
CS x Sk	90	19	9	118	0.83	0.66
Sch x Sk	95	20	10	125	1.13	0.57
N4 x Sk	88	20	10	118	1.14	0.57
CS x Sch	322	0	0	322		
CS x N4	494	0	0	494		
N4 x Sch	371	0	0	371		

^a Resistant (infection type 0-5B). ^b Moderately resistant (infection type 3GB-5GB).

^c Susceptible (infection type 3G-5G).

TABLE 4. Segregation of reactions on wheat cultivars in tetrad F₁ cultures derived from the cross between *Lolium* isolate TP2 and *Triticum* isolate Br48

Cultivar	No. of F ₁ cultures			χ^2 (2:1:1)	P
	A ^a	M ^b	V ^c	Total	
CS	28	13	15	56	0.93
Sch	28	13	15	56	0.93
N4	28	13	15	56	0.93
Sk	0	0	56	56	...

^a Avirulent (infection type 0-5B). ^b Moderately avirulent (infection type 3GB-5GB).
^c Virulent (infection type 3G-5G).

NOTE: The 28 cultures avirulent on CS were also avirulent on Sch/N4 while the 15 cultures virulent on CS were also virulent on Sch/N4. The 13 cultures moderately avirulent on CS were also moderately avirulent on Sch/N4.

TABLE 5. Segregation of reactions to F₁ cultures derived from TP2 x Br48 in F₂ populations derived from crosses between wheat cultivars

F ₁ culture (genotype)	Cross	No. of F ₂ seedlings			$\chi^2(3:1)$	$\chi^2(15:1)$
		R+M ^a	S ^b	Total		
305M2 (<i>Al;A2</i>)	CS x Hope	114	5	119	27.45**	0.85
	Sch x Hope	112	4	116	28.74**	1.55
	N4 x Hope	114	5	119	27.45**	0.85
305K1 (<i>Al;a2</i>)	CS x Hope	114	44	158	0.68	125.79**
	Sch x Hope	122	34	156	0.86	64.34**
	N4 x Hope	86	33	119	0.47	93.72**
	CS x Sch	372	0	372		
	CS x N4	504	0	504		
	N4 x Sch	414	0	414		

^a Resistant (infection type 0-5B) + Moderately resistant (infection type 3GB-5GB).

^b Susceptible (infection type 3G-5G).

** Significant at the 1% level.

Chapter 3

Genetic mapping of the resistance gene *R1* (*Rmg6*)

3.1 INTRODUCTION

Hexaploid wheat (*T. aestivum*, 2n=42, AABBDD) contains a huge genome including 21 pairs of chromosomes with seven pairs belonging to each of the A, B, and D genomes. The huge genome of hexaploid wheat had been a great obstacle in mapping or isolation of target genes from wheat genome. Some genetic tools were demonstrated to be useful for locating genes on different chromosome such as monosomic analysis in wheat or wheat chromosome substitution lines developed since the 1950s (Singh 1967, Doekes and Belderok 1976). Gene mapping in common wheat today is usually performed by using molecular markers such as RFLP (restriction fragment length polymorphism), RAPD (random amplification of polymorphic DNA), or microsatellite (simple sequence repeat, SSR). Among these, SSR markers show a higher level of polymorphism in hexaploid wheat compared with other marker systems, and widely used (Plaschke et al. 1995, Roder et al. 1998).

Tosa and his colleagues identified several genes for resistance to *P. oryzae* in common wheat, and their locations were identified using wheat monosomic lines or series of wheat chromosome substitution lines. *Rmg1* (*Rwt4*), controlling resistance to *Avena* isolates, was located on chromosome 1D using CS (Cheyenne) chromosome substitution lines (Hirata et al. 2005). Two resistance genes to *Triticum* isolates, *Rmg2* and *Rmg3*, were located on 7A and 6B, respectively, using CS (Thatcher) chromosome substitution lines (Zhan et al. 2008). Furthermore, *Rmg4* and *Rmg5*, resistance genes of wheat cultivars to *Digitaria* isolates were

located on 4A and 6B, respectively. *Rmg4* was located on chromosome 4A by a monosomic analysis of F₂ populations derived from crosses between 21 CS monosomic lines and N4, while *Rmg5* was located on chromosome 6B by using CS (Red Egyptian) chromosome substitution lines (Nga et al. 2009).

In this chapter, a hexaploid wheat resistance gene, *R1(Rmg6)*, to a *Lolium* isolate of *P. oryzae*, TP2, was located on chromosome 1D by CS (Hope) chromosome substitution lines and molecular mapping with SSR markers.

3.2 MATERIALS AND METHODS

3.2.1 Identification of the chromosome carrying the resistance gene *Rmg6*

Fungal materials

Lolium isolate TP2 and F₁ cultures derived from a cross between TP2 x Br48, 305K1 (*Al;a2*) and 306L2 (*Al;a2*), were used. TP2 isolate and its F₁ cultures have been stored in sterilized barley grains under dry conditions at 4°C at the Laboratory of Plant Pathology, Kobe University. Just before use for experiments, a grain was transferred to potato dextrose agar (PDA, 24 g of potato dextrose broth powder (Difco, Maryland) and 15 g of agar powder (Nacalai Tesque, Kyoto) in 1L of distilled water) in a test tube and incubated at 25°C.

Plant materials and infection assay

CS – Hope chromosome substitution lines were used to identify the chromosome carrying the resistance gene *Rmg6*. The chromosome substitution lines were stored at 22°C or 4°C at the Laboratory of Plant Pathology, Kobe University. Seeds were pre-germinated on moistened filter paper in plastic petri dishes for 24 h at 22°C, sown in a seedling case (5.5cm x 15cm x 10cm) filled with vermiculite (Asahi-Kyoto, Okayama), and grown at 22°C in a growth chamber with a 12h photoperiod for 7 days.

Infection assay was performed as described in chapter 2. The conidia produced were suspended in water and adjusted to a concentration of $1-2 \times 10^5$ conidia/mL. Ten mL of the conidial suspension supplied with 0.01% Tween 20 (Nacalai Tesque, Kyoto) were sprayed on the adaxial surface of 7-day-old primary leaves in a plastic case with an air compressor. The cases were sealed to maintain high humidity and placed in darkness for 36 h at 22°C. Infection types 0, 1B, 2B, 3B, 4B, 5B were considered as resistance, infection types 3G, 4G and 5G as susceptible, and 3GB, 4GB, 5GB as moderate resistance. Five seedlings of each chromosome substitution line were evaluated per replication. Each test had at least three trials. Hope and SK were used as a susceptible control, whereas CS as a resistance control.

3.2.2 Molecular mapping of the resistance gene *Rmg6*

Plant material

The common wheat cultivar, Norin 4, carries the resistance gene *Rmg6* to *Lolium* isolate TP2, whereas Hope does not. To map *Rmg6*, total 94 F₃ lines derived from a cross of N4 x Hope were used for infection assay and genomic DNA extraction. These F₃ lines were produced by self-pollinating 94 F₂ plants derived from N4 x Hope.

Disease resistance phenotype

Twenty seeds of each F₃ line derived from cross N4 x Hope were pre-germinated on moistened filter paper in plastic petri dishes for 24 h at 22°C, sown in a seedling case (5.5cm x 15cm x 10cm) filled with vermiculite (Asahi-Kyoto, Okayama), and grown at 22°C in a growth chamber with a 12h photoperiod for 7 days. They were inoculated with 305K1 (*Al;a2*) at a concentration of $1-1.5 \times 10^5$

conidia/mL as described previously (chapter 2) and scored for their disease resistance phenotype.

Genomic DNA isolation

Total genomic DNA was extracted from wheat leaves according to Appels and Moran (1984), and Koebner et al. (1986) with modifications. Twenty 5-day-old primary leaves (approximately 2 g) were collected, immediately frozen in liquid nitrogen, and store at - 80°C. The samples were taken out from - 80°C, quickly grounded in liquid nitrogen to a fine powder using a mortar and pestle, then transferred immediately into a 15 mL conical centrifuge tube (falcon) containing 4.67 mL of isolation buffer (Tris 6.1 g, NaCl 5.9 g, EDTA 74.5 g, adjust pH to 7.5, and add distilled water to 1000 mL), 0.3 mL of 10% SDS, and 30 µl of Proteinase K (20 mg/mL). The mixture was mixed well by vortex (Vortex Genie 2), placed in a water bath at 42°C for 2 h, and mixed every 30 min. Then, 1 g of sodium perchlorate (NaClO₄) was added to each sample, mixed gently at 100 rpm/10 min by Vortex Shaker VR-36D, and centrifuged at 8000 rpm for 5 min with a refrigerated centrifuge (HITACHI, homes SCR 20B). The upper phase was transferred to a new 15 mL conical tube by decanting or pipetting, mixed well with an equal volume of chloroform/ isoamylalcohol (24:1, v/v), and centrifuged at 10000 rpm for 5 min with a refrigerated centrifuge. The upper phase was transferred to a new 15 mL conical tube. Nine ml of EPR (a mixture of 320 ml of soln. A (100% ethanol added to 40g NaClO₄.H₂O) and soln. B (70ml of distilled water added to 120g NaClO₄.H₂O)) was added per tube, gently inverted, and precipitated DNA was collected and transferred to a new 1.5 mL tube by pipette or tip of glass micropipette. The precipitated DNA was washed with 1mL of 70% ethanol and centrifuged at 15000 rpm for 5 min. After removing the ethanol, the samples were dried for approximately 5 min. The pellet was then resuspended in a

mixture of 600 µl of TE (10mM Tris pH 8.0, and 1mM EDTA) and 1 µl of RNase (10 mg/mL), then incubated at 37°C for 1 h. An equal phenol/chloroform/isoamylalcohol (25:24:1, v/v/v) was added to each sample, gently inverted, centrifuged at 15000 rpm for 5 min (High speed micro refrigerated centrifuge 3740, Kubota, Japan), then the upper phase was transferred to a new 1.5 ml tube. One-tenth volume of 3M sodium acetate (CH₃COONa), and 2 volume of 100% ethanol were added to 1 volumes of transferred upper phase, mixed gently to precipitate DNA, centrifuged at 15000 rpm for 5 min, and the supernatant was removed. The DNA pellet was washed gently with 1mL of 70% ethanol, and centrifuged at 15000 rpm for 5 min. Ethanol was carefully removed and vacuum dried for 5 min. The DNA pellet was dissolved in 200-600 µl TE, and kept at 4°C or at – 20°C for long time storage.

Genetic mapping of *Rmg6* by using SSR markers

To identify SSR markers linked to *Rmg6*, bulked segregant analysis was performed as described by Michelmore et al. (1991). A total of 334 simple sequence repeat (SSR) loci reported by Somers et al. (2004) and Torada et al. (2006) were tested for polymorphisms between the parental cultivars, N4 and Hope. At least 10 SSR markers from each chromosome were chosen. These SSR loci were amplified by polymerase chain reaction (PCR) from total DNA of the parental cultivars (N4 and Hope) and two bulks, one containing aliquots (30 ng/µl) of 5 *R/r1* F₃ lines and the other containing 5 *r/r1* F₃ lines. The PCR reaction mixture contained 30 ng template DNA, 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.3 µM of each primer, and 1 unit of Taq polymerase. PCR condition were as follows: 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s between 51°C and 65°C depending on the primer combination, 1 min at 72°C, last step incubated 7 min at 72°C. The PCR amplicons were fractionated by electrophoresis through 2% agarose gels, or 6% denaturing polyacrylamide gels, and visualized by

ethidium bromide staining or silver staining. For polyacrylamide gel electrophoresis, the High Efficiency Genome Scanning system (Nippon Eido, Tokyo, Japan) was used. Once linked marker was found, all the 94 F₃ lines were tested to construct a linkage map.

Segregation data was analyzed using MAPMAKER Macintosh V2.0 with the F₂ intercross program. Thresholds of linkage for map construction were a 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 centimorgans (cM).

3.3 RESULTS

2.3.3. Identification of chromosomes carrying the resistance genes, *Rmg6*

The incompatibility between the *Lolium* isolate TP2 and common wheat is conditioned by two gene pairs, *R1 – A1* and *R2 – A2*, which interact in a gene-for-gene manner. Here, we focused only on *R1-A1* interaction resulting in strong resistance. To exclude the effect of the *R2-A2* interaction, a pilot experiment were performed with 305L2 (*a1;A2*) in different conditions. The resistant wheat cultivars CS, Sch, and N4, which carry *R1* and *R2*, became susceptible to 305L2 (*a1;A2*) when inoculated with 1~1.5 x 10⁵ conidia/mL of inoculum, and incubated for 36 h in a high humid box at 22°C (data not shown). This is a good condition to inactivate the weak resistance gene *R2* in wheat.

To identify the chromosome carrying *Rmg6*, chromosome substitution lines in which one of the 21 chromosomes of CS was substituted with its corresponding chromosome of Hope (Fig. 4), were inoculated with TP2 (*A1; A2*), 305K1 (*A1; a2*), and 306L2 (*A1; a2*) . CS (Hope 7A) line was susceptible, and CS (Hope 6B), CS

(Hope 1D) lines were moderately susceptible, while the other 18 lines were clearly resistant (Fig. 5, Table 6). These results indicate that *Rmg6* should be located on chromosome 7A, 6B, or 1D.

To map *Rmg6*, 94 F₃ lines derived from N4 x Hope were inoculated with 305K1 (*Al;a2*). All resistant, segregating, and all susceptible lines segregated in a 1:2:1 ratio as expected (Table 7). Among 45 SSR markers (Somers et al. 2004) on chromosome 7A, 16 markers were polymorphic between the parents (N4 and Hope). Segregation analysis of these markers with Mapmarker Macintosh V2.0 showed that *Rmg6* was unlinked to any of the markers on chromosome 7A with recombinant fraction (R) from 0.4 to 0.5, and LOD core 0.00. Similar results were obtained from an analysis of an F₂ population derived from CS x Hope (data not shown). Analysis with chromosome-specific molecular markers confirmed that our CS (Hope 7A) substitution line actually carries 7A chromosome of Hope (Fig. 6). These results suggest that *Rmg6* is not located on 7A chromosome.

According to the result of CS-Hope chromosome substitution lines, 6B and 1D chromosomes are other candidates. When 334 SSR markers on all 21 chromosomes reported by Somers et al. (2004) and Torada et al. (2006) were tested, 126 markers were polymorphic between the parents. Bulk segregant analysis with the polymorphic markers resulted in identification of a possible link marker, *Xwmc432*, on 1D chromosome (Fig. 7). Then, a linkage map around *Rmg6* was constructed by using 94 F₃ lines (Fig. 8). *Rmg6* was actually located on the short arm of 1D chromosome of common wheat.

3.4 DISCUSSION

Genetic resistance is one of effective methods to control blast disease, but rapid evolution of virulent blast races has been a major reason of loss of the

resistance. To successfully manage the disease, cultivars with durable resistance are really needed. *Rmg6* is the major gene controlling the resistance in common wheat cultivars against an inappropriate pathotype *Lolium* isolate TP2, under the control of gene-for-gene interaction. The wheat cultivars are not host of *Lolium* isolate of *P. oryzae*, so *Rmg6* would be more durable than the resistance genes to *Triticum* isolates. In this study, we successfully mapped *Rmg6* gene on the short arm of chromosome 1D by using chromosome substitution lines and bulked segregant analysis with SSR markers. *Rmg6* is regarded as the first resistance gene identified in common wheat against *Lolium* isolates. This result promises a great potential for a breeding program to improve the resistance to wheat blast disease in the near future, moreover, linked map of *Rmg6* will be useful to toward cloning the gene.

A question is “why CS-Hope(7A) was susceptible to A1 carriers”. Molecular analysis using SSR markers located on each chromosome led me to conclude that the CS-Hope chromosome substitution lines are valid as a whole (Fig. 6). Some chromosome rearrangements have occurred during the production of the lines.



FIGURE 4. Diagram of chromosome substitution line CS(Hope1A). The 1A chromosome pair (show in green) is derived from Hope and the other 20 chromosome pairs are from CS.

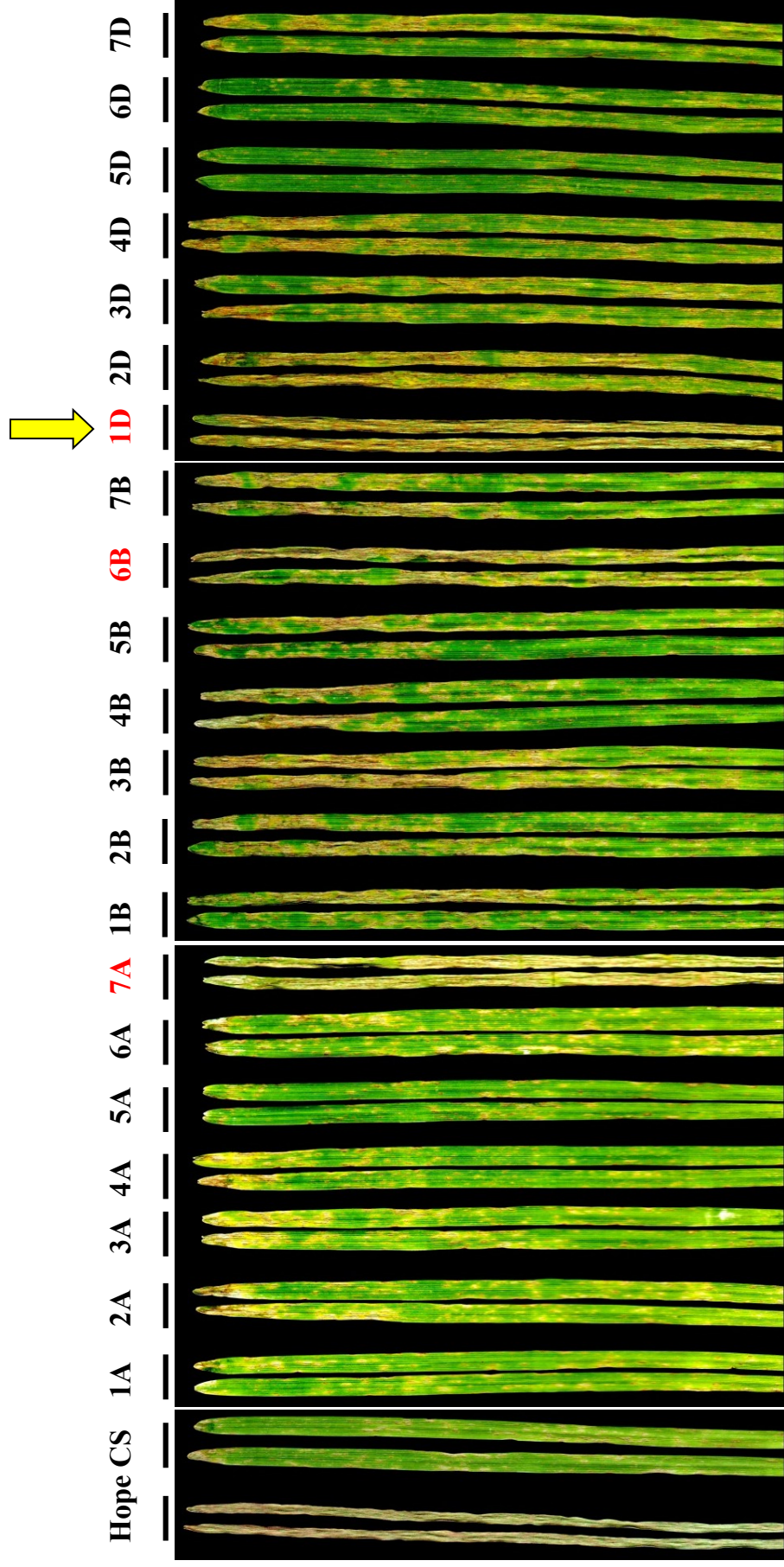


FIGURE 5. Responses of the CS-Hope chromosome substitution lines to 306L2 (*Al;a2*), derived from TP2 x Br48, 4 days after inoculation at 22°C. Names of substituted chromosomes are shown above the leaf pairs.

	gwm471				hbg238				hbg412			
	{				{				{			
M	1	2	3	4	1	2	3	4	1	2	3	4

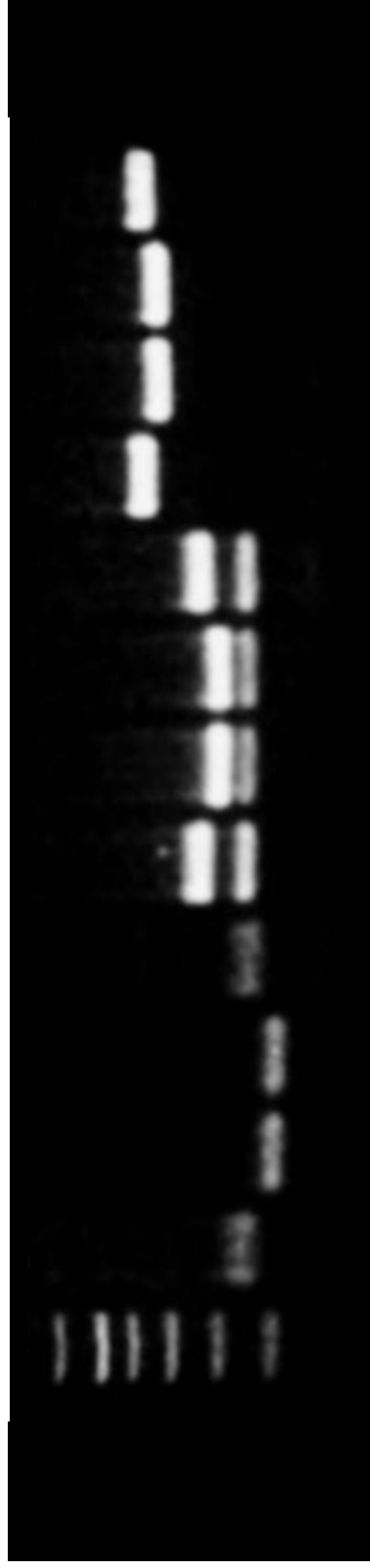


FIGURE 6. Confirmation of CS(Hope 7A), CS(Hope 7B) chromosome substitution lines using SSR markers on chromosome 7A.

1: CS; 2: Hope; 3: CS(Hope 7A); and 4: CS (Hope 7B)

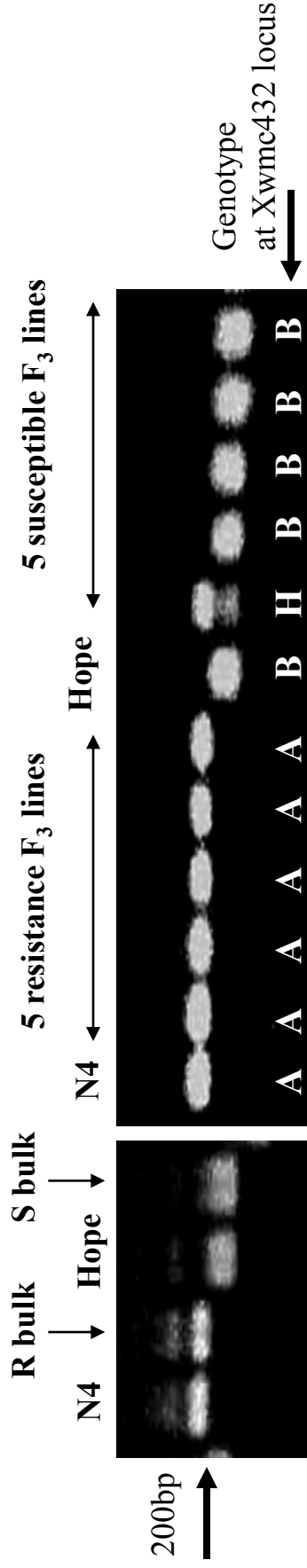


FIGURE 7. Bulked segregant analysis with two parents (N4 and Hope), an R bulk composed of 5 homozygous resistant F₃ lines, and an S bulk composed of 5 homozygous susceptible F₃ lines. The Xwmc432 locus was amplified, and checked on 3% agarose gel.

Chromosome 1D

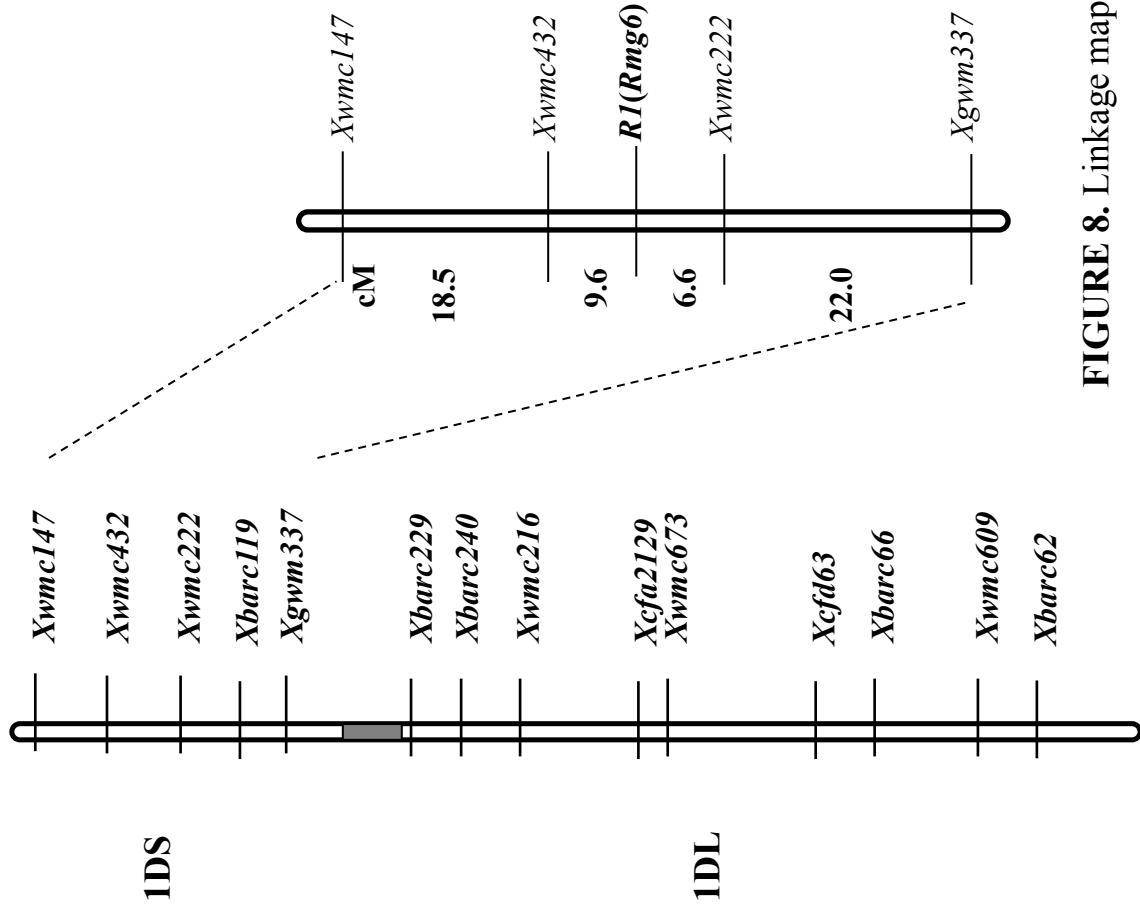


FIGURE 8. Linkage map around *Rmg6* constructed using a F₃ lines derived from the cross, N4 x Hope.

Somers et al. (2004)

Table 6. Response of CS-Hope chromosome substitution lines to *Lolium* isolate TP2, and 305K1 (*Al,a2*), 306L2 (*Al,a2*), F₁ progenies from Br48 x TP2.

Wheat lines	Infection type				
	TP2 (<i>Al;A2</i>)		F ₁ (TP2 x Br48)		
			305K1 (<i>Al; a2</i>)	306L2 (<i>Al; a2</i>)	
	1x10 ⁵ a	1.5x10 ⁵	3x10 ⁵	1-1.5x10 ⁵	1x10 ⁵
CS/Hope1A	1B	1-B	1+B	1B	1B
CS/Hope2A	1B	1+B	2B	2gB	1+B
CS/Hope3A	1B	1B	2-B	2B	2-B
CS/Hope4A	1-B	1B	1-B	1B	1B
CS/Hope5A	1B	1+B	1B	2B	1+B
CS/Hope6A	1B	1+B	1B	2B	2-B
CS/Hope7A	4-Gb	5G	5G	5Gb	5G
CS/Hope1B	2-B	2B	3gB	2B	2gB
CS/Hope2B	1B	1B	3GB	2B	2gB
CS/Hope3B	1B	1B	3+GB	2B	3gB
CS/Hope4B	1B	2B	2+B	2B	1B
CS/Hope5B	1B	2B	3gB	2B	1B
CS/Hope6B	3+GB	4+GB	4GB	4GB	3+GB
CS/Hope7B	1B	1+B	2B	2B	2gB
CS/Hope1D	3GB	4GB	5-GB	4GB	4GB
CS/Hope2D	2-B	2GB	3+GB	2B	3gB
CS/Hope3D	1-B	1-B	2-B	2B	2B
CS/Hope4D	1+B	2B	1+B	2B	2B
CS/Hope5D	1-B	1+B	2B	1B	1B
CS/Hope6D	1+B	2+B	2B	2B	1B
CS/Hope7D	0+B	0+B	1+B	2B	2-B

^a Concentration of inoculum

Table 7. Segregation of reactions to 305K1 (*Al;a2*) in F₃ population derived from N4 x Hope

All resistant	Number of F ₃ lines ^a		χ^2 (1:2:1)		P
	Segregating	All susceptible	Total		
28	47	19	94	1.7	0.4

^a Twenty seeds were retrieved from each F₃ lines.

Chapter 4

Identification of a resistance gene – avirulence gene pair involved in the resistance of wheat to both *Lolium* and *Avena* isolates of the blast fungus

4.1 INTRODUCTION

A genetic cross of a *Setaria* isolate with a *Triticum* isolate, and pathogenicity test of their F₁ cultures on wheat cultivars, revealed that the difference of pathogenicity on wheat between *Setaria* and *Triticum* isolates was controlled by 2 genes located at different loci (Murakami et al. 2000). They designated a locus associated with the lesion color and the other locus associated with the affected area as *Pwt1* and *Pwt2*, respectively (Murakami et al. 2000). Avirulence loci *Pwt3* and *Pwt4* were found in *Avena* isolate Br58 by crossing *Triticum* isolate Br48 with Br58 (Takabayashi et al. 2002). *Pwt1* was located on chromosome 2 and linked to the ribosomal DNA locus and a telomere (Chuma et al. 2010). *Pwt3* was located on chromosome 6A in a genetic map constructed using SSR markers (data not published).

Lolium isolate TP2 was collected in Tochigi, Japan in 1997. *Lolium* isolates are virulent on *Lolium* species and avirulent on common wheat (Tosa et al. 2004). In this study, two avirulence genes, *A1* and *A2*, were identified in TP2. Incompatibility between the *Lolium* isolate and common wheat was conditioned by two gene pairs, *R1 – A1* and *R2 – A2*, which interact in a gene-for-gene manner. The interaction between *R2* and *A2* was repressed by long time incubation (Chapter 3). Thus, the *R1(Rmg6)-A1* interaction plays an important role in

“communication” between *Lolium* isolates and common wheat cultivars. The main purpose of this chapter is to map the strong avirulent gene *Al* for its cloning. We successfully constructed a linkage map containing the *Al* gene using bulked segregant analysis with SSR markers.

4.2 MATERIALS AND METHODS

4.1.1 Genetic mapping of the avirulence gene *Al*

Infection assay

To map *Al* of *Lolium* isolate TP2, 56 tetrad and 79 random F₁ cultures derived from a cross Br48xTP2 were used for infection assay with three common wheat cultivars carrying the resistance gene *Rmg6*, CS, Sch, and N4, and a susceptible cultivar, Hope.

Infection assay was performed as described in Chapter 2. The conidia produced were suspended in water and adjusted to a concentration of $1-1.5 \times 10^5$ conidia/mL. After inoculation, the cases were sealed to maintain high humidity and placed in darkness for 24h with tetrad F₁ cultures, or 36h with random F₁ cultures at 22°C. Infection types 0, 1B, 2B, 3B, 4B, 5B were considered as avirulent, infection types 3G, 4G and 5G as virulent, and 3GB, 4GB, 5GB as moderately avirulent (resulting from the *R2-A2* interaction). Five seedlings of each line were evaluated per replication. Each test was performed at least three times.

Genomic DNA isolation and molecular mapping of *Al* using SSR markers

Genomic DNA of progenies was isolated as described by Nakayashiki et al. (1999). To identify SSR markers linked to the *Al* locus, bulked segregant analysis was performed as described by Michelmore et al. (1991). More than 90 SSR loci

on all 7 chromosomes of *P. oryzae* (Zheng et al. 2008) were amplified by PCR from genomic DNA of two parental isolates, *Triticum* isolates Br48 and *Lolium* isolate TP2, and two bulks, one containing aliquots (5 ng/μl) of 5 avirulent F₁ cultures, and the other containing 5 virulent F₁ cultures. These F₁ cultures for each bulk were chosen from different tetrads. The polymerase chain reaction (PCR) reaction mixture contained 5 ng template DNA, 1X PCR buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, 0.5 μM of each primer, and 1 unit of rTaq polymerase. PCR condition was as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, last step incubated 5 min at 72°C. The PCR amplicons were fractionated by electrophoresis through 2% or 3% agarose gels, or 6% denaturing polyacrylamide gels, and visualized by ethidium bromide staining or silver staining. For polyacrylamide gel electrophoresis, the High Efficiency Genome Scanning system (Nippon Eido, Tokyo, Japan) was used. Once linked marker was found, all genomic DNA of 56 tetrad F₁ cultures was tested. Then, a linkage map of *Al* was constructed by using 79 random F₁ cultures.

Segregation data were analyzed using MAPMAKER Macintosh V2.0 with the haploid program. Thresholds of linkage for map construction were a 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 centimorgans (cM).

4.1.2 Allelism tests

Allelism test of *Al* and *PWT3*

To check if *Al* and *PWT3* are located at the same locus on chromosome 6, a genetic cross between F₁ cultures carrying one of two avirulence genes was performed. The presence of two opposite mating types, *MAT1-1* and *MAT1-2*, are

essential for mating of *P. oryzae* isolates. A genetic cross is possible only between fertile isolates with different mating type. 73Q2 (*PWT3*; *pwt4*) with mating type *MAT1-2* was chosen from the F₁ population derived from a cross, Br58 x Br48. As its counterpart, therefore, an F₁ culture carrying *AI* alone and the opposite mating type *MAT1-1* is needed. To determine mating types of F₁ cultures from TP2 x Br48, PCR was performed with a specific primer set for *MAT1-1*, forward primer (5'-AGCCTCATCAACGGCAA-3') and reverse primer (5'-GGCACGAACATGCGATG-3'). Fifteen µl of PCR reaction contained 1X KOD plus buffer, 0.2 mM dNTPs, 1.5 mM MgSO₄, 0.5 µM each of forward and reverse primers, and 1 unit KOD plus. PCR condition was as follows: 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s between 60°C, 1 min at 72°C, and the last step incubated 7 min at 72°C. The PCR amplicons were electrophoresed in 1.5% agarose gels, and visualized by ethidium bromide staining.

One F₁ culture from a cross TP2 x Br48 was chosen for mating with 73Q2 on oatmeal agar medium as described in Chapter 2. All random F₁ cultures obtained from this genetic cross were used for infection assay with common wheat cultivars CS, N4, and Sch which carries both of the resistance genes, *Rwt3* and *Rmg6* (Fig. 13). Infection assay was performed as described in Chapter 2.

Allelism test of *Rmg6* and *Rwt3*

To examine whether *Rmg6* and *Rwt3* are located at the same locus, the same set of F₃ lines derived from N4 x Hope was inoculated with 306L2 (*AI;a2*) and 73Q2 (*PWT3*; *pwt4*). Infection assay was performed as described in Chapter 2.

4.3 RESULTS

Molecular mapping of *AI*

The common wheat cultivars, CS, Sch, N4, and Hope, were inoculated with the tetrad and random F₁ cultures derived from the cross TP2 x Br48. Segregation of avirulent and virulent/moderately avirulent F₁ cultures on CS, Sch, and N4 fitted a 1:1 ratio (Table 8). This segregation is attributed to the strong avirulence gene *A1* (Fig. 3 in Chapter 2). In addition, we successfully found an experimental condition to suppress the weak interaction between *R2-A2* as described in Chapter 3. This condition was used for infection assay with random F₁ cultures derived from the cross TP2 x Br48 on resistance wheat cultivars.

As a result of the screening of SSR markers through bulked segregant analysis, two SSR markers on chromosome 6, MGM130 and MGM402, were found to be linked to the *A1* locus. Segregation analysis with the 56 tetrad F₁ cultures revealed that MGM130 and MGM402 are actually linked to *A1* (Fig. 9), indicating that the strong avirulence gene *A1* is located on chromosome 6. A new SSR marker MoSSR6-1 was designed based on the genomic sequence of *M. oryzae* 70-15. Using the 79 random F₁ cultures derived from TP2 x Br48, a linkage map around the *A1* locus was constructed with three linked markers, MGM402, MGM130, and MoSSR6-1. The most closely linked marker was MoSSR6-1 (Fig. 10, Supplemental Table 2).

PWT3 (A1)* is shared by two distinct subgroups of *P. oryzae

Interestingly, *A1* gene was located around a genomic region on chromosome 6 that contained *PWT3* (Fig. 11) corresponding to a wheat resistance gene, *Rwt3*, against *Avena* isolates of *P. oryzae* (Takabayashi et al. 2002). To demonstrate whether *A1* and *PWT3* are located at the same locus on chromosome 6, a genetic cross between an F₁ culture carrying *A1* alone and 73Q2 (*MAT1-2*) carrying *PWT3* alone is needed (Fig. 13). Mating types of the F₁ cultures derived from TP2 x Br48 were determined using *MAT1-1*-specific primers. Among the 79 random F₁

cultures, 46 cultures carried the *MAT1-1* allele (Fig. 12). Similar result was obtained with the tetrad F₁ progenies derived from the same cross (data not shown). Two *MAT1-1* carriers, 305K1 and 306L2, were chosen, which had been revealed to carry *Al* alone (305K1, Table 5 in Chapter 2; 306L2 data not shown). 306L2 (*Al;a2*) was successfully crossed with 73Q2 (*PWT3;pwt4*). When 63 F₁ cultures derived from this cross was sprayed on CS, Sch, and N4, all these F₁ cultures were avirulent on all cultivars (Table 9). This result suggests that *Al* may be *PWT3*.

If the resistance of wheat to the two inappropriate subgroups, *Lolium* isolates and *Avena* isolate, is conditioned by gene-for-gene interactions, *Rmg6* should be *Rwt3*. This was confirmed by inoculating the same set of F₃ lines derived from N4 x Hope with 305K1 (*Al;a2*) and 73Q2 (*PWT3; pwt4*). When the F₃ populations derived from N4 x Hope were inoculated with 305K1, all R : segregating : all S segregated in a 1:2:1 ratio (Table 10). This result confirmed that the resistance of N4 to 305K1 is controlled by one gene, *R1*. Furthermore, this segregation against 305K1 was perfectly concordant to that with 73Q2 (Table 10), suggesting that *R1* recognizes the avirulence gene *PWT3* in 73Q2. These results suggest that *Rmg6* (*R1*) is *Rwt3*. Now, I designate *Rmg6* with *Rwt3* listed as a synonym – *Rmg6* (*Rwt3*).

From these results I suggest that *PWT3* is shared by *Lolium* isolates and *Avena* isolates and that *Rmg6*(*Rwt3*), located on the 1D chromosome, controls the resistance of wheat to these two distinct subgroups of *P. oryzae*.

4.4 DISCUSSION

Avena isolate Br58 collected in Brazil in 1990 is only one *Avena* isolate preserved in our lab. The avirulence of Br58 on wheat is conditioned by *PWT3* and *PWT4* (Takabayashi et al. 2002). *PWT4* is temperature-insensitive and recognized by resistance gene *Rwt4* in N4 whereas *PWT3* is temperature-sensitive and

recognized by resistance gene *Rwt3* in N4 and CS (Takabayashi et al. 2002). Interestingly, genetic analysis in this study suggested that *Al* from a *Lolium* isolate could be *PWT3*. We suggest that the *PWT3 (Al)* gene is commonly involved in the avirulence of two distinct subgroups of *P. oryzae*, *Avena* isolates and *Lolium* isolates. This, in turn, suggests that *Rmg6(Rwt3)* corresponding to *Al(PWT3)* is widely involved in the resistance gene of common wheat against *Avena* isolates and *Lolium* isolates (Fig. 14). Moreover, *Lolium/Avena* isolates are indicated to be close to *Triticum*, and *Eluesine* isolates based on sequence analysis of the rDNA-ITS-2 region (Oh et al. 2002, Tosa et al. 2004). Once the avirulence gene *Al* is cloned, we will be able to elucidate the origin of these isolates.

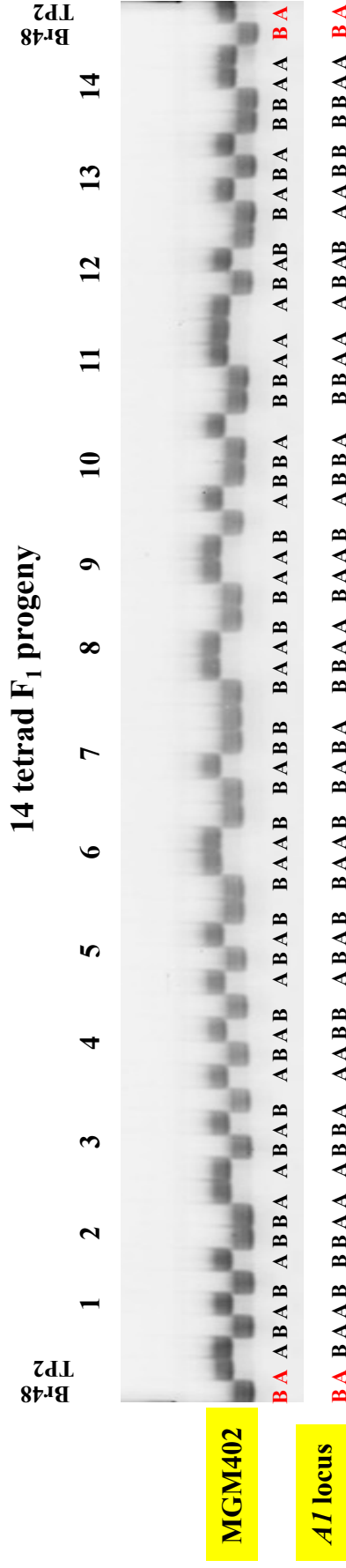


FIGURE 9. Segregation of the MGM402 and *AI* loci in the 14 tetrad F_1 progeny derived from a cross Br48 x TP2. Genomic DNA of the F_1 cultures were amplified with MGM402 primers, and were checked on 6% denaturing polyacrylamide gels. A: TP2 type signal, B: Br48 type signal.

Chromosome 6

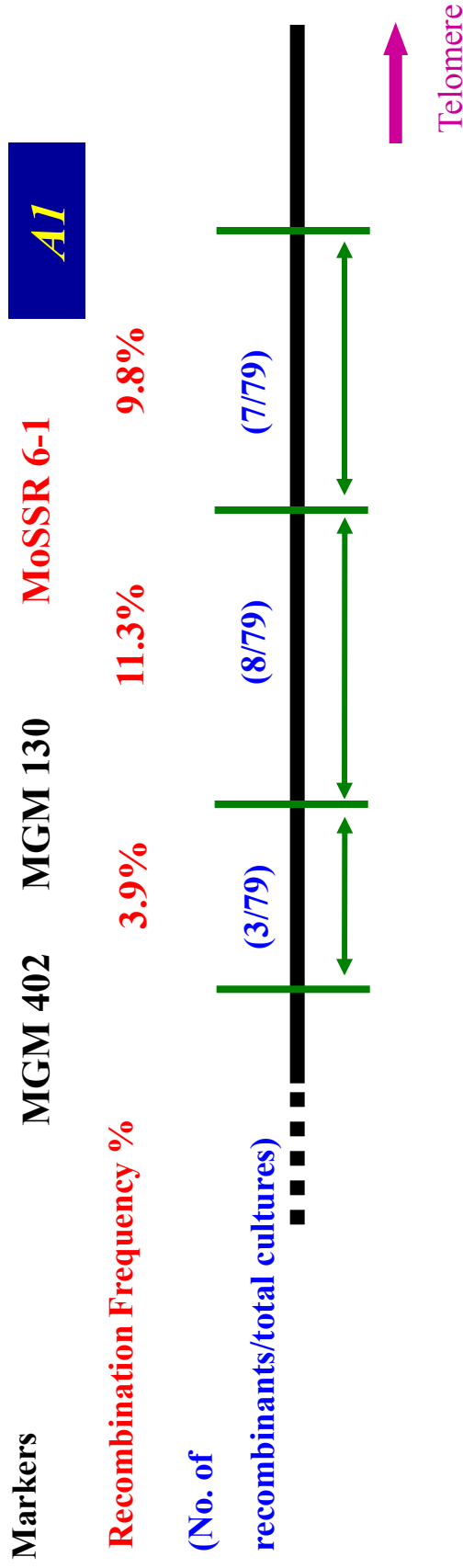


FIGURE 10. Linkage map around *Al* constructed using an F₁ population derived from the cross, TP2 x Br48. Markers in black are from Zheng et al. (2008). MoSSR 6-1 is designed using the database of *M. oryzae* 70-15 genomic sequence.

Chromosome 6

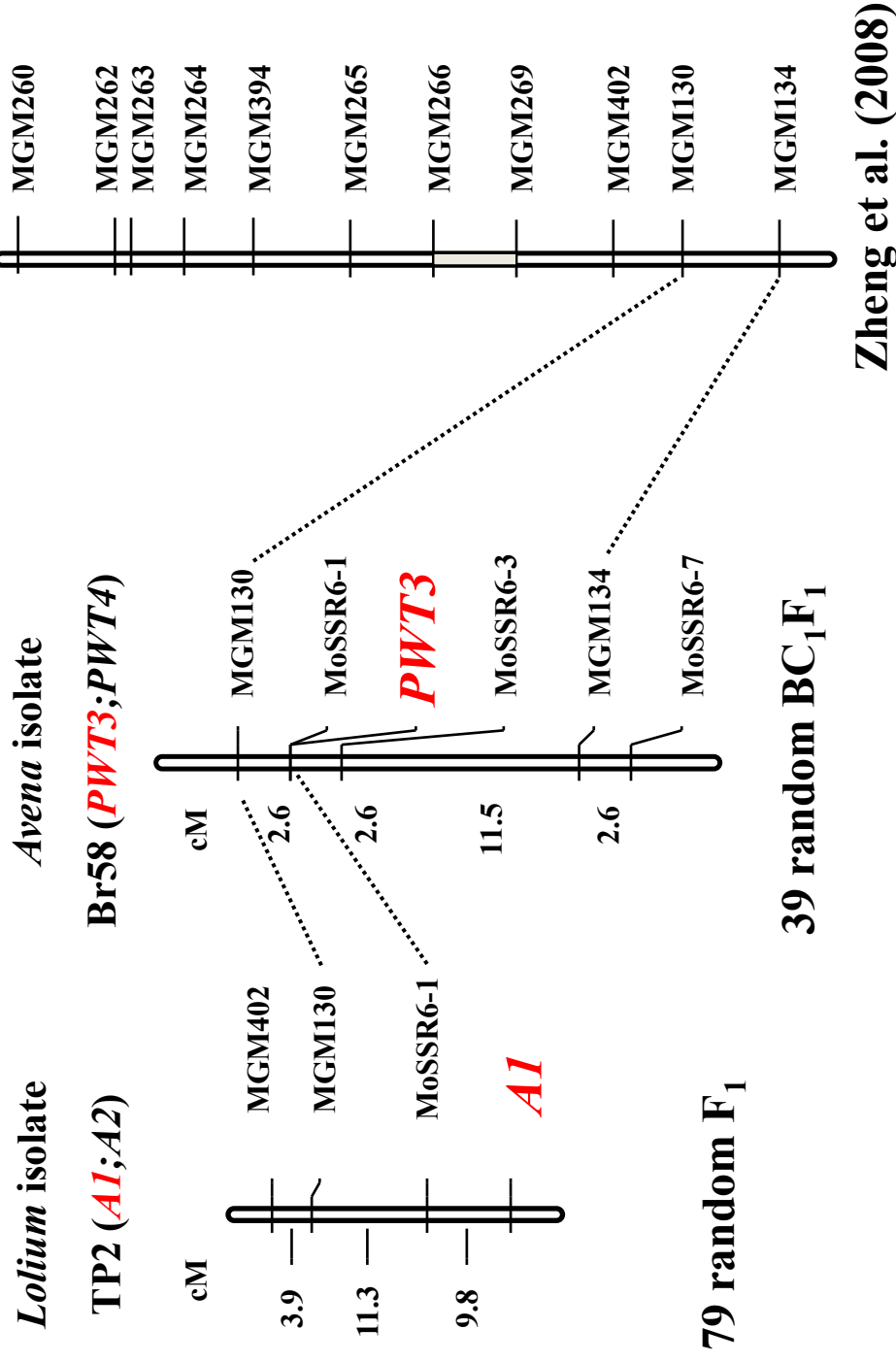


FIGURE 11. Linkage map of *A1* and *PWT3* on chromosome 6 of *P. oryzae*

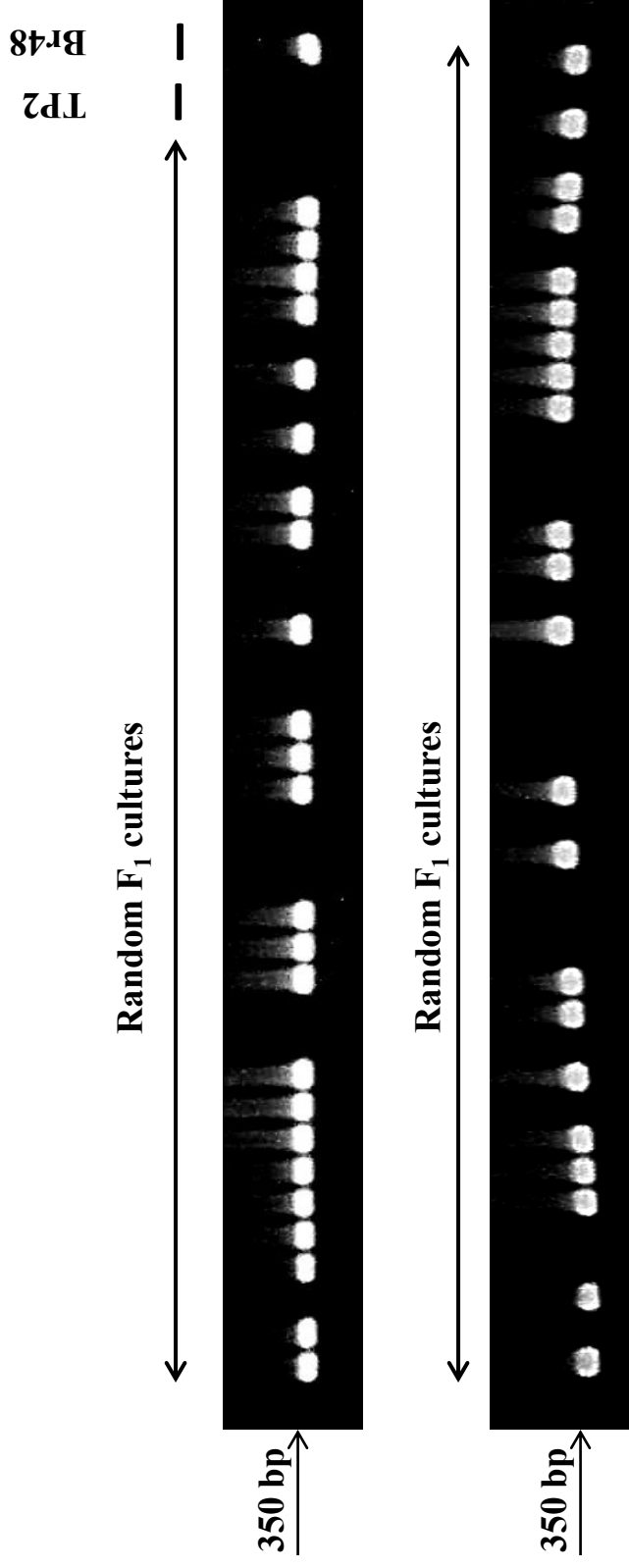


FIGURE 12. Determination of mating types of 79 random F_1 progeny derived from the cross, Br48 x TP2, using specific primers for the MAT1 gene of *P. oryzae*. PCR products was checked on 1.5% agarose gel. The presence of PCR amplicon indicates that the F_1 culture carries MAT1-1.

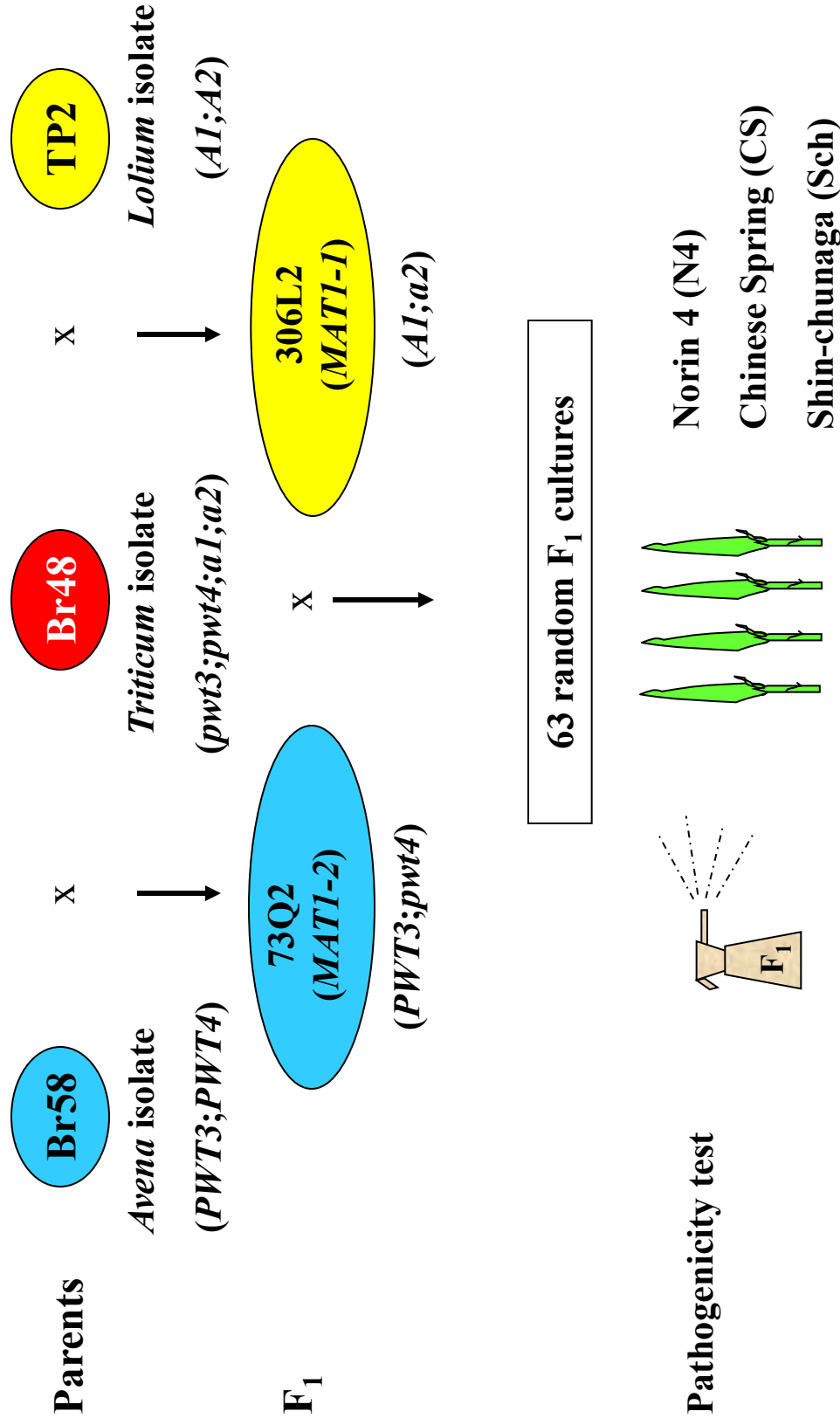


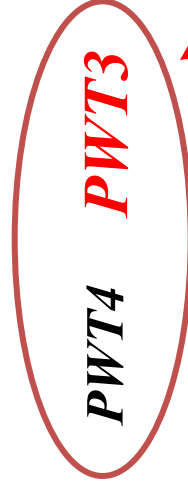
FIGURE 13. Genetic cross to check allelic relationships between two avirulence genes

PWT3 of *Avena* isolate Br58 and *A1* of *Lolium* isolate TP2

Pyricularia oryzae

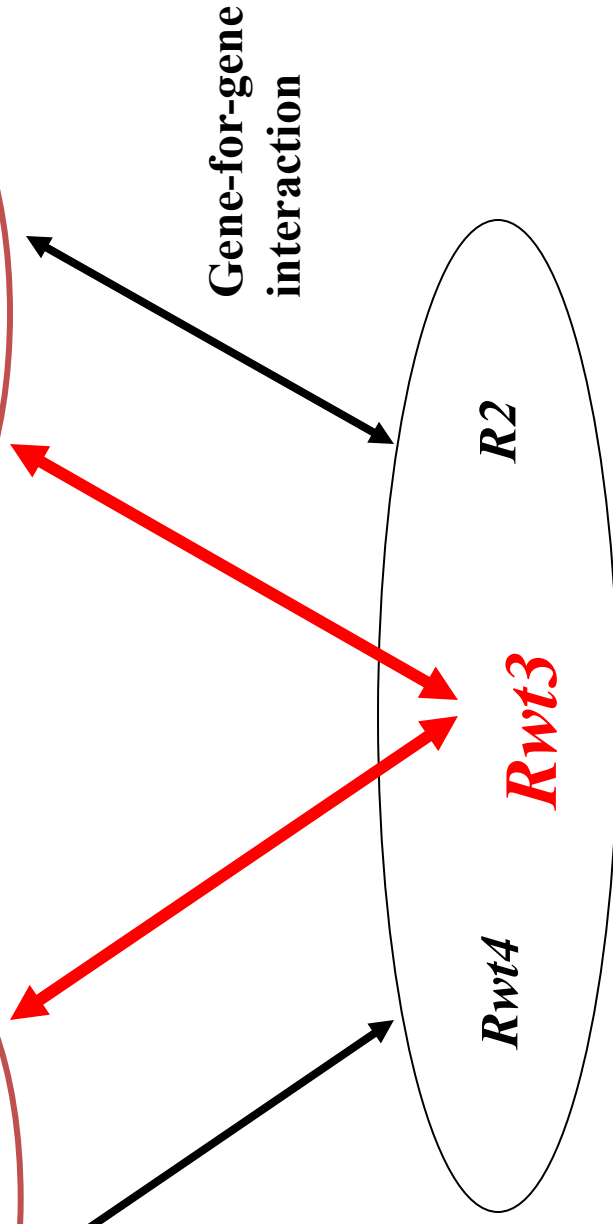
Avena isolate Br58

Parana, Brazil (1990)



Lolium isolate TP2

Tochigi, Japan (1997)



Common wheat (*Triticum aestivum*)

FIGURE 14. Incompatibility between a *Lolium* isolate, an *Avena* isolate, and wheat cultivars

TABLE 8. Segregation of pathogenicity on wheat cultivars in tetrad and random F₁ cultures derived from the cross, Br48 x TP2

F ₁ progeny	Cultivar	No. of F ₁ cultures			χ^2 (2:1:1)	P
		A ^a	M ^b + V ^c	Total		
Tetrad	CS	28	28	56	0.14	0.93
	Sch	28	28	56	0.14	0.93
	N4	28	28	56	0.14	0.93
	Hope	0	56	56	...	...
Random	CS	40	39	79	0.01	0.91
	Sch	40	39	79	0.01	0.91
	N4	40	39	79	0.01	0.91
	Hope	0	79	79	...	...

^a Avirulent (infection type 0-5B)

^b Moderately avirulent (infection type 3GB-5GB)

^c Virulent (infection type 3G-5G)

TABLE 9. Virulence on wheat of 63 random progenies derived from the cross between 73Q2(*PWT3*) and 306L2(*AI*)

Cultivar	Infection type ^a at 22°C		No. of F ₁ cultures	
	F ₁ TP2xBr48	F ₁ Br58xBr48	Avirulent ^b	Virulent ^c
	306L2 (<i>AI;a2</i>)	73Q2 (<i>PWT3;pwt4</i>)		
CS	2B	2B	63	0
Sch	2B	3B	63	0
N4	1B	1B	63	0
Hope	5G	5G	0	63

^a Refer to the text

^b avirulence (infection type 0-5B)

^c virulence (infection type 3G-5G)

TABLE 10. Segregation of reactions to F₁ cultures in F₃ lines derived from a cross between N4 and Hope

Isolate	Number of F ₃ lines			χ^2 (1:2:1)	P	
	All resistance	Segregating	All susceptible Total			
F ₁ from Br58xBr48 73Q2 (<i>PWT3</i> ; <i>pwt4</i>)	28	47	19	94	1.7	0.4
F ₁ from TP2xBr48 305K1 (<i>Al</i> ; <i>a2</i>)	28	47	19	94	1.7	0.4

Supplemental Table 2. Genotypes and phenotypes of random F₁ cultures derived from the cross,
Br48 x TP2.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
Code No. of F ₁ culture	1	3	8	10	14	16	22	24	32	33	36	37	42	43	45	46	48	50	51	52	54	57	61	64	65	66	68	71	79	81	83	40	53	56	59	18	23	34	74	13	21	
MGM 402	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	
MGM 130	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A
MoSSR 6-1	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A
AI	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	A	A	A	A	A	A	A	A	A	

No.	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	
Code No. of F ₁ culture	21	2	4	6	7	9	17	19	20	25	26	27	28	29	30	31	38	41	44	47	49	55	58	62	67	69	70	72	73	80	82	39	60	77	5	11	63	78	35	
MGM 402	B	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
MGM 130	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B
MoSSR 6-1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	
AI	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	B	B	B	B	B	B	B	B

B: Br48 type signal

A: TP2 type signal

Chapter 5

Molecular cloning of *PWT3*

5.1 INTRODUCTION

Wheat blast disease caused by *Pyricularia oryzae* has become a serious threat for wheat production, especially in South America. After first reported in 1985 in the State of Parana in Brazil, it has rapidly spread to neighboring countries including Bolivia, south and southeastern Paraguay, and northeastern Argentina. In Uruguay scattered infections were recognized during wet and warm years (Kohli et al. 2011). Its outbreaks occurred in the southern cone region of South America just in a short time after the first report. Movement of infected seeds is the most possible way to spread wheat blast from the original outbreak site in Brazil to other parts of Brazil and other neighboring countries as Bolivia and Paraguay (<http://www.k-state.edu/wheatblast/about/pathogen/index.html>). In addition, wheat blast disease appeared in Kentucky, USA, in 2011. Its causal agent was suggested to have arisen through the evolution and establishment of new strains of *P. oryzae* from indigenous population of grass species such as the annual ryegrass pathogen in favorite environment for disease development (<http://news.ca.uky.edu/article/uk-researchers-find-important-newdisease>). This hypothesis is supported by the close relationship between wheat pathogen and other grass pathogens causing severe diseases in perennial (*Lolium perenne*), annual ryegrasses (*Lolium multiflorum*), finger millet (*Eleusine coracana*), and oat (*Avena sativa*) (Oh et al. 2002, Tosa et al. 2004, Kusaba et al. 2006). However, there are many gaps in understanding the process of evolution of the wheat blast pathogen. (i) The origin of *Triticum* pathogen has not been elucidated. (ii) *Triticum* isoaltes may be able to hide within

other plant species which have not yet been determined. Host plant resistance is one significant management plan to prevent yield loss by wheat blast. To date, however, durable resistance has been lacking.

A close relationship between *Triticum* isolates (pathogenic on wheat) and *Lolium* isolates (pathogenic on perennial ryegrass) was demonstrated. Comparative analyses with repetitive elements and ITS sequences of rDNA showed a close relationship between isolates from perennial ryegrass and those from wheat (Viji et al. 2001). Fingerprinting with transposons revealed that the perennial ryegrass pathogen was similar to the wheat pathogen (Farman 2002). *Lolium* isolates collected in Japan shared a 100% identical ITS2 sequence with *Triticum* and *Eleusine* isolates (Tosa et al. 2004). The present study showed that the incompatibility between a *Lolium* isolate and wheat cultivars is under simple genetic control. Therefore, there seems a potential risk that *Lolium* isolates jump hosts and become a threat to wheat. In addition, the avirulence gene *Al* (*PWT3*) located on chromosome 6 is shared by two distinct subgroup of *P. oryzae*, *Lolium* and *Avena* isolates, and is recognized by the same resistance gene *Rmg6*(*Rwt3*) located on chromosome 1D. Once the avirulence gene *PWT3* is cloned, we will be able to understand how *Triticum*, *Avena*, and *Lolium* isolates have evolved from their common ancestor.

Here, we tried map-based cloning and characterization of *PWT3*. A candidate gene of *PWT3* encoded a protein (141 amino acids) with unknown function. Distribution of *PWT3* in *Pyricularia* populations was also examined. Sequence analysis of *PWT3* homologues from various *Pyricularia* isolates suggested that positive selection may play a role in the evolution of this gene.

5.2 MATERIALS AND METHODS

5.2.1 Fungal strains and genetic cross

To construct a high-resolution map of *PWT3*, a genetic backcross 73Q2 x Br48 was performed. 73Q2 (*PWT3;pwt4*) with *MAT1*-2 is an F₁ culture derived from a cross between *Avena* isolate Br58 (*PWT3;PWT4*) and *Triticum* isolate Br48 (*pwt3;pwt4*) (Fig.15). Ninety-nine wild-type isolates from various hosts, e.g. *Oryza*, *Setaria*, *Panicum*, *Eluesine*, *Avena*, *Triticum*, *Lolium*, *Digitaria*, *Zizania*, *Zingiber*, *Sasa*, and *Kyllinga* spp., were used to examine the distribution of *PWT3* homologs in *Pyricularia* populations (Table 11). For long term storage, these cultures were transferred to sterilized barley seeds in vials, grown at 25°C until mycelia covered the barley seeds (~1 month), and kept in a case with silica gel at 4°C or room temperature at the Laboratory of Plant Pathology, Kobe University. A grain was transferred to PDA in a test tube and incubated at 25°C just before use for experiments as described in Chapter 2.

5.2.2 Pathogenicity assay

The infection assay was performed as described in Chapter 2. The conidia produced were suspended in water and adjusted to a concentration of 1.5×10^5 conidia/mL. Ten mL of the conidia suspension with 0.01% Tween 20 (Nacalai Tesque, Kyoto) were sprayed on the adaxial surface of 7-day-old primary leaves in a plastic case with an air compressor. The cases were sealed to maintain high humidity and placed in darkness for 36 h at 22°C. Infections were scored 4 days after inoculation. Five seedlings of each common wheat cultivar were evaluated per replication. Each inoculation test had at least three trials. Hope and SK were used as susceptible cultivars, whereas CS, Sch and N4 as resistant.

To test expression of selected genes *in vivo*, Tat14 (*Triticum paleocolchicum* – KU156) and Bar14 (*Hordeum vulgare* – cv. Nigrate), non-carriers of *Rwt3*, were inoculated with a high concentration (5×10^5 conidia/mL) of conidial suspension. Ten mL of the conidial suspension with 0.01% Tween 20 (Nacalai Tesque, Kyoto) were sprayed on the adaxial surface of 7-day-old primary leaves in a plastic case with an air compressor. The cases were sealed to maintain high humidity and placed in darkness at 22°C. Infected leaves were harvested at 30h and 48h after inoculation (Supplemental Fig. 2). Twenty seedlings were used for each cultivar.

5.2.3 Microscopy

Inoculated primary leaves were collected at 30h or 48h after inoculation, and boiled in alcoholic lactophenol (lactic acid/phenol/glycerol/distilled water/ethanol – 1:1:1:1:8) for 2 min for fixation. Cytological responses were observed with BX51 microscope (Olympus, Tokyo, Japan) under bright field or fluorescence field with an exciter filter B.

5.2.4 DNA isolation

Fungal DNA was isolated by the method described by Amer et al. (2011) with some modifications. Fungal isolates were incubated on PDA (test tube or plastic plate) for 7 days. The mycelia were picked up with sterile toothpick, transferred to 1.5ml eppendorf tube, and homogenized by a plastic pestle (Kontes pellet pestle). The sample was supplied with 400 µl of isolation buffer (1M KCl, 100mM Tris-HCl pH 8, 10mM EDTA pH 8), vortexed for 30 sec at maximum speed, and incubated at 70°C in a water bath for 30 min. The lysate was centrifuged at 15000 rpm for 10 min and the upper phase (300 µl) was transferred to a fresh 1.5 ml eppendorf tube. An equal volume of isopropanol (300 µl) was added to each sample, mixed well, and the samples were centrifuged at 13000 rpm

for 30 mins at 4°C. DNA pellets were washed 2 times with 400µl of 70% ethanol by centrifuging the sample at 13000 rpm for 4 mins at 4°C and draining the upper phase. DNA pellets were vacuum-dried for 5 min, then resuspended in 50 µl of TE (10 mM Tris-HCl, 1 mM EDTA) buffer (pH 8.0).

5.2.5 Identification of recombinants

To identify recombinants between flanking markers of the *PWT3* locus, genomic DNA of 273 BC₁F₁ cultures derived from the cross 73Q2 x Br48 were screened with two flanking markers, MGM130 and MoSSR6-23, and a co-segregating marker, MoSSR6-1. The primer sequences for the screening are shown in Table 12. The polymerase chain reaction (PCR) mixture and PCR condition were as described in Chapter 4.

5.2.6 Marker development and genetic mapping of the avirulence gene *PWT3*

A DNA sequence of genomic region between two flanking markers of *PWT3*, MGM130 and MoSSR6-3, was obtained from *M. oryzae* database MG8 (Magnaporthe comparative Sequencing Project, Broad Institute of Harvard and MIT, <http://www.broadinstitute.org>). In order to identify the corresponding genomic region in *Avena* isolate Br58 and *Triticum* isolate Br48, the DNA sequence obtained from the database was used for blastn search against the whole-genome sequences of Br58 and Br48 (unpublished data). Putative secreted protein genes in this region were identified by a gene prediction software, FGENESH trained on *Magnaporthe* sequences (SoftBerry), and signal peptide prediction software, SignalP version 4.0 (Petersen et al. 2011). In a total, six putative secreted protein genes were picked up as *PWT3* candidates.

For a high-resolution mapping of the *PWT3* locus, candidate gene-specific primers including single nucleotide polymorphism (SNP) primers, cleaved amplified polymorphic sequence (CAPS) primers, and derived cleaved amplified polymorphic sequence (dCAPS) primers were designed (Table 13). CAPS sites were identified by CAPS Designer (Bombarely et al. 2011). dCAPS sites were identified by dCAPS Finder 2.0 (Neff et al. 2002). Primers were picked up by Primer3web version 4.0.0 (Koressaar and Remm 2007, Untergrasser et al. 2012). The polymerase chain reaction (PCR) reaction mixture and PCR condition were described in Chapter 4. PCR products of CAPS or dCAPS primers were digested with restriction enzymes (Takara, Tokyo) and electrophoresed on a 2% agarose gel (Nacalai Tesque, Kyoto, Japan).

5.2.7 Reverse transcription – PCR

Total RNA was prepared from infected leaves harvested 30h and 48h after inoculation, and from mycelia grown in a complete liquid medium for 3 days. Total RNA was extracted from the samples by using Sepasol RNA I super G (Nacalai, Tesque, Kyoto, Japan) following the manufacturer's instructions. Total RNA samples were treated with DNase I Amplification Grade (Invitrogen) prior to reverse transcription. Reverse transcription was carried out using the Prime Script RT reagent kit (Takara, Tokyo). Specific primers for *PWT3* candidate genes were designed by using Primer3. Actin gene primers were used as an internal control. The primers used were shown in Table 14. The reaction mixture of PCR was prepared as described in Chapter 4. PCR condition was as follows: 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C, and the last step with incubation for 5 min at 72°C. The PCR amplicons were electrophoresed in 1.5% agarose gels and visualized by ethidium bromide staining.

5.2.8 Plasmid and Fosmid DNA extraction and DNA sequencing

E. coli cultures carrying plasmid or fosmid clones were incubated in LB liquid medium containing 50ug/ml ampicillin (plasmid) or 12.5ug/ml chloramphenicol (fosmid) overnight at 37°C. Plasmid and fosmid DNAs were extracted by using QIAprep Spin Miniprep Kit (QIAGEN) following the manufacturer's instructions. Plasmid and fosmid clones were sequenced as described by Tosa et al. (2005). PCR products were directly sequenced after purification with ExoSAP-IT (Affymetrix, Inc, USA) or MinElute PCR purification kit (QIAGEN).

5.2.9 Screening of Br58 fosmid library

A fosmid library of Br58 was constructed with CopyControl Fosmid Library Production Kit (EPICENTRE, Madison, Wisconsin) by Takara Bio (Shiga, Japan). Total DNAs were sheared into approximately 40 kb fragments, blunt-ended, and ligated into the pCC1FOS vector. Fosmid clones containing two *PWT3* candidate genes, Br58_scaf36_210 and Br58_scaf36_211, were selected from the Br58 fosmid library by colony hybridization. The fosmid clones were plated onto LB agar medium (20 g of Plusgrow II, 10 g of agar in 500 ml of distilled water) containing 12.5 µg/ml of chloramphenicol. Colonies were transferred onto a nylon membrane, Hybond N+ (GE healthcare UK, Amersham Place, Little Chalfont, Buckinghamshire). The bacterial cells on the membrane were lysed by 0.5M NaOH, neutralized by neutralization buffer (1M Tris, 1.5M NaCl, pH 7.5), washed with 2XSSC, and crosslinked with 70mJ of UV radiation. A labeled probe was added to the matrix and hybridization was performed by using the ECL direct nucleic acid labeling and detection system (GE healthcare UK, Amersham Place, Little Chalfont, Buckinghamshire) according to the manufacturer's instructions.

The labeled probe was a PCR product amplified from genomic DNA of Br58 by using a pair of primer Br58_scaf36_210_probe_F1 (5'-CATAAGTTCAAATTGTTCAAGGAC-3'), and Br58_scaf36_210_probe_R1 (5'-CTGCGGTAGAATTTTCATTATCG-3'). Positive colonies hybridized with the labeled probe were further screened by colony PCR with the primer set used to make the DNA probe. Once the positive colonies were identified, fosmid DNAs were extracted and end-sequenced with primers, pCC1FOS-F (5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3') and pCC1FOS-R (5'-CTCGTATGTTGTGTGGAATTGTGAGC-3').

5.2.10 Plasmid construction and transformation of *E. coli* DH5 α competent cells

A 2.3kb fragment containing Br58_scaf36_210 was PCR-amplified from a fosmid clone, FAv_210_1, with primers, Br58_scaf36_210_NotI_F (5'-TTGCGGCCGCCAACACCGTGATTGACTTGG-3') and Br58_scaf36_210_XbaI_R (5'-GGTCTAGATTTCTGTGGGAAGGCTCAAC-3'). A 1.9kb fragment containing Br58_scaf36_211 was PCR-amplified from a fosmid clone, FAv_210_1, with primers, Br58_scaf36_211_XbaI_F (5'-CCTCTAGACCGGAGTCACCGATACTGTT-3') and Br58_scaf36_211_NotI_R (5'-TTGCGGCCGCTGGTGGTCGTTGCTTTGATA-3'). PCRs were performed using KOD Plus-Neo (Toyobo, Osaka, Japan). A 50 μ l reaction mixture contained 0.05 ng fosmid DNA, 1X Buffer for KOD-Plus-Neo, 0.2 mM dNTPs, 1.5 mM MgSO₄, 0.5 μ M of each primer, and 1 unit of KOD plus Neo. PCR condition was as follows: 2 min at 94°C, 35 cycles of 10 s at 98°C, 30 s at 60°C, 3 min at 68°C, followed by a final extension of 68°C for 5 min. The PCR products were purified by using a MinElute PCR purification kit (QIAGEN).

The purified PCR products and plasmid DNA of pBluescript II SK (+) were cut with two restriction enzymes, *NotI* and *XbaI* (TAKARA BIO INC, Shiga, Japan). Digested DNAs were separated by electrophoresis on 0.7% agarose gels in 1X TAE buffer. After the desired bands were cut from the gels, DNA was extracted and purified by using MinElute Gel Extraction Kit (QIAGEN). The sticky-ended fragments were ligated individually into opened vector pBluescript II SK (+) by using DNA Ligation Kit ver.2.1 (Takara, Japan). The ligation product was used to transform *E. coli* DH5 α competent cells (TAKARA BIO INC, Japan) following the manufacturer's instructions. Positive colonies were selected by PCR with KOD FX (TOYOBO, Japan) and two primer pairs used to amplify insert fragments. A 10 μ l reaction mixture contained 1X PCR buffer for KOD FX, 0.4 mM dNTPs, 0.5 μ M of each primer, and 0.01 unit of KOD FX. PCR condition was as follows: 2 min at 94°C, 35 cycles of 10 s at 98°C, 30 s at 60°C, 3 min at 68°C, followed by a final extension of 68°C for 5 min. The plasmid DNA of selected colonies was sequenced on both strands as described by Tosa et al. (2005).

5.2.11 Transformation of *P. oryzae*

Preparation and transformation of competent cells of Br48 was performed as described by Tosa et al. (2005). One hundred and fifty μ l of the protoplast suspension of Br48 was mixed with 40-50 μ l of fosmid clones or plasmid clones containing candidates of the avirulence gene *PWT3* and 20 μ l of plasmid DNA pSH75 (Kimura and Tsuge 1993) which carries the hygromycin B phosphotransferase gene, respectively.

5.2.12 PCR screening for *PWT3* homologs in *Pyricularia* populations

PCR screening for *PWT3* homologs in the *Pyricularia* populations was performed with primers PWT3_ORF_F (5'-AGGCCTGAAAACCTCCTCAT-3') and PWT3_ORF_R (5'-CTGCAAAATTCCGCCTTTACT-3'). A 15 μ l of PCR

reaction mixture contained 1X PCR buffer for KOD-Plus-Neo, 0.2 mM dNTPs, 1.5mM MgSO₄, 0.5 µM of each primer, 5 ng DNA template, and 0.02 Unit of KOD-Plus. PCR condition was mentioned above. The PCR products were electrophoresed in 0.7% agarose gel and stained with ethidium bromide.

The PCR products were purified with ExoSAP – IT (Affymetrix, Inc, USA) or MinElute PCR purification kit (QIAGEN). The purified DNA was sequenced directly with PWT3_ORF primers using the BigDye terminator cycle sequencing ready reaction kit and ABI Prism 3100 or 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, U.S.A.). Nucleotide sequences were aligned using MEGA 5.1 program (Tamura et al. 2011). A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) in MEGA 5.1.

5.3 RESULTS

5.3.1 A high-resolution mapping of *PWT3*

A genetic map around *PWT3* locus was constructed by using 39 random BC₁F₁ cultures derived from 73Q2 x Br48 (Fig. 15). *PWT3* was located between two flanking markers MGM130 and MoSSR6-3 on chromosome 6, and co-segregated with a new marker, MoSSR6-1 (Fig.16). The distance between two flanking markers was ~283 kb in supercontig 8.5 of *M. oryzae* database MG8.

To further narrow down the region cosegregating with *PWT3*, a larger BC₁F₁ population is needed. A genetic cross between F₁ culture 73Q2 and *Triticum* isolate Br48 was performed. Out of the resulting 273 random BC₁F₁ cultures, 45 recombinants between the two *PWT3* flanking markers, MGM130 and MoSSR6-23, were picked up (Fig. 17). When common wheat cultivars, CS, Sch, and N4, carrying the resistance gene *Rwt3* were inoculated with the 45 recombinants, 27

cultures were avirulent while 18 cultures were virulent (Fig. 18). Among six *PWT3* candidate genes, Br58_scaf16_21, Br58_scaf36_214, Br58_scaf36_211, Br58_scaf36_210, Br58_scaf36_204, and Br58_scaf36_195, only Br58_scaf36_211 and Br58_scaf36_210 co-segregated with *PWT3*, while Br58_scaf36_214 and Br58_scaf36_204 were mapped to flanks of the *PWT3* locus with 1 recombinant (Fig. 18). As a result, the genomic region cosegregating with *PWT3* was narrowed down to a ~23kb region (Fig. 18). A physical map of this region is shown in Fig. 19.

5.3.2 Selection of candidate genes using gene expression

Most of AVR proteins have functions as pathogenicity effectors on susceptible hosts. Based on this knowledge, gene expression analysis was performed. *Triticum* isolate Br48 and *Avena* isolate Br58 were sprayed on susceptible host Tat14 and Bar14 (non-carrier of *Rwt3*). RNA was extracted from the infected leaves and subjected to RT-PCR. The 214 and 204 genes were not expressed *in planta*, while 211 and 210 genes were expressed at 30h after inoculation with Br58, and 48h after inoculation with Br48. Moreover, the 211 and 210 genes were expressed *in planta*, but not expressed in mycelia (Fig. 20). They were considered to be the best candidates for *PWT3*.

5.3.3 Identification of a 2.3 kb fragment carrying the avirulence gene *PWT3*

To clone *PWT3* gene, two fosmid clones were picked up from fosmid library of Br58 by colony hybridization. Fosmid 1 (38 kb) and Fosmid 2 (43 kb) contained both 210 and 211 genes. Two subclones were constructed from Fosmid 1; subclone-211 (1.9 kb) carried the 211 gene alone, while subclone-210 (2.3 kb) carried the 210 gene alone (Fig. 21).

The two fosmid clones (FAv_210_1, FAv_210_2) and the two plasmid clones (pBS_210_1_15, and pBS_211_1_7) were used to transform a *Triticum* isolate Br48 (non-carrier of *PWT3*) together with pSH75, a plasmid containing a hygromycin resistance gene cassette. Ten, 19, 17, and 20 transformants were obtained from transformation with FAv_210_1, FAv_210_2, pBS_210_1_15, and pBS_211_1_7, respectively. In addition 10 transformants carrying pBluescript II SK (+) empty vector were also obtained.

Five transformants were arbitrary chosen from each for infection assay. Transformants carrying of FAv_210_1, FAv_210_2, and pBS_210_1_15 were avirulent on *Rwt3* carriers, CS, Sch, and N4, while transformants carrying pBS_211_1_7 or empty vector were virulent on the *Rwt3* carriers (Fig. 22). All the transformants tested were virulent on a *Rwt3* noncarrier, Hope, suggesting that avirulence of transformants carrying of FAv_210_1, FAv_210_2, or pBS_210_1_15 were *Rwt3*-specific. As a result, the avirulence gene *PWT3* was localized to a 2.3 kb DNA fragment containing a putative secreted protein gene, Br58_scaf36_210 (426 bp). This gene encoded a protein of 141 amino acids (Fig. 23). No homologs were identified by blastn search of Br58_scaf36_210 nucleotide sequence against NCBI nucleotide collection database.

5.3.4 Distribution of *PWT3* homologs in *Pyricularia* populations

A total of 99 wild isolates of *Pyricularia* spp. was used to survey the distribution of *PWT3* homologs. One primer pair PWT3_ORF_F and PWT3_ORF_R was used to amplify a region about 570 bp containing an ORF region of *PWT3* gene (Table 15, Supplemental Fig. 1). *PWT3* homologs were present within *Pyricularia oryzae* populations, but absent in other species of *Pyricularia* (Table 16). However, nine *Oryza* isolates, one *Panicum* isolate, and one *Triticum* isolate showed no band in PCR analysis (Table 16).

A phylogenetic tree of *PWT3* homologs was constructed by using nucleotide sequences of the *PWT3* ORF region. Interestingly, *PWT3* homologs were grouped into four types. *PWT3* homologs of *Oryza*, *Setaria* isolates were designated as A type, *Panicum* isolates as B type, *Avena*, *Triticum*, *Lolium*, and *Eleusine* isolates as C type, and *Triticum*, *Brachiaria*, and *Eragrostis* as D type (Figs. 24, 25). Original *PWT3* gene sequence from the *Avena* isolate Br58 (A-1B) was included in the C type. This type was considered to be an avirulent type. *PWT3* homolog in the *Triticum* isolate Br48 (T-4B) was included in D type. This type was considered to be a virulent type. One important result was that *Triticum* isolates were divided into two types, C and D types. The C type (avirulent type) included Br49 (T-5B), Br115.7 (T-6B), Br116.5 (T-7B), and Br118.2D (T-8B) while the D type (virulent type) included Br3 (T-1B), Br8 (T-3B), and Br48 (T-4B). Interestingly, *Triticum* isolates were grouped into two types in a phylogenetic tree constructed with the sequences of the beta-tubulin gene (Fig. 25).

The *Triticum* isolate Br48 (T-4B) was virulent on most of the wheat (*Triticum* spp.) cultivars tested, including CS, Sch, and N4 (*Rwt3* carriers). However, four *Triticum* isolates belonged to the C type (avirulent type), leading us to hypothesize that these isolates might lack the avirulence function of *PWT3* due to some mutations of other than its ORF. To test this hypothesis, upstream region of the *PWT3* ORF was characterized using PCR with two primer pairs. Among four *Triticum* isolates belonging to C type, T-6B and T-8B yielded amplicons with both primer pairs for upstream region, whereas T-5B and T-7B did not yield an amplicon with a primer pairs of upstream region just adjacent to the ORF region of *PWT3* gene (Fig. 26). As structural variation was detected in the upstream region of *PWT3* ORF, *PWT3* homologs of T-5B and T-7B were renamed as the C* type.

An infection assay was performed to test whether *Triticum* isolates harboring the C and C* type were virulent on wheat cultivars carrying *Rwt3*. Unfortunately, T-5B and T-6B lost the ability of sporulation. T-8B (C type) was avirulent on *Rwt3* carriers, while T-7B (C* type) and T-4B (D type) were virulent. Hypersensitive responses were observed in epidermal cells of N4 48h after inoculation with T-8B (C type), but not with T-7B (C* type) (Fig.27).

5.4 DISCUSSION

Several avirulence genes of *Pyricularia oryzae* have been cloned to date such as *Avr-Pita*, *Ace1*, *AvrPiz-t*, *AVR1-CO39*, *Avr-Pik/km/kp*, and *PWL2* (Rouxel et al. 2010). *PWL2*, *Avr-Pik*, and *AvrPiz-t* were cloned based on the genetic mapping (Sweigard et al. 1995, Lou et al. 2005). Sometimes positional cloning was difficult due to low fertility of rice isolates employed. *Avr-pia* was isolated by using a mutant strain (Miki et al. 2009). In the present study, *PWT3* was cloned based on its map position. A large BC₁F₁ population more than 270 cultures was utilized for constructing a high-resolution map of *PWT3*. A genetic cross was performed advantageously owing to the high fertility of *Triticum* isolate Br48 (*MAT1-1*).

The high-resolution mapping and gene expression analysis are points for the successful cloning of the *PWT3* gene. The genetic distance between two flanking markers MGM130 and MoSSR6-3 was around ~283kb in supercontig8.5 of *M. oryzae*, where one recombinant (one map unit) represents ~140 kb in a rough map of *PWT3* constructed using 39 BC₁F₁ cultures. Therefore, a large BC₁F₁ population was produced for a high – resolution mapping with additional markers designed from the selected candidate genes in the region around *PWT3*. Finally, the target

region was narrowed down to approximately 20kb (Figs. 18, 19). Among six candidate genes found in this region, the 211 and 210 genes were expressed in infected host plant cells, and considered to be the best candidates of *PWT3*.

PWT3 was localized to a 2.3 kb DNA fragment containing a putative secreted protein gene, Br58_scaf36_210 with 426 bp ORF encoding a protein of 141 amino acids. Although the 2.3kb fragment contains several ORFs, the biggest one is expected to have function of *PWT3*. The 426 bp gene is expressed in host leaves, and contains a signal peptide (54 bp) which is a typical feature of most of cloned fungal avirulence genes. To confirm that this ORF is actually *PWT3*, however, we need to do additional experiments such as complementation with a construct with the ORF combined with an authentic promoter or disruption of this ORF in Br58 isolate. If this ORF is really *PWT3*, the disruptant will be virulent on wheat cultivars carrying *Rwt3*.

PWT3 homologs were distributed widely in *P. oryzae* populations but were grouped into four types. Interestingly, homologs of *Triticum* isolates were grouped into two types, C type (avirulent type) and D type (virulent type) (Figs. 24, 25). *Triticum* isolates with the C type was further divided into two subgroups, T-6B and T-8B carrying the authentic C type and T-5B and T-7B carrying the C* type. The difference of these subgroups were determined by structural variation of the upstream region of *PWT3* (426 bp) and an infection assay. These results led us to hypothesize that there may be two ways for the evolution of *Triticum* isolates in Brazil. (i) Some of *P. oryzae* isolates from *Avena*, *Eleusine* or *Lolium* spp. jumped hosts and first infected susceptible wheat cultivars which do not carry the resistance gene *Rwt3* (C type). (ii) Some lost function of *PWT3* through mutation in the upstream region (C* type), and/or mutation in the ORF region (D type). It should be noted that two distinct populations with different sexual characteristics

and DNA fingerprints were identified in *Triticum* isolates of *P. oryzae* collected in Brazil (Urashima et al. 2005).

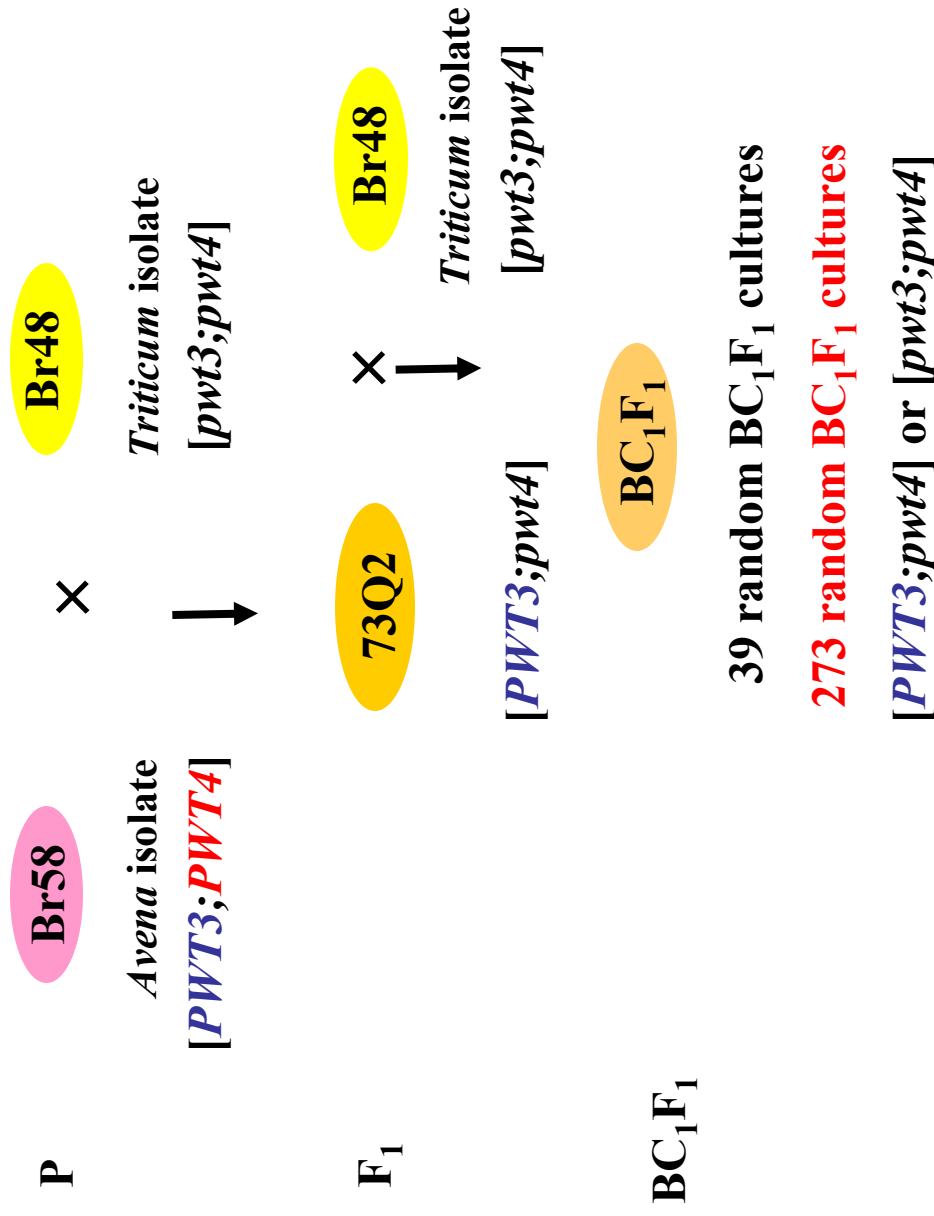


FIGURE 15. Genetic cross to construct a high-resolution map of *PWT3*

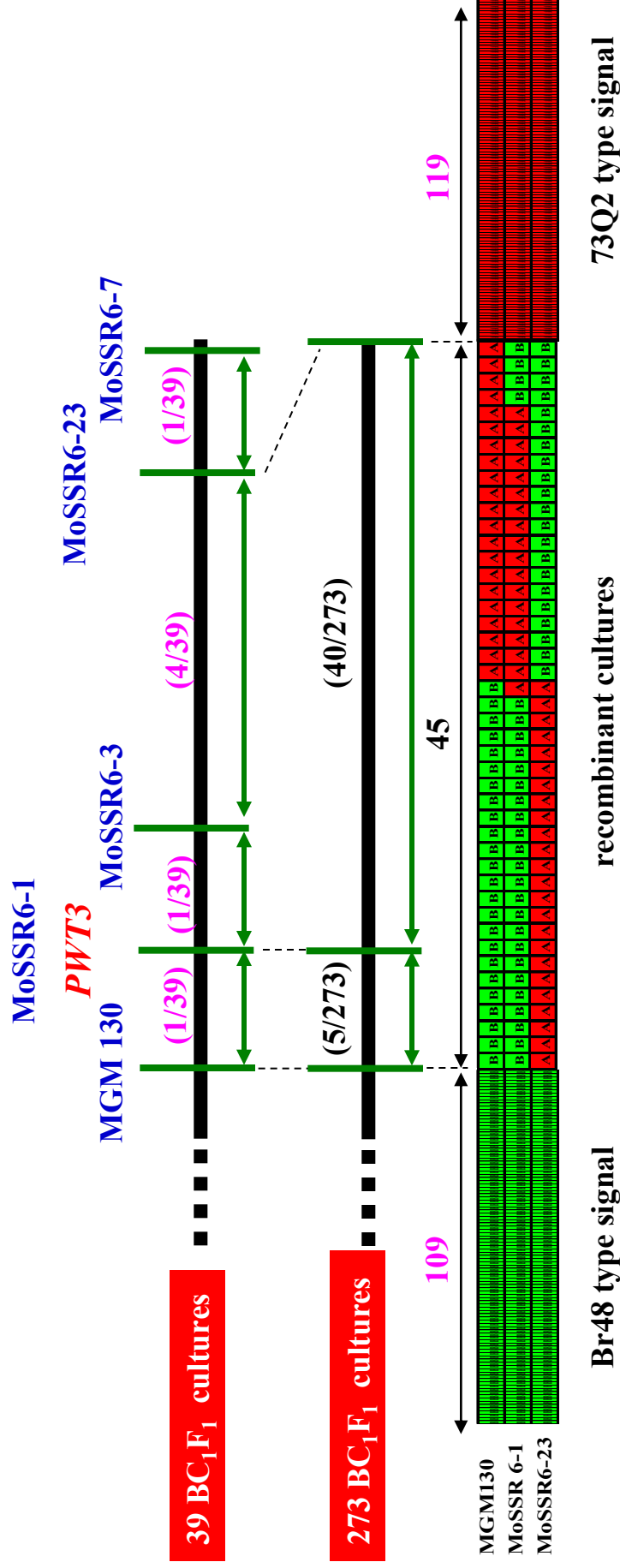


FIGURE 17. Screening of 273 BC₁F₁ cultures derived from the backcross, 73Q2 x Br48, for recombinants using two flanking markers of *PWT3*, MGM130 and MoSSR6-23. B: Br48 type signal; A: 73Q2 type signal.

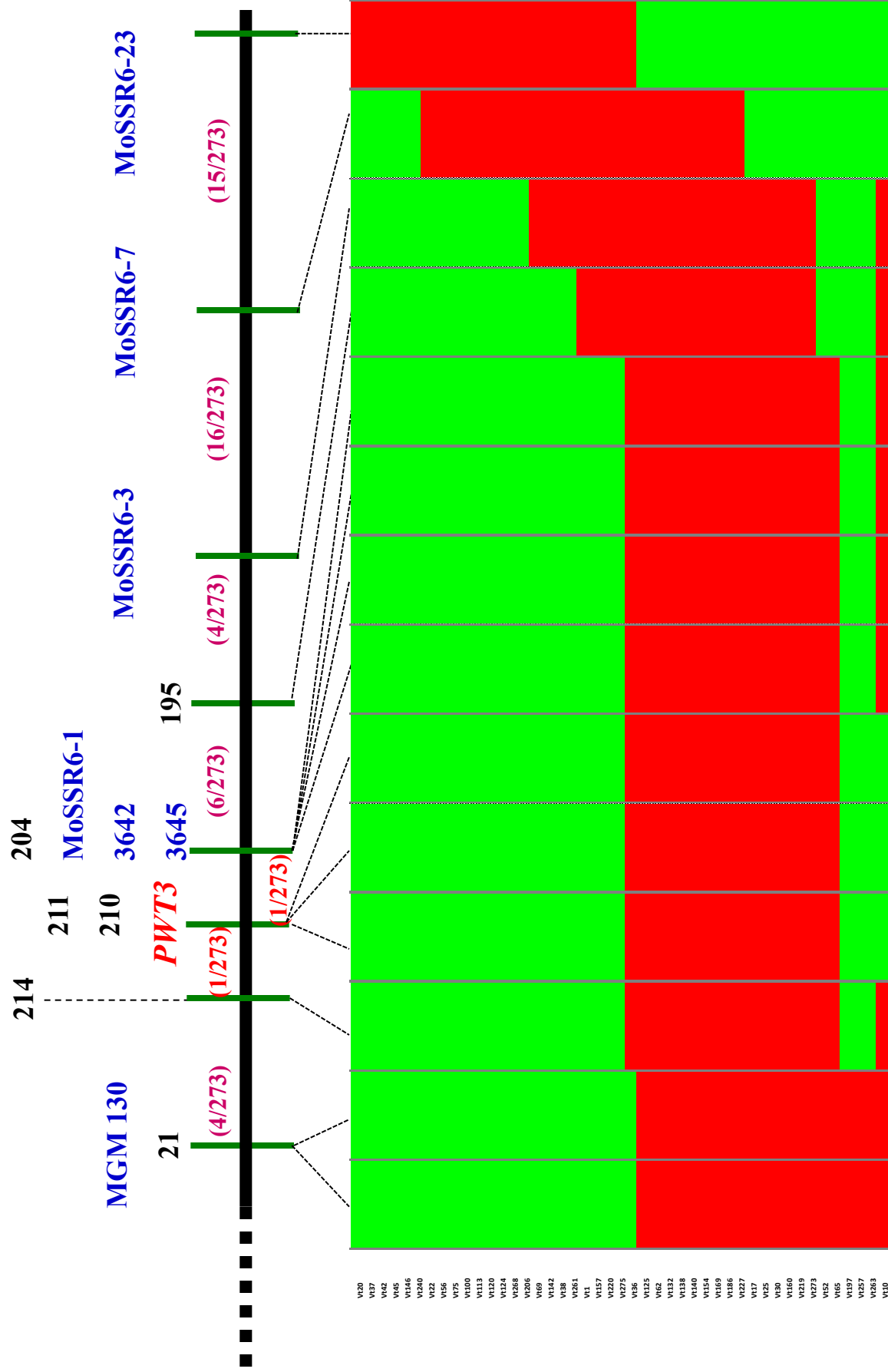


FIGURE 18. A high-resolution map of *PWT3* region on chromosome 6. The linked markers, 21, 214, 211, 210, 204, and 195 markers were designed based on the genome sequence of Br48 and Br58. green: Br48 type signal; red: 73Q2 type signal.

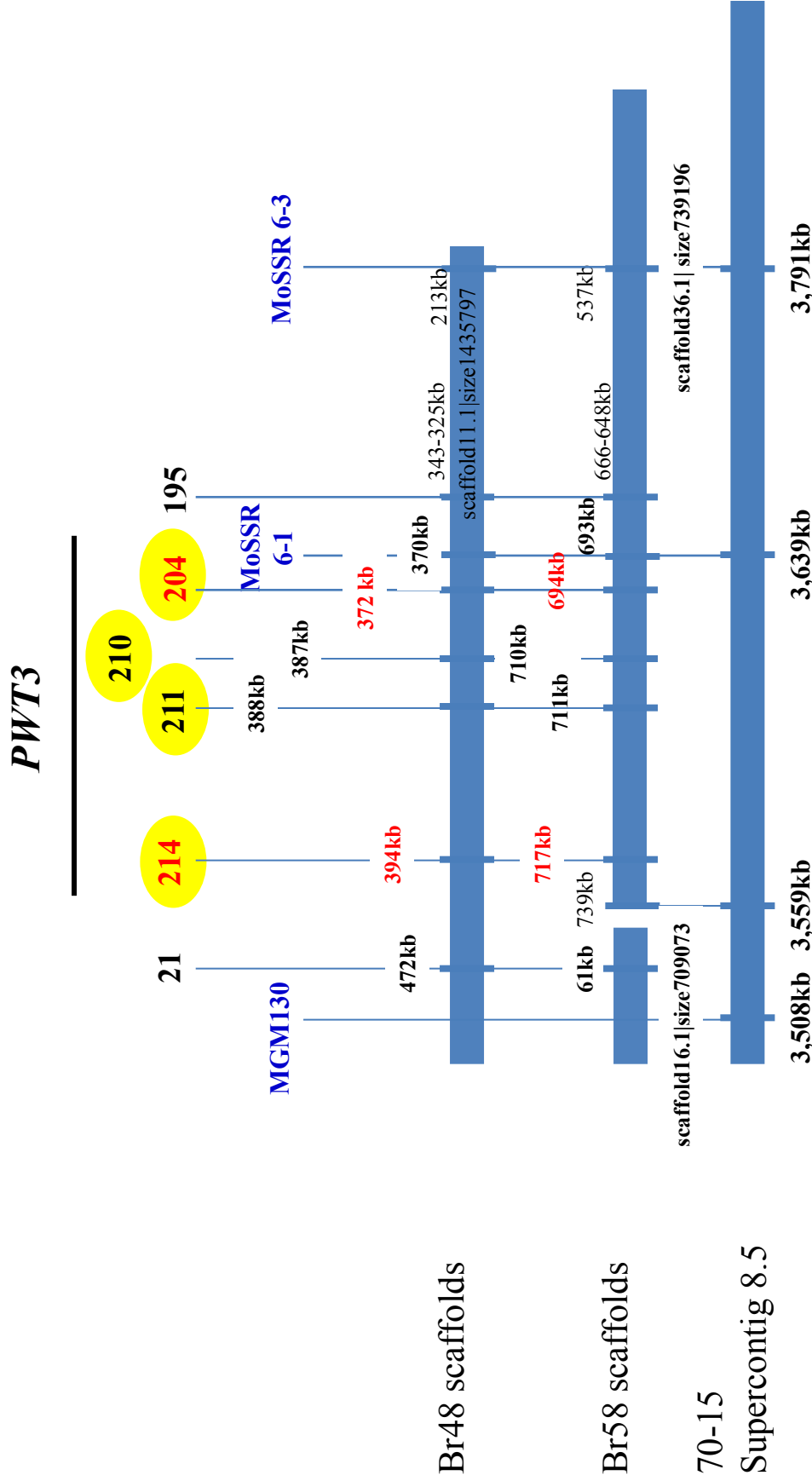


FIGURE 19. Physical map around *PWT3* based on the scaffolds of Br48 and Br58 isolates, and supercontig 8.5 of *M. oryzae* 70 – 15 from database.

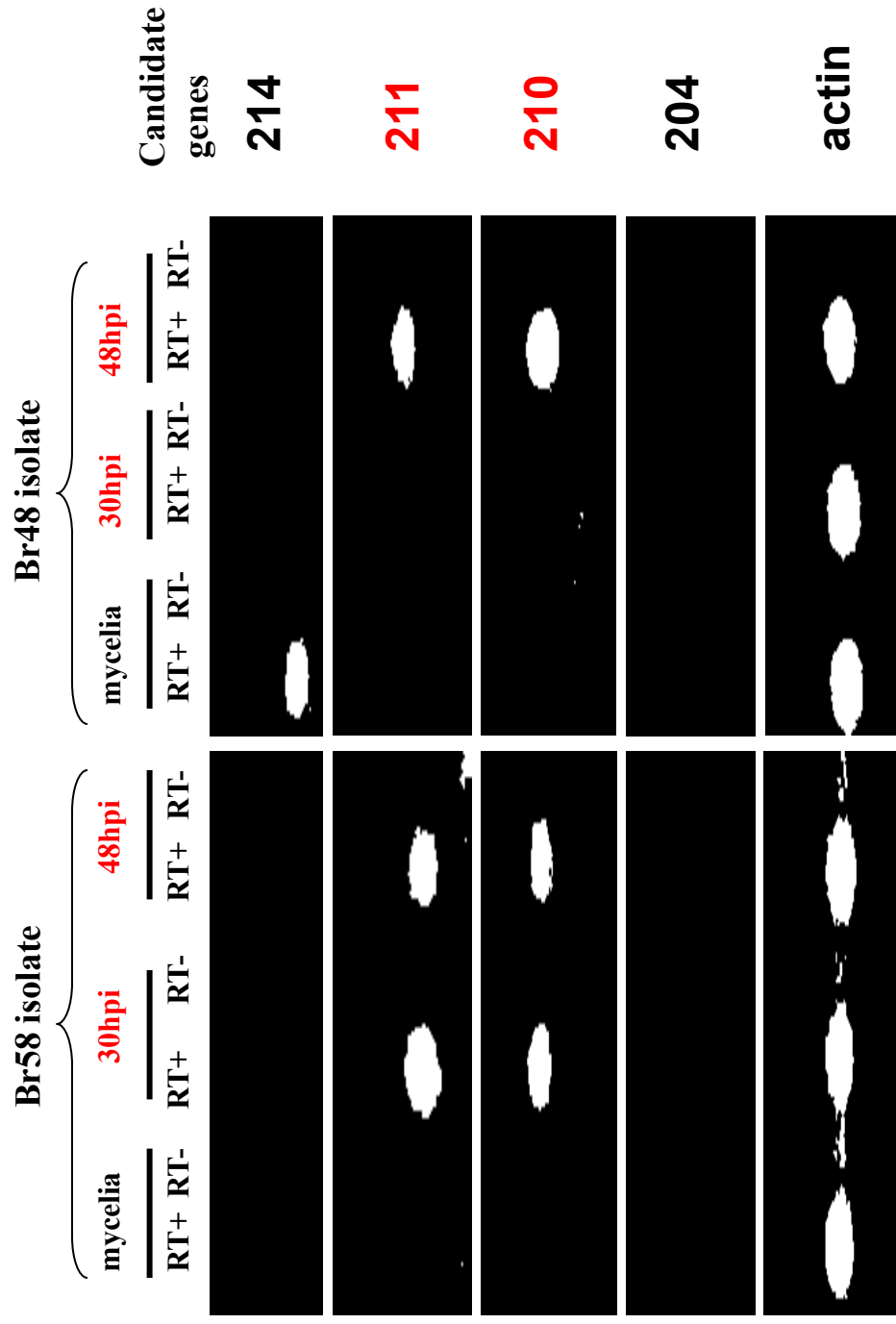


FIGURE 20. Expression of *PWT3* candidates invitro and in planta in *P. oryzae* wild isolates. Total RNA were extracted from 3 day old mycelia grown in CM media, or infected leaves of Tat14 at 30h or 48h post inoculation (pi). cDNAs were amplified with specific primers for each genes, and actin gene used as a control. These PCR products were checked on 1.5% agarose gel.

RT+: with reverse transcription ; RT-: without reverse transcription

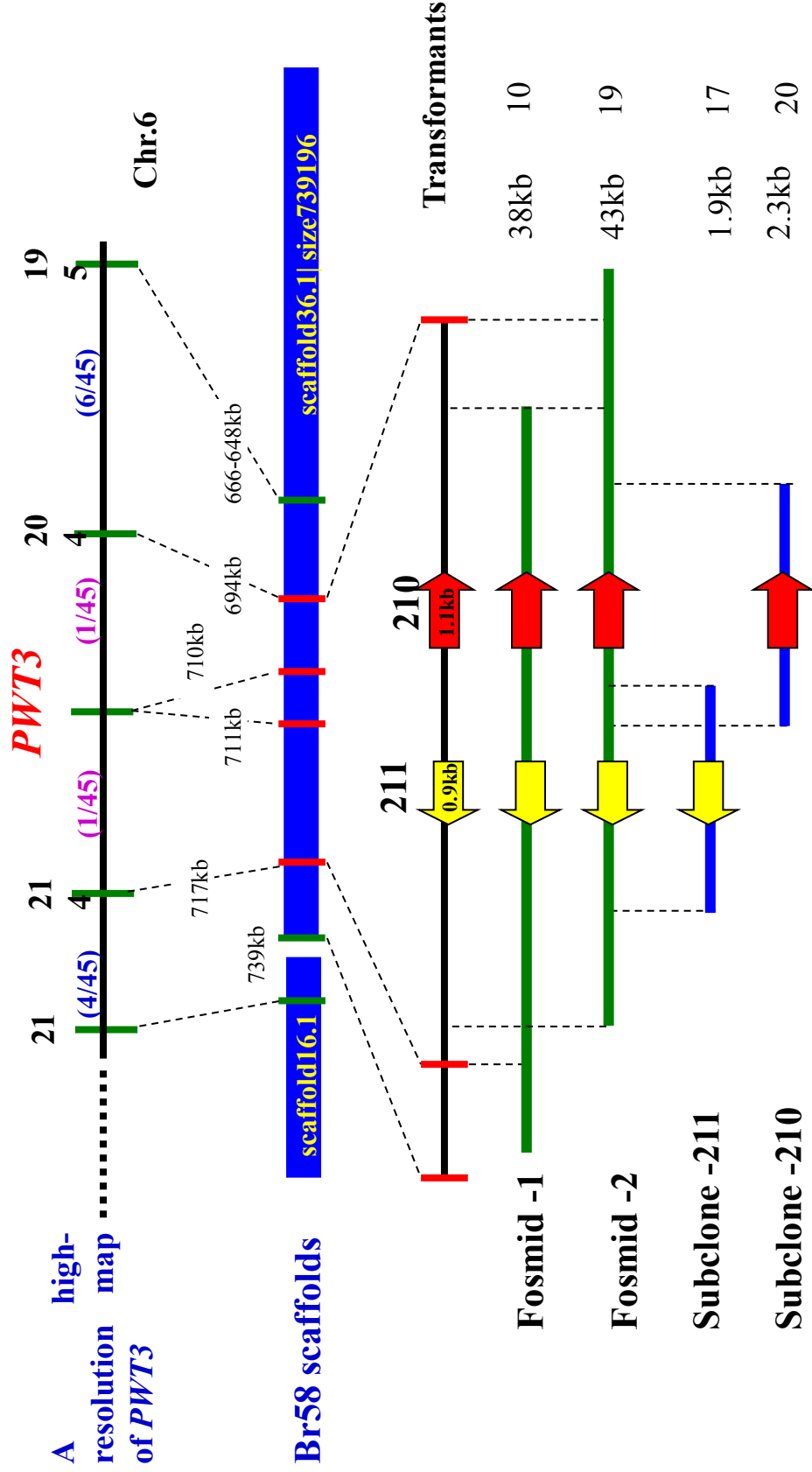


FIGURE 21. Constructs for complementation test. Fosmid-1 (38kb), and Fosmid-2 (43kb) selected from fosmid library of *Avena* isolate Br58 containing two candidate genes, 211 (in yellow arrow) and 210 (in red arrow), and two subclones for each gene are shown. The total number of transformants is indicated on the right of each construct.

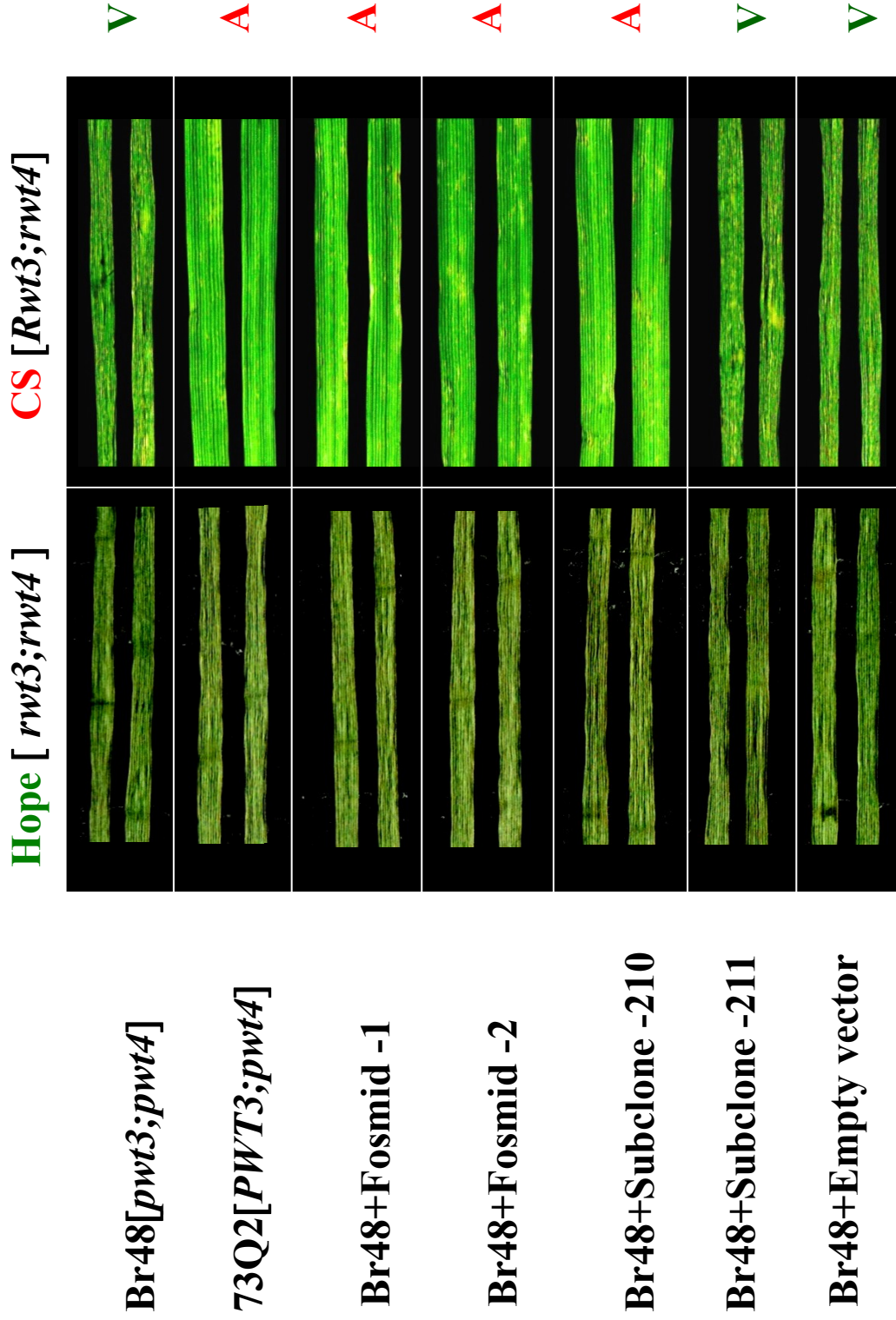


FIGURE 22. Pathogenicity of Triticum isolate Br48 and its transformants carrying fosmids or subclones on common wheat cultivars, Hope and CS, 4 days after inoculation. 73Q2 is a positive control showing the *PWT3* phenotype.

Signal peptide



1	ATGAACCTCAGACTTATTACTTTTAAATGACCTCCGTGGCCGGCGGTAGTGAC	60
	M N L R L I T F L M T S V A G A V A S D	
61	TTTTGAAAGTAGACCTTTATGGACCCCAAGGGTCACTCCAACGTCAAAAGTGAAATA	120
	F W K V D L Y G P P Q G H S N V K S E I	
121	GTGGCCACGGTGGTCTGCGGGGACGGACGAAAAGCAAATTTGAGTTACACTCATAGCTGG	180
	V A T V V C G D G R K A N L S Y T H S W	
181	CGCGGTGATAGTACAAACCAAGACTATTACCATCAGATGTCGAATTACACCCAAAGGTTATT	240
	R G D S T T K T I T I R C R I T P K V I	
241	AAGGTGGACCACTACCCCCCTACTACGAATTGAAAGCGGAACCCATATGGGCCGCGGAT	300
	K G G P L P P Y Y E L K A E P I W A G D	
301	GAGCGAGCCATAGCGGCTGCTaAGAGGGGACGAGGAAGAACGGGTCCTTGAACAAATTTGAA	360
	E R A I A A A K R E Q E E R V L E Q F E	
361	CTTATGAACCTGATGATGGACGCATCGGAGGATCCTAAGCGCGGCGGTGTTGCATCG	420
	L M N L M M D A S E D P K A R R L F A S	
421	CCGTAA 426	
	P *	

FIGURE 23. The coding region of the 210 gene, a candidate of *PWT3*. Its DNA and predicted amino acid sequence were determined by sequencing of subclone 210.

a)	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
	Position	57	97	194	199	201	233	241	261	262	283	289	290	313	322	343	347	361	364	389
	<i>PWT3</i>	T	C	C	A	G	A	A	C	T	C	T	G	C	A	G	T	C	A	A
	<i>Oryza</i> isolates	.	T	.	.	C	.	.	T	A	.	.	.	A	.	T	A	.	.	C
	<i>Setaria</i> isolates	.	T	.	.	C	.	.	T	A	.	.	.	A	.	T	A	.	.	C
	<i>Lolium</i> isolate - L-6	.	T	.	.	C	.	.	T	A	.	.	.	A	.	T	A	.	.	C
	<i>Panicum</i> P-6	.	T	.	.	C	.	.	T	A	.	.	.	A	.	T	A	.	.	C
	<i>Panicum</i> isolates	.	T	.	.	T	C	.	.	.	.	.	.	A	.	T	A	A	.	C
	<i>Triticum</i> isolates (group 1)	A	T	G	G	.	.	G	.	.	T	C	C	.	G	T	A	.	G	C
	<i>Brachiaria</i> isolates B-1, B-2	A	T	G	G	.	.	G	.	.	T	C	C	.	G	T	A	.	G	C
	<i>Eragrostis</i> isolate X-1	A	T	G	G	.	.	G	.	.	T	C	C	.	G	T	A	.	G	C
	<i>Triticum</i> isolate (group 2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	<i>Avena</i> isolate	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	<i>Lolium</i> isolates	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	<i>Eleusine</i> isolates	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

A type

B type

D type

C type

b)	No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Position	19	33	65	67	78	81	88	95	97	105	108	115	116	121	122	130
	<i>PWT3</i>	S	H	T	K	K	K	Y	P	W	A	K	V	L	L	M	E
	<i>Oryza</i> isolates	.	Y	.	N	.	.	N	.	.	E	.	F	H	.	.	A
	<i>Setaria</i> isolates	.	Y	.	N	.	.	N	.	.	E	.	F	H	.	.	A
	<i>Lolium</i> isolate - L-6	.	Y	.	N	.	.	N	.	.	E	.	F	H	.	.	A
	<i>Panicum</i> P-6	.	Y	.	N	.	.	N	.	.	E	.	F	H	.	.	A
	<i>Panicum</i> isolates	.	Y	.	N	T	.	.	.	.	E	.	F	H	I	.	.
	<i>Triticum</i> isolates (group 1)	R	Y	R	E	.	E	.	S	P	.	R	F	H	.	V	A
	<i>Brachiaria</i> isolates B-1, B-2	R	Y	R	E	.	E	.	S	P	.	R	F	H	.	V	A
	<i>Eragrostis</i> isolate X-1	R	Y	R	E	.	E	.	S	P	.	R	F	H	.	V	A
	<i>Triticum</i> isolate (group 2)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	<i>Avena</i> isolate	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	<i>Lolium</i> isolates	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	<i>Eleusine</i> isolates	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

A type

B type

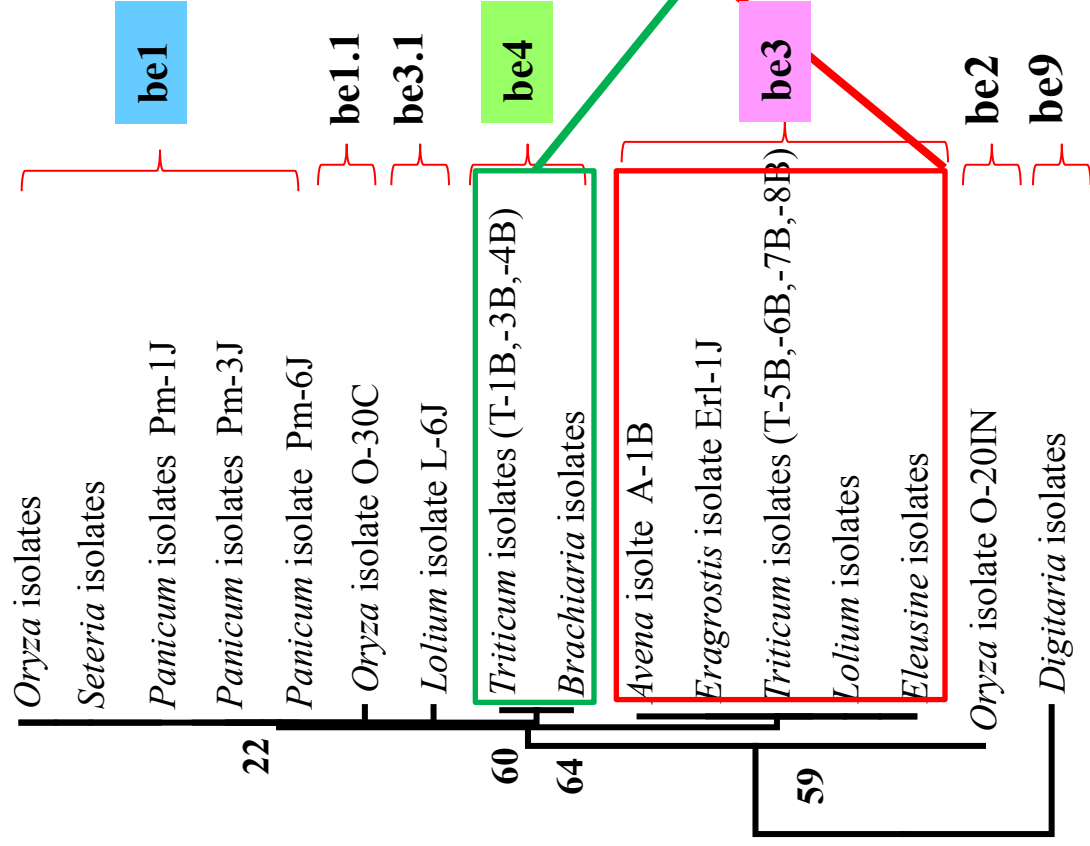
D type

C type

FIGURE 24. Nucleotide and amino acid sequence alignment of the coding region of *PWT3* and its homologs.

a) Nucleotide sequence alignment; b) Amino acid sequence alignment

β -tubulin gene



ORFs of the *PWT3* gene

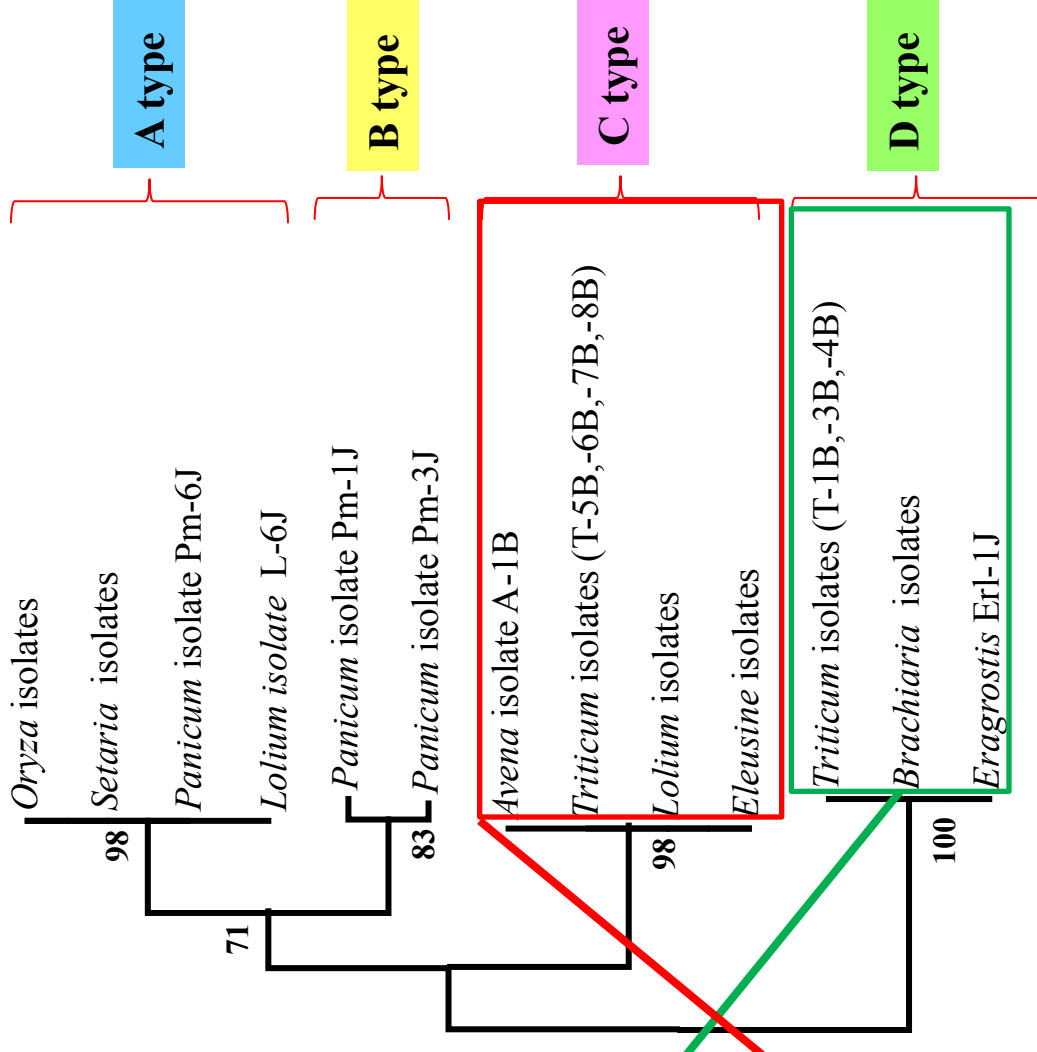


FIGURE 25. Phylogenetic trees of *PWT3* homologues (right) and *Pyricularia* isolates (left) constructed from nucleotide sequences of the ORF of *PWT3* homologues and the β -tubulin gene, respectively.

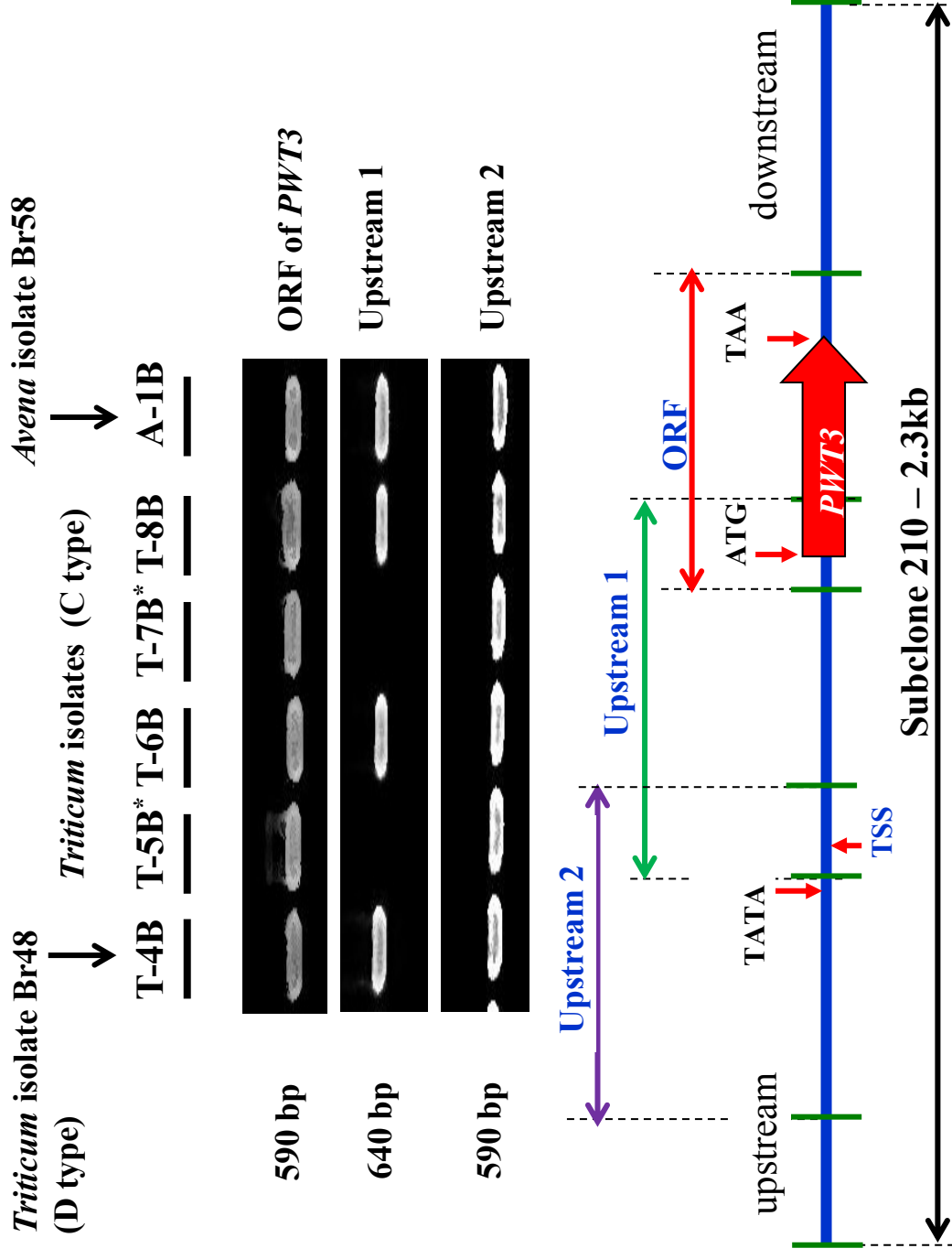
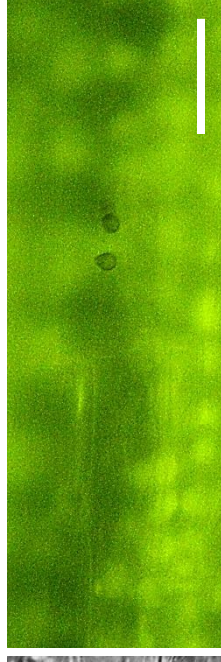
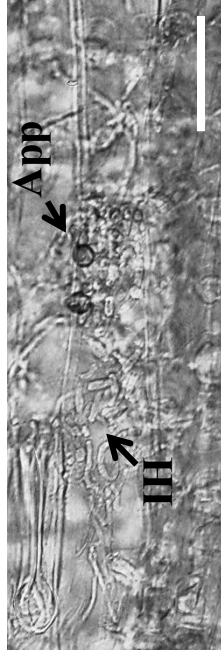
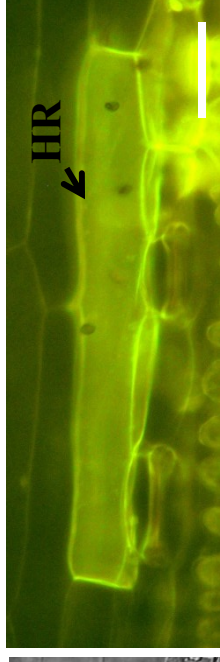
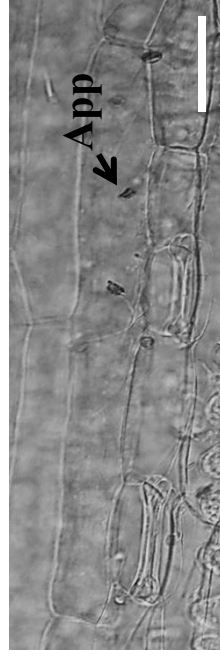


FIGURE 26. Characterization of *PWT3* upstream and ORF regions of *Triticum* isolates with the C type homologues. The ORF, upstream 1, and upstream 2 regions were amplified and checked on 1.5% agarose gel.

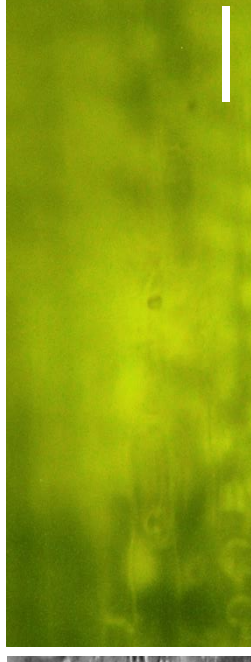
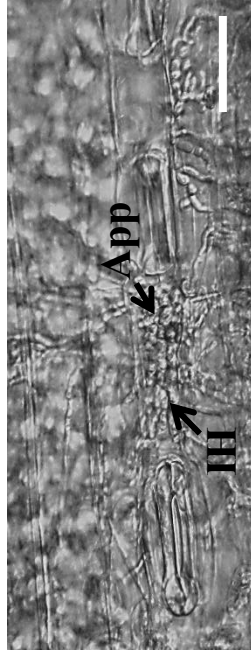
Br48 (T-4B)
D type



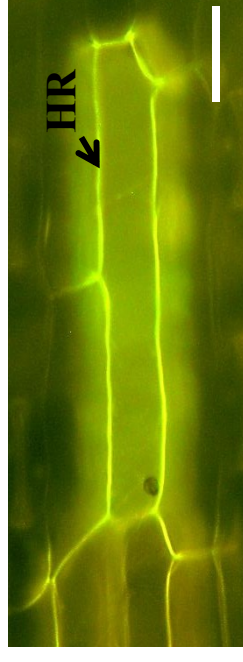
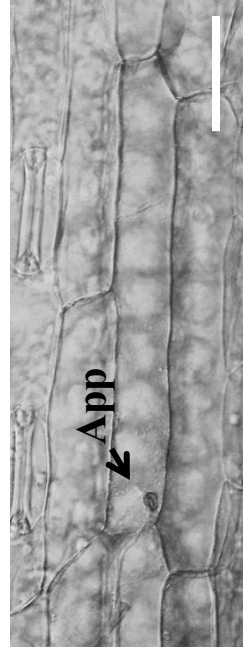
73Q2
(F₁ of Br58xBr48)



Br116.5 (T-7B)
C* type



Br118.2D (T-8B)
C type



Bright field

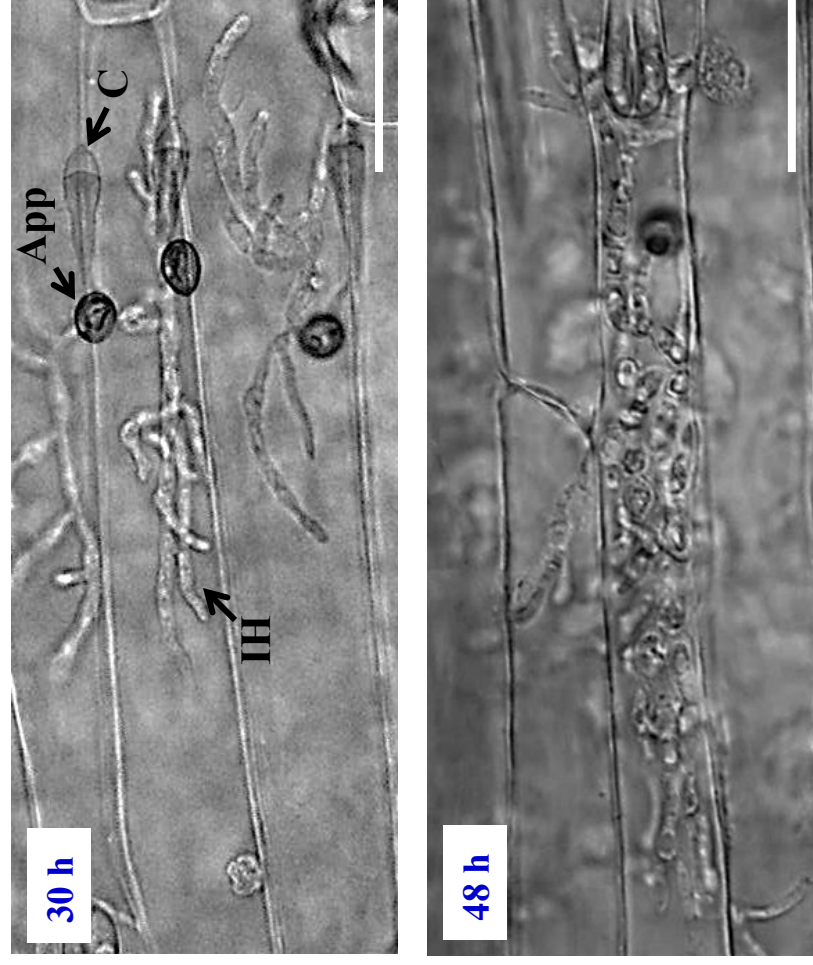
Fluorescence field

FIGURE 27. Cytological responses observed under bright field and fluorescence field in epidermal cells of hexaploid wheat N4 inoculated with *Triticum* isolates, Br48 carrying the D type, Br116.5 carrying the C* type, and Br118.2D carrying the C type and 73Q2 (F₁) derived from a cross, Br58 x Br48, 30h and 48h after inoculation. App: appressoria; IH: infection hyphae; HR: hypersensitive reaction. Bars = 50 μ m.

→

attccgcctt tactctttta taattgtttc aacaacctct catatatatt tgacatatata 1260
acatt atg aac ctg aga ctt att act ttt tta atg acc tcc gtg gcc gcc 1310
Met Asn Leu Arg Leu Ile Thr Phe Leu Met Thr Ser Val Ala Gly
1 5 10 15
gcg gtg gct agt gac ttt tgg aaa gta gac ctt tat gga ccc cca cag 1358
Ala Val Ala Ser Asp Phe Trp Lys Val Asp Leu Tyr Gly Pro Pro Gln
20 25 30
ggt cac tcc aac gtc aaa agt gaa ata gta gtc gcc acg gtg gtc tgc ggg 1406
Gly His Ser Asn Val Lys Ser Glu Ile Val Ala Thr Val Val Cys Gly
35 40 45
gac gga cga aaa gca aat ttg agt tac act cat agc tgg cgc ggt gat 1454
Asp Gly Arg Lys Ala Asn Leu Ser Tyr Thr His Ser Trp Arg Gly Asp
50 55 60
agt aca acc aag act att acc atc aga tgt cga att aca ccc aag gtt 1502
Ser Thr Thr Lys Thr Ile Thr Ile Arg Cys Arg Ile Thr Pro Lys Val
65 70 75
att aag ggt gga cca cta ccc ccc tac tac gaa ttg aaa gcg gaa ccc 1550
Ile Lys Gly Gly Pro Leu Pro Pro Tyr Tyr Glu Leu Lys Ala Glu Pro
80 85 90 95
ata tgg gcc gcc gat gag cga gcc ata gcc gct gct aag agg gag cag 1598
Ile Trp Ala Gly Asp Glu Arg Ala Ile Ala Ala Lys Arg Glu Gln
100 105 110
gaa gaa cgg gtc ctt gaa caa ttt gaa ctt atg aac ctg atg atg gac 1646
Glu Glu Arg Val Leu Glu Gln Phe Glu Leu Met Asn Leu Met Met Asp
115 120 125
gca tgc gag gat cct aag gcg cgg cgg ctg ttt gca tgc ccg taa gtagg 1696
Ala Ser Glu Asp Pro Lys Ala Arg Arg Leu Phe Ala Ser Pro Stop
130 135 140
agcagataact ggttttttaga ctggatcggc tgcgcgcttt accgatttgg attaatggag 1756
ggcgggcatg aggagggtttt caggcccttg tcccatgcga atagtaaaga gggggaaaaat 1816
←

SUPPLEMENTAL FIGURE 1. Nucleotide and amino acid sequences of the position of a pair of primers used for PCR analysis of the *Pyricularia* population are shown in boxes.



SUPPLEMENTAL FIGURE 2. Cytological responses observed under a bright field in epidermal cells of tetraploid wheat Tat14 (KU156) inoculated with *Avena* isolate Br58, 30h and 48h after inoculation. App: appressoria; IH: infection hyphae; C: conidium. Bars = 20 μ m.

TABLE 11. List of *Pyricularia* isolates used

No.	Species	Code	Isolate	Original host	locality	Isolation year	Collector or reference
1	<i>P. oryzae</i>	O-1G	Guy11	<i>Oryza sativa</i>	Guiana (Combi)	1978	J.L. Notteghem
2	<i>P. oryzae</i>	O-2J	Ken54-04	<i>Oryza sativa</i>	Japan (Gifu)	1954	Kiyosawa (1974)
3	<i>P. oryzae</i>	O-3J	Ken54-20	<i>Oryza sativa</i>	Japan (Yamaguchi)	1954	Kiyosawa (1974)
4	<i>P. oryzae</i>	O-4J	Hoku1	<i>Oryza sativa</i>	Japan (Hokkaido)	1948	Kiyosawa (1974)
5	<i>P. oryzae</i>	O-5J	Ina72	<i>Oryza sativa</i>	Japan(Nagano)	1957	Kiyosawa (1974)
6	<i>P. oryzae</i>	O-6J	Ina168	<i>Oryza sativa</i>	Japan (Aichi)	1958	Kiyosawa (1974)
7	<i>P. oryzae</i>	O-7J	Ken53-33	<i>Oryza sativa</i>	Japan (Aichi)	1953	Kiyosawa (1974)
8	<i>P. oryzae</i>	O-8J	P-2b	<i>Oryza sativa</i>	Japan (Niigata)	1948	Kiyosawa (1974)
9	<i>P. oryzae</i>	O-9J	0903-4	<i>Oryza sativa</i>	Japan (Tochigi)	1976	Yamada et al. (1976)
10	<i>P. oryzae</i>	O-10J	2012-1	<i>Oryza sativa</i>	Japan (Ishikawa)	1976	Yamada et al. (1976)
11	<i>P. oryzae</i>	O-11J	2403-1	<i>Oryza sativa</i>	Japan (Mie)	1976	Yamada et al. (1976)
12	<i>P. oryzae</i>	O-12J	1836-3	<i>Oryza sativa</i>	Japan (Niigata)	1976	Yamada et al. (1976)
13	<i>P. oryzae</i>	O-13J	88A	<i>Oryza sativa</i>	Japan (Ibaraki)	1976	Yamada et al. (1976)
14	<i>P. oryzae</i>	O-14C	CHNOS 59-6-1	<i>Oryza sativa</i>	China (Yunnan)	1989	N. Hayashi
15	<i>P. oryzae</i>	O-15C	CHNOS 60-8-1	<i>Oryza sativa</i>	China (Yunnan)	1989	N. Hayashi
16	<i>P. oryzae</i>	O-16B	Br10	<i>Oryza sativa</i>	Brazil (Parana)	1990	S. Igarashi
17	<i>P. oryzae</i>	O-17B	Br13	<i>Oryza sativa</i>	Brazil (Parana)	1990	S. Igarashi
18	<i>P. oryzae</i>	O-18B	Br15	<i>Oryza sativa</i>	Brazil (Parana)	1990	S. Igarashi
19	<i>P. oryzae</i>	O-19B	Br18	<i>Oryza sativa</i>	Brazil (Parana)	1990	S. Igarashi
20	<i>P. oryzae</i>	O-20IN	PO-02-7306	<i>Oryza sativa</i>	Indonesia (Jawa Barat)	1973	A. Mukelar
21	<i>P. oryzae</i>	O-21IN	PO-02-7501	<i>Oryza sativa</i>	Indonesia (Jawa Barat)	1975	A. Mukelar
22	<i>P. oryzae</i>	O-22IN	PO-04-7501	<i>Oryza sativa</i>	Indonesia (Jawa Timur)	1975	A. Mukelar
23	<i>P. oryzae</i>	O-23IN	PO-12-7301-2	<i>Oryza sativa</i>	Indonesia (Lampung)	1973	A. Mukelar
24	<i>P. oryzae</i>	O-24IN	PO-12-7301	<i>Oryza sativa</i>	Indonesia (Lampung)	1973	A. Mukelar
25	<i>P. oryzae</i>	O-25V	VHT6.1	<i>Oryza sativa</i>	Vietnam (Ha Tay)	1998	L.D. Don
26	<i>P. oryzae</i>	O-26V	VTB6.1	<i>Oryza sativa</i>	Vietnam (Thai Binh)	1998	L.D. Don
27	<i>P. oryzae</i>	O-27V	VHG4.5	<i>Oryza sativa</i>	Vietnam (Hau Giang)	1996	L.D. Don
28	<i>P. oryzae</i>	O-28V	VHT3.3	<i>Oryza sativa</i>	Vietnam (Ha Tay)	1998	L.D. Don
29	<i>P. oryzae</i>	O-29J	84R-62B	<i>Oryza sativa</i>	Japan (Aichi)	1984	H. Naito
30	<i>P. oryzae</i>	O-30C	Y93-245c-2	<i>Oryza sativa</i>	China (Yunnan)	1993	Y. Fujita
31	<i>P. oryzae</i>	Si-1J	GFSI1-7-2	<i>Setaria italica</i>	Japan (Gifu)	1977	H. Kato
32	<i>P. oryzae</i>	Si-2J	NRSI2-2-2	<i>Setaria italica</i>	Japan (Nara)	1977	H. Kato
33	<i>P. oryzae</i>	Si-3J	NRSI3-1-1	<i>Setaria italica</i>	Japan (Nara)	1977	H. Kato
34	<i>P. oryzae</i>	Si-4J	NNSI3-2-1	<i>Setaria italica</i>	Japan (Nagano)	1984	H. Kato
35	<i>P. oryzae</i>	Si-5I	IN77-16-1-1	<i>Setaria italica</i>	India (Mysore)	1977	H. Kato
36	<i>P. oryzae</i>	Si-6I	IN77-20-1-1	<i>Setaria italica</i>	India (Mysore)	1977	H. Kato
37	<i>P. oryzae</i>	Sv-7J	KANSV1-4-1	<i>Setaria viridis</i>	Japan (Kanagawa)	1975	H. Kato
38	<i>P. oryzae</i>	Sv-8J	NI913	<i>Setaria viridis</i>	Japan (Chiba)	1974	N. Nishihara
39	<i>P. oryzae</i>	Pm-1J	NNPM1-2-1	<i>Panicum miliaceum</i>	Japan (Nagano)	1984	N. Hayashi
40	<i>P. oryzae</i>	Pm-2J	NRPM1-1-1	<i>Panicum miliaceum</i>	Japan (Nara)	1990	H. Kato
41	<i>P. oryzae</i>	Pm-3J	STPM1-3-2	<i>Panicum miliaceum</i>	Japan (Saitama)	1981	H. Kato
42	<i>P. oryzae</i>	Pm-4J	STPM4-2-2	<i>Panicum miliaceum</i>	Japan (Saitama)	1981	H. Kato
43	<i>P. oryzae</i>	Pm-5J	SZPM1-1-1	<i>Panicum miliaceum</i>	Japan (Shizuoka)	1978	H. Kato
44	<i>P. oryzae</i>	Pm-6J	YNPM4-1-1	<i>Panicum miliaceum</i>	Japan (Yamanashi)	1983	H. Kato
45	<i>P. oryzae</i>	Pb-7J	NI922	<i>Panicum bisulcatum</i>	Japan (Tochigi)	1974	N. Nishihara
46	<i>P. oryzae</i>	Ec-1J	G10-1	<i>Eleusine coracana</i>	Japan (Tochigi)	1977	S. Itoi
47	<i>P. oryzae</i>	Ec-2J	Z2-1	<i>Eleusine coracana</i>	Japan (Kagawa)	1977	S. Itoi
48	<i>P. oryzae</i>	Ec-3J	MZ5-1-6	<i>Eleusine coracana</i>	Japan (Miyazaki)	1976	H. Kato
49	<i>P. oryzae</i>	Ec-4J	Ken15-15-1	<i>Eleusine coracana</i>	Japan (Tokyo)	1976	H. Kato
50	<i>P. oryzae</i>	Ec-5J	SZEC1-1-1	<i>Eleusine coracana</i>	Japan (Shizuoka)	1978	H. Kato

TABLE 11. List of *Pyricularia* isolates used (continued)

No.	Species	Code	Isolate	Original host	locality	Isolation year	Collector or reference
51	<i>P. oryzae</i>	Ec-6J	GFEC1-5-1	<i>Eleusine coracana</i>	Japan (Gifu)	1977	H. Kato
52	<i>P. oryzae</i>	Ec-7I	IN77-31-1-1	<i>Eleusine coracana</i>	India (Bangalore)	1977	H. Kato
53	<i>P. oryzae</i>	Ec-8I	IN77-39-1-2	<i>Eleusine coracana</i>	India (Bangalore)	1977	H. Kato
54	<i>P. oryzae</i>	Ec-9N	NP10-17-4-1-3	<i>Eleusine coracana</i>	Nepal (Syabru)	1975	S. Sakamoto
55	<i>P. oryzae</i>	Ec-10N	NP10-28-1-1-1	<i>Eleusine coracana</i>	Nepal (Pokara)	1975	S. Sakamoto
56	<i>P. oryzae</i>	Ea-11J	NI1006	<i>Eleusine africana</i>	Japan (Tochigi)	1975	N. Nishihara
57	<i>P. oryzae</i>	Eb-12J	NI1011	<i>Eleusine boranensis</i>	Japan (Tochigi)	1975	N. Nishihara
58	<i>P. oryzae</i>	Ei-13I	IN77-36-1-1	<i>Eleusine indica</i>	India (Mysore)	1977	H. Kato
59	<i>P. oryzae</i>	A-1B	Br58	<i>Avena sativa</i>	Brazil (Parana)	1990	S. Igarashi
60	<i>P. oryzae</i>	T-1B	Br3	<i>Triticum aestivum</i>	Brazil (Parana)	1990	S. Igarashi
61	<i>P. oryzae</i>	T-2B	Br7	<i>Triticum aestivum</i>	Brazil (Parana)	1990	S. Igarashi
62	<i>P. oryzae</i>	T-3B	Br8	<i>Triticum aestivum</i>	Brazil (Parana)	1990	S. Igarashi
63	<i>P. oryzae</i>	T-4B	Br48	<i>Triticum aestivum</i>	Brazil (Mato Grosso do Sul)	1990	S. Igarashi
64	<i>P. oryzae</i>	T-5B	Br49	<i>Triticum aestivum</i>	Brazil (Mato Grosso do Sul)	1990	S. Igarashi
65	<i>P. oryzae</i>	T-6B	Br115.7	<i>Triticum aestivum</i>	Brazil (Parana)	1992	A.S. Urashima
66	<i>P. oryzae</i>	T-7B	Br116.5	<i>Triticum aestivum</i>	Brazil (Parana)	1992	A.S. Urashima
67	<i>P. oryzae</i>	T-8B	Br118.2D	<i>Triticum aestivum</i>	Brazil (Parana)	1992	A.S. Urashima
68	<i>P. oryzae</i>	L-1J	TP1	<i>Lolium perenne</i>	Japan (Tochigi)	1997	A. Tanaka, T. Tani
69	<i>P. oryzae</i>	L-2J	TP2	<i>Lolium perenne</i>	Japan (Tochigi)	1997	A. Tanaka, T. Tani
70	<i>P. oryzae</i>	L-3J	AK1	<i>Lolium perenne</i>	Japan (Akita)	1998	A. Tanaka, T. Tani
71	<i>P. oryzae</i>	L-4J	LW3	<i>Lolium perenne</i>	Japan (Yamanashi)	1999	A. Tanaka, T. Tani
72	<i>P. oryzae</i>	L-5J	FI5	<i>Lolium perenne</i>	Japan (Chiba)	1998	A. Tanaka, T. Tani
73	<i>P. oryzae</i>	L-6J	WK3-1	<i>Lolium perenne</i>	Japan (Yamaguchi)	1996	A. Tanaka, T. Tani
74	<i>P. oryzae</i>	B-1B	Br35	<i>Brachiaria plantaginea</i>	Brazil (Parana)	1990	S. Igarashi
75	<i>P. oryzae</i>	B-2B	Bp3a	<i>Brachiaria plantaginea</i>	Brazil (Sao Paulo)	1996	A.S. Urashima
76	<i>P. oryzae</i>	Erl-1J	NI986	<i>Eragrostis lehmanniana</i>	Japan (Kumamoto)	1975	N. Nishihara
77	<i>Pyricularia</i> sp. (LS) ^c	Leo-1J	NI919	<i>Leersia oryzoides</i>	Japan (Chiba)	1974	N. Nishihara
78	<i>Pyricularia</i> sp. (LS) ^c	Sg-1B	Br37	<i>Setaria geniculata</i>	Brazil (Parana)	1990	S. Igarashi
79	<i>Pyricularia</i> sp. (CE) ^c	Cc-1J	NI981	<i>Cenchrus ciliaris</i>	Japan (Kumamoto)	1975	N. Nishihara
80	<i>Pyricularia</i> sp. (CE) ^c	Ce-1B	Br36	<i>Cenchrus echinatus</i>	Brazil (Parana)	1990	S. Igarashi
81	<i>Pyricularia</i> sp. (CE) ^c	Ecc-1B	Br38	<i>Echinochloa colonum</i>	Brazil (Parana)	1990	S. Igarashi
82	<i>P. grisea</i>	Dsa-1J	Dig41	<i>Digitaria sanguinalis</i>	Japan (Hyogo)	1990	Y. Iwamoto
83	<i>P. grisea</i>	Dsa-2J	NI907	<i>Digitaria sanguinalis</i>	Japan (Tochigi)	1974	N. Nishihara
84	<i>P. grisea</i>	Dsa-3J	IBDS4-1-1	<i>Digitaria sanguinalis</i>	Japan (Ibaraki)	1985	N. Hayashi
85	<i>P. grisea</i>	Dsm-4J	NI980	<i>Digitaria smutsii</i>	Japan (Kumamoto)	1975	N. Nishihara
86	<i>P. grisea</i>	Dh-5B	Br29	<i>Digitaria horizontalis</i>	Brazil (Sao Paulo)	1990	S. Igarashi
87	<i>P. grisea</i>	Dh-6B	Br33	<i>Digitaria horizontalis</i>	Brazil (Parana)	1990	S. Igarashi
88	<i>P. zizaniaecola</i>	Zz-1J	IBZL3-1-1	<i>Zizania latifolia</i>	Japan (Ibaraki)	1985	N. Hayashi
89	<i>P. zizaniaecola</i>	Zz-2J	KYZL201-1-1	<i>Zizania latifolia</i>	Japan (Kyoto)	2003	K. Yoshida, K. Hirata
90	<i>P. zingiberi</i>	Z-1J	HYZiM101-1-1-1	<i>Zingiber mioga</i>	Japan (Hyogo)	1990	M. Ogawa
91	<i>P. zingiberi</i>	Z-2J	HYZiM201-0-1	<i>Zingiber mioga</i>	Japan (Hyogo)	2002	H. Kato
92	<i>P. zingiberi</i>	Z-3J	HYZiM202-1-2	<i>Zingiber mioga</i>	Japan (Hyogo)	2003	I. Chuma
93	<i>P. zingiberi</i>	Z-4J	HYZiM201-1-1	<i>Zingiber mioga</i>	Japan (Hyogo)	2003	I. Chuma
94	<i>Pyricularia</i> sp. (SsPb) ^c	Ss-1J	INA-B-92-45	<i>Sasa</i> sp.	Japan (Aichi)	1992	S. Koizumi
95	<i>Pyricularia</i> sp. (SsPb) ^c	Ph-1J	INA-B-93-19	<i>Phyllostachys bambusoides</i>	Japan (Aichi)	1993	S. Koizumi
96	<i>Pyricularia</i> sp. (Kb) ^c	K-1J	HYKB202-1-2	<i>Kyllinga brevifolia</i>	Japan (Hyogo)	2003	I. Chuma
97	<i>Pyricularia</i> sp. (Kb) ^c	K-2J	FKKB201-1-5	<i>Kyllinga brevifolia</i>	Japan (Fukuoka)	2003	I. Chuma
98	<i>Pyricularia</i> sp. (Kb) ^c	K-3J	FKKB201-3-2	<i>Kyllinga brevifolia</i>	Japan (Fukuoka)	2003	I. Chuma
99	<i>P. higginsii</i>	Ci-1J	HYCI201-1-1	<i>Cyperus iria</i>	Japan (Hyogo)	2002	H. Kato

TABLE 12. New SSR markers designed using database of *M. oryzae* 70-15

Locus	Primer_name	Primer_sequence(5' -> 3')	Product_size(bp)	Tm(°C)
MG8_supercont8.5_3639859_3639905_(TG)n	MoSSR_6_1_F	TCAACATGCCTCTATTCTGTC	144	55
	MoSSR_6_1_R	CTTCTACGGCTGTCAAAGATAC		
MG8_supercont8.5_3791509_3791545_(T)n	MoSSR_6_3_F	TTGATATGCTTGTTCGATATG	149	55
	MoSSR_6_3_R	TGTACGTTGACACCTTCTAGG		
MG8_supercont8.5_4190127_4190150_(TTG)n	MoSSR_6_7_F	ATGACGGTCTTTTGGTG	158	55
	MoSSR_6_7_R	AGGGTGCTGTTCGACACT		
MG8_supercont8.5_4314317_4314352_(GAAAA)n	MoSSR_6_23_F	CATGCGGGTAGACCACTA	245	55
	MoSSR_6_23_R	TGGGAAAACGCATAAAAAG		
MG8_supercont8.5_4468851_4468881_AT-rich	MoSSR_6_24_F	TATACGACGCACAACACG	246	55
	MoSSR_6_24_R	ACGGGCATTTATTTTGTG		
MG8_supercont8.5_3645k_indel_F1	3645_F	CTGTCGTGGTCGTCTTCTCA	250	55
MG8_supercont8.5_3645k_indel_R1	3645_R	CTTCAACTGCCTCTCTCAACC	280	55
MG8_supercont8.5_3642k_indel_F1	3642_F	CTAGACGACGAGCTGGAA		
MG8_supercont8.5_3642k_indel_R1	3642_R	GCTCTGCCCTCTTCAACAA		

TABLE 13. Gene-specific primers for a high – resolution map of *PWT3*

Primer_name	Primer_sequence(5' -> 3')
Br58_scaf16_21_Hinfl_F1	ACCGAGAAAGAGCGTCAGC
Br58_scaf16_21_Hinfl_R1	ACAGTGTGCACGGTCGAGT
Br58_scaf36_195_indel_F1	GAGACTGGCACACAGGTGGTTC
Br58_scaf36_195_indel_R1	CTCCCAGTTCCCCGATCCT
Br58_scaf36_202_PA_F1	AACTCACCGTTCTCGCCTCT
Br58_scaf36_202_PA_R1	CCTCAGGGTCAACGCATCT
Br58_scaffold36.1_202_PA_F2	CTCACGAGCGTAGGGAGT
Br58_scaffold36.1_202_PA_R2	CGAGGTTTCCAGACTGC
Br58_scaf36_204_PA_F1	CCGACTGTTCCAGCTTCC
Br58_scaf36_204_PA_R1	GAGGGTGTTCACCTCGTGAT
Br58_scaf36_210_PA_F1	CTACTACGAATTGAAAGCGGAAC
Br58_scaf36_210_PA_R1	GCGTCCATCATCAGGTTTCAT
Br58_scaf36_210_Hinfl_F1	ACCTTTATGGACCCCCACAG
Br58_scaf36_210_Hinfl_R1	CGCTATGGCTCGCTCATC
Br58_scaf36_211_EcoRI_F1	CCTTTCTTCAACACTACCGTGA
Br58_scaf36_211_EcoRI_R1	GGAGGTGATGATGCGAGGT
Br58_scaf36_214_indel_F1	CTCGGTAACCAGTCTTACAACG
Br58_scaf36_214_indel_R1	GCTTGATGCAGCAGAGGAG

TABLE 14. Primers designed for detection of transcripts of *PWT3* candidates

Primer_name	Primer_sequence(5' -> 3')
Br58_scaf36_204_qPCR_F	CTTGCTTACATTGACTCTGACCAC
Br58_scaf36_204_qPCR_R	ATTATCCCGTAGGTCGGAAGAAC
Br58_scaf36_210_qPCR_F	CTACTACGAATTGAAAGCGGAAC
Br58_scaf36_210_qPCR_R	CCATCATCAGGTTCATAAGTTCA
Br58_scaf36_211_qPCR_F	GTGTCTTATACCAACCGCATCTGAG
Br58_scaf36_211_qPCR_R	GTAGAAGTGGTTCCTATGCCATT
Br58_scaf36_214_qPCR_F	GTCGGTCTGTGGGAAAGCTAC
Br58_scaf36_214_qPCR_R	GATGGTTGTAATCGGGACAAAC
Actin-F	GCGGTTACACCTCTCTACCAC
Actin-R	AGTCTGGATCTCCTGCTCAAAG

TABLE 15. Primer pairs used for PCR analysis of *PWT3* homologs in *Pyricularia* populations

Primer_name	Primer_sequence(5' -> 3')	Product_size(bp)	Tm(°C)
PWT3_ORF_F	AGGCCTGAAAACCTCCTCAT	589	55
PWT3_ORF_R	CTGCAAAATTC CGCCTT TACT		
Br58_PWT3_up_F1	GTGGGGGTCCATAAAGGTCT	700	55
Br58_PWT3_up_R1	GCTTTGCCGACTTTGGTAAT		
Br58_PWT3_up_F2	ACCCCTTGTAACCGTGAT	584	55
Br58_PWT3_up_R2	CTTTCGTGGTAGTCTCCTTG		

TABLE 16. Distribution of *PWT3* homologs in the *Pyricularia* populations

Species	Host	Distribution of <i>PWT3</i> homologs		
		Total	Present	Absent
<i>Pyricularia oryzae</i>	<i>Oryza sativa</i>	30	21	9
<i>P. oryzae</i>	<i>Setaria</i> spp.	8	8	0
<i>P. oryzae</i>	<i>Panicum miliaceum</i>	6	6	0
<i>P. oryzae</i>	<i>Panicum bisulcatum</i>	1	0	1
<i>P. oryzae</i>	<i>Eleusine</i> spp.	13	13	0
<i>P. oryzae</i>	<i>Avena sativa</i> (Br58)	1	1	0
<i>P. oryzae</i>	<i>Triticum aestivum</i>	8	7	1
<i>P. oryzae</i>	<i>Lolium perenne</i>	6	6	0
<i>P. oryzae</i>	<i>Brachiaria plantaginea</i>	2	2	0
<i>P. oryzae</i>	<i>Eragrostis lehmanniana</i>	1	1	0
<i>Pyricularia</i> sp.	<i>Leersia oryzoides</i>	1	0	1
<i>Pyricularia</i> sp.	<i>Setaria geniculata</i>	1	0	1
<i>Pyricularia</i> sp.	<i>Cenchrus</i> spp.	2	0	2
<i>Pyricularia</i> sp.	<i>Echinochloa colonum</i>	1	0	1
<i>P. grisea</i>	<i>Digitaria</i> spp.	6	0	6
<i>P. zizaniaecola</i>	<i>Zizania latifolia</i>	2	0	2
<i>P. zingiberi</i>	<i>Zingiber mioga</i>	4	0	4
<i>Pyricularia</i> sp.	<i>Sasa</i> sp.	1	0	1
<i>Pyricularia</i> sp.	<i>Phyllostachys bambusoides</i>	1	0	1
<i>Pyricularia</i> sp.	<i>Kyllinga brevifolia</i>	3	0	3
<i>Pyricularia</i> sp.	<i>Cyperus iria</i>	1	0	1

Chapter 6

General Discussion

In the present study we successfully identified two resistance genes *R1* and *R2* as a common wheat gene conferring resistance to a *Lolium* isolate. To our knowledge they are first resistant genes of common wheat against an inappropriate subgroup of *P. oryzae*, *Lolium* isolates. Incompatibility between the *Lolium* isolate and common wheat was conditioned by two gene pairs, *R1* – *A1* and *R2* – *A2* following a gene-for-gene interaction. A question is whether they are identical to some of the previously identified resistance/avirulence genes. Based on genetic analysis, we successfully demonstrated that *A1* could be *PWT3*. *PWT3* is one of the two fungal genes involved in the avirulence of the *Avena* isolate (Br58) on wheat cultivars, and corresponds to the resistance gene *Rwt3* (Takabayashi et al. 2002). These results suggest that *PWT3* (*A1*) is commonly involved in the avirulence of the two distinct subgroups of *P. oryzae*, and is recognized by the resistance gene *Rwt3*(*R1*) widely distributed in common wheat cultivars.

PWT3 gene was successfully cloned into a 2.3kb fragment. This fragment contained some possible ORFs. However, the 426 bp ORF (gene 210) fulfilled all the requirements for an avirulence gene. For instance, this gene had a signal peptide (54 bp) and its transcript was detected in infected plant cells by RT-PCR or transient assay (data not shown).

An interaction between resistance (*R*) gene and avirulence (*AVR*) gene products induces defense responses including hypersensitive response (HR) in infected host cells. In this interaction, the *R* gene product acts as a receptor that directly or indirectly recognizes a ligand or elicitor produced by the *AVR* gene.

Indirect interactions have been observed in several cases. Nucleotide-binding site (NBS) - leucine-rich repeat (LRR) proteins may recognize pathogen effectors indirectly through an accessory protein. In the guard model, an effector modifies an accessory protein; then, a NB-LRR receptor recognizes the modified accessory protein. For example, in *Arabidopsis thaliana*, RPM1-interacting protein 4 (RIN4) associates with resistance gene *RPM1* (Resistance to *Pseudomonas syringae* pv. *maculicola* 1). RIN4 may be a target of effectors encoded by two avirulence genes *AvrRpm1* and *AvrB*. Phosphorylation of RIN4 mediated by those effectors activates *RPM1*. Thus, the modification of RIN4 by these effectors can explain how *RPM1* recognizes more than one effector. Direct interactions between R gene and AVR gene products have also been reported. The *AVR-Pita* protein of the rice blast fungus binds directly to the rice resistance gene *Pi-ta* – LRD (leucine-rich domain) region inside the plant cell to initiate *Pi-ta* mediated resistance (Jia et al.2000). In this study, I showed that the interaction between *Rwt3* and the cloned *PWT3* resulted in strong resistance in common wheat cultivars (*Rwt3* carriers). The predicted protein product encoded by the potential *PWT3* gene (426 bp) was expressed in host cells. Cell death caused by interactions between *PWT3* and *Rwt3* was confirmed through a transient assay with wheat protoplasts as reduced luciferase activity (data not shown). However, further studies are needed to determine whether the *PWT3* product is either secreted into host cells or not, and whether the *PWT3* and *Rwt3* gene products interact directly or indirectly.

PWT3 homologs were widespread in *P. oryzae* populations, indicating that *PWT3* was present before *P. oryzae* differentiated into several subgroups. Tanaka et al. (2010) reported that TP2 carried *PWLI*, a gene conditioning the avirulence of *Eleusine* isolates on weeping lovegrass. In addition, Peyyala and Farman (2006) demonstrated that a copy of *AVR1-CO39* in perennial ryegrass isolates, designed as

AVR1-CO39^{LP}, was recognized by *Pi-CO39(t)* in rice. *AVR1-CO39* was lost during the early stage of evolution of the *Oryza* -specific subgroup of *P. oryzae* (Tosa et al. 2005), whereas *PWL1* may have been present in the ancestral population of *P. grisea/oryzae* species complex before the speciation of cryptic species (Tanaka et al. 2010).

By analyzing *PWT3*, we may be able to understand how host-jump from annual ryegrass to wheat occurred in Kentucky in 2011. *Lolium* isolates are close to *Triticum*, *Avena*, and *Eluesine* isolates (Oh et al. 2002, Tosa et al. 2004). Therefore, it is reasonable to hypothesize that *PWT3* should be shared by these subgroups of *P. oryzae*, probably those belonging to the TELE group (Hirata et al. 2007). The TELE group may share characteristics with a “mother population” which gave birth to the wheat pathogen in South and North Americas.

SUMMARY

Lolium isolate TP2 of *Pyricularia oryzae*, a causal agent of gray leaf spot of perennial ryegrass (*Lolium perenne*), is virulent on perennial ryegrass, but avirulent on wheat cultivars. Genetic analysis of wheat F₂ populations revealed that the resistance of wheat cultivars, Chinese Spring, Shin-chunaga, and Norin 4, to TP2 was conditioned by two genes, *R1* and *R2*. *R1* was highly effective while *R2* was less effective. The strong resistance gene *R1*, designated as *Rmg6*, was mapped on chromosome 1D using microsatellite markers. To reveal genetic mechanisms of avirulence, TP2 was crossed with *Triticum* isolate Br48. Segregation analysis of their F₁ progenies revealed that the avirulence of TP2 on the three wheat cultivars was conditioned by two unlinked genes, one (*A1*) highly effective and the other (*A2*) less effective. These results suggest that the incompatibility between TP2 and the common wheat cultivars is conditioned by two gene pairs, the *Rmg6* – *A1* interaction resulting in strong resistance and the *R2* – *A2* interaction resulting in moderate resistance.

The strong avirulence gene *A1* was located at a region on chromosome 6, which also contained another avirulence gene *PWT3* corresponding to wheat resistance gene *Rwt3* against *Avena* isolate Br58. Genetic analysis suggested that *A1* should be *PWT3*. Thus, *PWT3* (*A1*) was considered to be commonly involved in the avirulence of the two distinct subgroups of *P. oryzae*, *Lolium* isolates and *Avena* isolates.

To clone *PWT3*, a high-resolution map was constructed using a larger population with 273 random BC₁F₁ cultures. A total of 45 recombinants were obtained from a screening with two flanking markers. Finally, *PWT3* was delimited

to a 23 kb region carrying two co-segregating genes, No.211 and No.210. Both genes were expressed *in planta*, but not expressed in mycelia. Two fosmid clones containing this region were isolated from a fosmid library of *Avena* isolate Br58 by colony hybridization, and two subclones were constructed from one of them so that each subclone carried either of the No.211 and No.210 genes. These clones were transformed into *Triticum* isolate Br48 (non *PWT3* carrier). Transformants with the two fosmid clones and a subclone-No.210 were avirulence on *Rwt3* carriers, while those with subclone-No.211 and empty vector were still virulent. Thus, we successfully localized *PWT3* on a 2.3 kb fragment of Br58 genomic DNA containing a potential open reading frame with 426 bp.

PWT3 homologs were present within *P. oryzae* populations, but absent in other species of *Pyricularia*. Those homologs were grouped into four types. *Oryza*, *Setaria* isolates carried A type while *Panicum* isolates carried B type. *Avena*, *Lolium*, and *Eleusine* isolates carried C type (avirulence type), while *Brachiaria* and *Eragrostis* isolates carried D type (virulence type). Interestingly, *Triticum* isolates were split into two subgroups, one with the C type and the other with the D type. Some of the C type homologs in *Triticum* isolates had some rearrangements in its upstream region. These results suggest that there are two distinct populations with different origins in wheat blast isolates collected in Brazil.

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