***PWT1*, an Avirulence Gene of *Magnaporthe oryzae* Tightly Linked to the rDNA Locus, Is Recognized by Two Staple Crops, Common Wheat and Barley**

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ABSTRACT

Chuma, I., Zhan, S.-W., Asano, S., Nga, N. T. T., Vy, T. T. P., Shirai, M., Ibaragi, K., and Tosa, Y. 2010. *PWT1*, an avirulence gene of *Magnaporthe oryzae* tightly linked to the rDNA locus, is recognized by two staple crops, common wheat and barley. *Phytopathology* 100:436-443.

The pathogenicity to wheat (*Pwt1*) locus conditions host species specificity of *Magnaporthe oryzae* on wheat. GFSII-7-2 (*Setaria* isolate) carries the avirulence allele (*PWT1*) at this locus while Br48 (*Triticum* isolate) carries the virulence allele (*pwt1*). An F₁ culture derived from a cross between GFSII-7-2 and Br48 was backcrossed with Br48 to produce a tester population in which *PWT1* alone segregated. When hexa-

ploid wheat lines were inoculated with the BC₁F₁ testers, they were all resistant to all *PWT1* carriers and susceptible to all *pwt1* carriers, suggesting that they recognize *PWT1*. When barley cultivars were inoculated with the testers, they showed the same pattern of reactions as the hexaploid lines, suggesting that the barley cultivars also recognize *PWT1*. These results suggest that *PWT1* is a fundamental gene that universally conditions the avirulence of *Setaria* isolates on two staple crops, hexaploid wheat and barley. Interestingly, tetraploid wheat lines did not recognize *PWT1*. Molecular mapping using the F₁ and BC₁F₁ populations revealed that the *Pwt1* locus is located on chromosome 2 and tightly linked to the ribosomal DNA locus and a telomere.

The filamentous ascomycete fungus *Magnaporthe oryzae* (2) causes blast disease on many gramineous plants. This fungus exhibits a high degree of host specificity. On the basis of host range, mating ability, and genetic relatedness, *M. oryzae* isolates are classified into several host-specific subgroups or pathotypes; for example, the *Oryza* pathotype, pathogenic on rice (*Oryza sativa* L.); the *Setaria* pathotype, pathogenic on foxtail millet (*Setaria italica* Beauv.); the *Triticum* pathotype, pathogenic on wheat (*Triticum aestivum* L.); and the *Avena* pathotype, pathogenic on oat (*Avena sativa* L.) (10,16,22). The *Oryza* and *Setaria* pathotypes contain physiological races which show various patterns of virulence on cultivars within their host species.

Race-cultivar specificity (or cultivar specificity) (5) has long been a major research topic in the *M. oryzae*-gramineous plant system. Silue et al. (19) demonstrated that race-cultivar specificity is controlled by gene-for-gene interactions. Valent and coworkers cloned *Pita*, a rice gene that confers resistance to races of the rice pathogen (1), and its corresponding avirulence gene, *AVR-Pita* (17). They suggested that their products bind together inside the plant cell to initiate a defense response (8).

On the other hand, genetic mechanisms of host species specificity (or plant species specificity) (5) remain to be elucidated. A preliminary genetic analysis was performed by Yaegashi (27). He crossed *Eleusine* isolates (isolates from finger millet, *Eleusine coracana*) with *Eragrostis* isolates (isolates from weeping love-

grass, *Eragrostis curvula*), inoculated finger millet and weeping lovegrass with their F₁ cultures, and suggested that a single gene was involved in the specific parasitism on each host. Valent and coworkers (24) identified *PWL1* and *PWL2*, major genes involved in the species specificity of the *Eleusine* pathotype and the *Oryza* pathotype, respectively, toward weeping lovegrass. These genes were successfully cloned (9,20), and were revealed to function as avirulence genes.

Our laboratory started a systematic genetic analysis of host species specificity. We chose a *Triticum* isolate (Br48) as a common parent for crossing because it is highly fertile with other isolates of *M. oryzae*. Murakami et al. (12) crossed the *Triticum* isolate with a *Setaria* isolate (GFSII-7-2), and inoculated a wheat cultivar with their F₁ cultures. Segregation analyses revealed that two loci, designated as *Pwt1* and *Pwt2*, were involved in the specific parasitism of the parental isolates on wheat. Subsequently, Takabayashi et al. (21) crossed the *Triticum* isolate with an *Avena* isolate (Br58) and identified two other loci, *Pwt3* and *Pwt4*, which were involved in the specific parasitism of the parental isolates on wheat. Further, Tosa et al. (23) crossed the *Triticum* isolate with an *Oryza* isolate (PO12-7301-2) and showed that three loci, *Pwt1*, *Pwt2*, and *Pwt5*, were involved in the specific parasitism of the parental isolates on wheat. From circumstantial evidence, they assumed that *Pwt1* to 5 are avirulence loci or, in other words, that avirulence alleles are functional. Based on this assumption, the avirulence/virulence alleles at these loci were designated as *PWT1/pwt1*, *PWT2/pwt2*, *PWT3/pwt3*, *PWT4/pwt4*, and *PWT5/pwt5*, respectively (21,23).

The F₁ hybrids carrying *PWT4* were avirulent on wheat cv. Norin 4 but virulent on another wheat cultivar, Chinese Spring (21). By using this "cultivar specificity", Takabayashi et al. (21) successfully identified the first wheat gene for resistance to *M. oryzae* and designated it as *Rmg1*, with *Rwt4* listed as a synonym. *Rwt4* corresponded to *PWT4* in a gene-for-gene manner (7). These results suggested that *Pwt4* is actually an avirulence locus

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*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains a table showing the marker descriptions and segregation patterns in 78 F₁ cultures and a figure showing the structure of the supercontig 1 of the genome sequence of *M. oryzae* strain 70-15.

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and that gene-for-gene interactions also underlie host species specificity (21). Then, a question arises: what is the difference between avirulence genes involved in cultivar specificity and those involved in species specificity?

In the present study, we focused on the *PWT1* gene which conditions avirulence of the *Setaria* and *Oryza* pathotypes on wheat. One possible difference between the avirulence genes involved in cultivar specificity and those involved in species specificity may be the range of plant species that recognize them. To determine the range of plant species which recognize *PWT1*, various accessions of wheat and barley were inoculated with a tester population of *M. oryzae*. Another possible difference between the two categories of avirulence genes may be in their stability. Avirulence genes involved in cultivar specificity tend to be located on subtelomeric regions, which are known to be hyper-variable (3,18). To identify the chromosomal region carrying *PWT1*, a linkage analysis was performed using chromosome-specific restriction fragment length polymorphism markers and telomere repeats as probes. The analysis revealed that *PWT1* was tightly linked to the ribosomal DNA (rDNA) locus and a telomere.

MATERIALS AND METHODS

Fungal strains. *M. oryzae* strains used in the present study were descendants of GFSII-7-2 (a *Setaria* isolate collected in Japan in 1977) and Br48 (a *Triticum* isolate collected in Brazil in 1990). Genotypes of these field isolates are *PWT1*; *PWT2*; *pwt3*; *pwt4*; *pwt5* and *pwt1*; *pwt2*; *pwt3*; *pwt4*; *pwt5*, respectively (23). GFSII-7-2 has the *MAT1*-2 allele at the mating-type locus while Br48 has the *MAT1*-1 allele. Murakami et al. (12) crossed these isolates and produced 80 F₁ cultures in which *PWT1* and *PWT2* segregated. To produce a tester population in which *PWT1* alone segregated, 3M2 (*PWT1*; *pwt2*; *pwt3*; *pwt4*; *pwt5*) with the *MAT1*-2 allele was chosen from the F₁ cultures and backcrossed with Br48 on oatmeal agar medium (20 g of oatmeal, 10 g of agar, and 2.5 g of sucrose in 500 ml of water) as described previously (12). One ascospore was arbitrarily chosen from ascospores in each ascus and isolated. The resulting BC₁F₁ population was composed of 63 cultures (derived from 63 asci).

To produce an F₁ population for fine mapping, GFSII-7-2 was again crossed with Br48 on oatmeal agar medium. The progeny was isolated as described above (i.e., one ascospore from each ascus). The resulting F₁ population was composed of 78 cultures (derived from 78 asci).

These cultures were maintained on sterilized barley seed media (4) under dry condition at 4°C for long-term storage. Just before use for experiments, a barley grain with mycelia was transferred to potato dextrose agar (PDA) (24 g of potato dextrose broth powder [Difco, Sparks, MD] and 15 g of agar powder [Nakalai Tesque, Kyoto, Japan] in 1 liter of water) in a test tube, and incubated at 25°C.

Plant materials. *T. aestivum* Norin 4 was used for the determination of genotypes of the BC₁F₁ cultures. In addition, 28 hexaploid wheat cultivars or accessions (2n = 6X = 42, genome AABBDD), 28 tetraploid wheat accessions (2n = 4X = 28, genome AABB or AAGG), and four barley cultivars (*Hordeum vulgare* L., 2n = 2X = 14, genome HH) (Tables 1 and 2) were used to examine the effect of *PWT1*. Seed were pregerminated on moistened filter paper for 1 day, then sown in a seedling case (5.5 by 15 by 10 cm) filled with vermiculite (Asahi-Kogyo, Okayama, Japan) and grown at 21°C in a growth chamber with a 12-h photoperiod for 7 days.

Infection assay. *M. oryzae* cultures were grown on oatmeal agar media in petri dishes 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 (360 nm, 40W) at 25°C for 4 days to induce

sporulation (12). The conidia produced were suspended in water and adjusted to 1 × 10⁵ spores/ml (low concentration) or 2 × 10⁵ spores/ml (high concentration). Spore suspension (10 ml) with 0.01% Tween 20 (Nakalai Tesque) were sprayed on the adaxial surface of the 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 23 to 24°C. The inoculated plants were then transferred to a growth chamber at 23 to 24°C with a 12-h photoperiod. At 4 to 6 days after inoculation, symptoms were evaluated based on the color and the size of lesions (29). The size was rated using six 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, brown (B) and green (G). Infection types were represented by the combination of the size and color. For example, infection type 3B represents brown lesions of size 3. When several types of lesions appeared on a single leaf, the infection type was determined according to the predominant type. Six plants per line were evaluated for each isolate per replication. The pathogenicity test was repeated at least three times.

Southern blot analysis. Total genomic DNA was isolated as described by Nakayashiki et al. (13). The DNA samples were digested with restriction enzymes (Takara, Kyoto, Japan), electrophoresed on an agarose gel in 0.5× Tris-borate-EDTA buffer, blotted onto a Hybond N+ filter (GE Healthcare, Buckinghamshire, U.K.) and fixed by UV irradiation.

Probes were an rDNA clone (pZS9D10-rDNA-S); chromosome-specific markers (15) provided by S. A. Leong, University of Wisconsin-Madison, and M. L. Farman, University of Kentucky; cosmid clones derived from a genomic library of Br48; rDNA-associated genes (MGG_11011 and MGG_11012), *pex* genes (28); and a synthetic oligonucleotide (TTAGGG)₁₀ for telomere repeats. The rDNA clone, pZS9D10-rDNA-S, was constructed by liberating a 8.4-kb *Sal*I fragment with a single repeat of rDNA from a BAC clone, ZS9D10, (among a genomic library of GFSII-7-2) and then by inserting the fragment into the *Sal*I site of pBluescriptII SK(+). This rDNA clone, the chromosome-specific markers, and the cosmid clones of Br48 were labeled using the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare). The rDNA-associated genes were chosen from supercontig 1 of the *M. oryzae* (strain 70-15) genome sequence in the database (e-Xtra). They were amplified from genomic DNA of GFSII-7-2 with primers listed in Table 3 in a 50-μl reaction containing 1 unit of KOD-plus DNA polymerase (TOYOBO, Osaka, Japan), 1× polymerase chain reaction (PCR) buffer for KOD-plus provided by the manufacturer, 200 μM each dNTP, 0.3 μM each primer, 1 mM MgSO₄, and 5 ng of template DNA. This reaction was run by Mastercycler gradient (Eppendorf, Hamburg, Germany) programmed for 2 min at 94°C and 30 cycles of 15 s at 94°C, 30 s at 56°C, and 1 min at 68°C. Amplified fragments were directly labeled using the ECL Direct kit described above. *Pex* genes were genes encoding putative secreted proteins which were present in Ina168, an *Oryza* isolate of *M. oryzae* collected in Japan but absent in the assembled sequence of strain 70-15 in the database. In a preliminary screening of ≈300 *pex* genes using primers reported by Yoshida et al. (28), we found three *pex* genes (*pex054*, *pex150*, and *pex317*) that were closely linked to the rDNA locus. These genes were amplified from genomic DNA of Ina168 with primers listed in Table 3 in the same condition as for the rDNA-associated genes, and the amplicons were directly labeled using the ECL Direct kit. The telomere probe was labeled using the Alkphos direct labeling and detection system (GE Healthcare) because a high sensitivity was needed. Southern hybridization and detection of the genomic DNA was performed using these kits according to the manufacturer's instructions.

PCR analysis. Segregation analysis of the rDNA locus was also conducted using PCR. Based on a single-nucleotide polymorphism in the rDNA repeat of GFSII-7-2 and Br48 (6), two primer sets were designed (Table 3); one was specific for the rDNA repeat of GFSII-7-2 and the other was specific for that of Br48. Using these specific primers, rDNA was amplified in a 50- μ l reaction containing 1 unit of HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), 1 $\times$ PCR buffer provided by the manufacturer, 200 μ M each dNTP, 0.1 μ M each primer, and 5 ng of template DNA. This reaction was run by Mastercycler gradient (Eppendorf) programmed for 15 min at 95°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C; followed by 10 min at 72°C.

Amplified fragment length polymorphism analysis. Amplified fragment length polymorphism (AFLP) analysis was conducted using AFLP Analysis System for Microorganisms (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, with modifications in the selective amplification primers. Briefly, total DNA (200 ng) was digested with *Eco*RI and *Mse*I and the adaptors were ligated to the ends of the restriction fragments. In total, 20 cycles of PCR were performed for preamplification (30 s at 94°C, 1 min at 56°C, and 1 min at 72°C). Selective amplification was performed using *Eco*RI and *Mse*I primers that additionally contained one to three selective nucleotides at the 3' ends (*e*-Xtra). The first phase of the selective amplification was in one

TABLE 1. Responses of wheat cultivars or accessions to BC₁F₁ testers derived from the backcross, 3M2 $\times$ Br48^a

Code	Species	Cultivar or accession	Genomes	Infection type with			No. of BC ₁ F ₁ cultures			
				GFSII-7-2	3M2	Br48	<i>PWT1</i> carriers		<i>pwt1</i> carriers	
							Avirulent	Virulent	Avirulent	Virulent
1502	<i>Triticum aestivum</i>	Strain P168	ABD	2B	5B	5G	20	0	0	20
1503	<i>T. aestivum</i>	Chinese Spring	ABD	1B	2B	5G	20	0	0	20
1504	<i>T. aestivum</i>	Norin 26	ABD	0	4B	5G	20	0	0	20
1505	<i>T. aestivum</i>	Strain Salmon	ABD	0	2B	5G	20	0	0	20
1507	<i>T. aestivum</i>	Selkirk	ABD	0	1B	5G	20	0	0	20
1509	<i>T. spheerococcum</i>	KU161	ABD	0	1B	4G	20	0	0	20
1510	<i>T. compactum</i>	KU151	ABD	1B	3B	5G	20	0	0	20
1512	<i>T. macha</i>	KU154	ABD	0	5B	5G	20	0	0	20
1513	<i>T. aestivum</i>	Norin 29	ABD	1B	5B	5G	20	0	0	20
1515	<i>T. aestivum</i>	Shin-chunaga	ABD	0	2B	5G	20	0	0	20
1601	<i>T. aestivum</i>	Hope	ABD	0	3B	5G	20	0	0	20
1602	<i>T. aestivum</i>	Timstein	ABD	1B	5B	5G	20	0	0	20
1603	<i>T. aestivum</i>	Cheyenne	ABD	0	1B	3G	20	0	0	20
1835	<i>T. aestivum</i>	Red Egyptian	ABD	0	2B	5G	20	0	0	20
H-1	<i>T. aestivum</i>	Chancellor	ABD	0	1B	3G	20	0	0	20
H-11	<i>T. aestivum</i>	Renown	ABD	0	2B	5G	20	0	0	20
H-13	<i>T. aestivum</i>	Spica	ABD	0	0	5G	20	0	0	20
H-14	<i>T. aestivum</i>	Mendos	ABD	0	3B	5G	20	0	0	20
H-15	<i>T. aestivum</i>	Timvera	ABD	0	0	5G	20	0	0	20
H-16	<i>T. aestivum</i>	Transec	ABD	0	2B	4G	20	0	0	20
H-17	<i>T. aestivum</i>	Transfed	ABD	0	4B	5G	20	0	0	20
H-20	<i>T. aestivum</i>	Normandie	ABD	2B	5B	5G	20	0	0	20
H-21	<i>T. aestivum</i>	Little Club	ABD	1B	3B	5G	20	0	0	20
H-22	<i>T. aestivum</i>	Hard Federation	ABD	0	5B	5G	20	0	0	20
H-23	<i>T. aestivum</i>	Ramona 50	ABD	0	4B	5G	20	0	0	20
H-24	<i>T. aestivum</i>	Mayo 64	ABD	0	2B	5G	20	0	0	20
Tat-17	<i>T. aestivum</i>	KU163	ABD	0	4B	4G	20	0	0	20
Tat-19	<i>T. vavilovi</i>	KU192	ABD	0	0	3G	20	0	0	20
St-11	<i>T. dicoccoides</i>	KU108-1	AB	2B	5G	5G	0	20	0	20
St-12	<i>T. dicoccoides</i>	KU109	AB	2B	5G	5G	0	20	0	20
St-13	<i>T. dicoccoides</i>	KU110	AB	1B	5G	5G	0	20	0	20
St-14	<i>T. dicoccoides</i>	KU195	AB	0	5G	5G	0	20	0	20
St-16	<i>T. dicoccum</i>	KU111	AB	2B	5G	5G	0	20	0	20
St-27	<i>T. dicoccum</i>	KU124	AB	2B	5G	5G	0	20	0	20
St-28	<i>T. dicoccum</i>	KU189	AB	1B	5G	5G	0	20	0	20
Tat-14	<i>T. paleocolchicum</i>	KU156	AB	4B	5G	5G	0	20	0	20
St-29	<i>T. paleocolchicum</i>	KU190-1	AB	2B	5G	5G	0	20	0	20
St-30	<i>T. paleocolchicum</i>	KU191	AB	3B	5G	5G	0	20	0	20
Tat-6	<i>T. durum</i>	KU125	AB	1B	5G	5G	0	20	0	20
St-31	<i>T. durum</i>	KU126	AB	3B	5G	5G	0	20	0	20
St-35	<i>T. durum</i>	KU130	AB	1B	5G	5G	0	20	0	20
St-36	<i>T. durum</i>	KU132	AB	3B	5G	5G	0	20	0	20
St-38	<i>T. durum</i>	KU136	AB	1B	5G	5G	0	20	0	20
St-39	<i>T. durum</i>	KU301	AB	1B	5G	5G	0	20	0	20
St-41	<i>T. durum</i>	KU329	AB	1B	5G	5G	0	20	0	20
St-43	<i>T. durum</i>	KU459	AB	1B	5G	5G	0	20	0	20
Tat-11	<i>T. turgidum</i>	KU147	AB	1B	5G	5G	0	20	0	20
St-46	<i>T. turgidum</i>	KU149	AB	2B	5G	5G	0	20	0	20
Tat-7	<i>T. orientale</i>	KU137	AB	4B	5G	5G	0	20	0	20
Tat-9	<i>T. polonicum</i>	KU141	AB	3B	5G	5G	0	20	0	20
St-48	<i>T. polonicum</i>	KU142	AB	2B	5G	5G	0	20	0	20
St-49	<i>T. polonicum</i>	KU143	AB	3B	5G	5G	0	20	0	20
St-50	<i>T. polonicum</i>	KU144	AB	2B	5G	5G	0	20	0	20
Tat-18	<i>T. abyssinicum</i>	KU185	AB	0	5G	5G	0	20	0	20
St-57	<i>T. abyssinicum</i>	KU186	AB	2B	5G	5G	0	20	0	20
Tat-3	<i>T. timopheevi</i>	KU107-1	AG	2B	5G	5G	0	20	0	20

^a Conidial suspension was adjusted to the low concentration (1×10^5 spores/ml).

cycle (30 s at 94°C, 30 s at 65°C, and 1 min at 72°C). The second touchdown phase was in 12 cycles, with the annealing temperature decreasing stepwise from 64.3 to 56.6°C. The last phase was in 23 cycles (30 s at 94°C, 30 s at 56°C, and 1 min at 72°C). Amplified products were fractionated by electrophoresis through 6% denaturing polyacrylamide gels and stained using SILVER SEQUENCE DNA Staining Reagents (Promega Corp., Madison, WI).

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

RESULTS

Production of BC₁F₁ cultures and determination of their genotypes. *Pwt1* was identified by inoculating a hexaploid wheat cultivar, Norin 4, with the F₁ cultures derived from the cross GFSII-7-2 × Br48 (12). Briefly, GFSII-7-2 was avirulent on Norin 4 (infection type 0) while Br48 was virulent (infection type 5G). In the F₁ population, cultures producing brown lesions and those producing green lesions segregated in a 1:1 ratio (35:34, χ^2 [1:1] = 0.01, 0.9 < *P*). This locus associated with the lesion color was designated *Pwt1* as described above. However, the segregation of avirulence and virulence in the F₁ population was complex due to the involvement of another locus, *Pwt2*, which was associated with the lesion number (12). To exclude the effect of *Pwt2* from the segregation, 3M2, an F₁ culture with the *PWT1;pwt2* genotype, was backcrossed with the *Triticum* isolate Br48 (*pwt1;pwt2*). In total, 63 BC₁F₁ cultures were obtained from this cross. Each of these cultures was derived from a distinct ascus and, therefore, from an independent meiotic event.

To determine an appropriate inoculum concentration and the border between avirulent and virulent reactions, Norin 4 and

Chinese Spring were inoculated with conidial suspensions of 3M2 prepared at various concentrations. Murakami et al. (12) performed their infection assays using suspensions adjusted to 2×10^5 spores/ml. When inoculated at this concentration (the high concentration), Norin 4 showed the 5B type (the B+ type in Murakami et al. [12]) (Fig. 1). When the concentration was decreased, the size of the predominant lesions decreased concomitantly. At 1×10^5 spores/ml (the low concentration), the infection type on Norin 4 was 4B (Fig. 1). Interestingly, the 4B type was observed on Chinese Spring inoculated at the high concentration and, in turn, the infection type on this cultivar changed to 2B with the decrease of the inoculum concentration (Fig. 1). These results suggest that the lesion size score fluctuates with the inoculum concentration.

Leaves with the 5B type were completely shriveled and heavily damaged (Fig. 1). However, when these leaves were incubated in a moist condition, no conidia were produced, supporting the idea that type 5B is a resistant reaction. Consequently, infection types 0, 1B, 2B, 3B, 4B, and 5B were considered avirulent (or resistant) while infection types with “G” were considered virulent (or susceptible). When various wheat cultivars or accessions were inoculated with 3M2 and Br48, infection types 3G, 4G, and 5G were observed but 1G or 2G never occurred (Table 1). Consequently, possible infection types were considered to be 0 to 5B (resistant) and 3G to 5G (susceptible). The subsequent infection assays were performed using the low concentration unless otherwise described.

When Norin 4 was inoculated with the 63 BC₁F₁ cultures, avirulent (infection type 0-5B) and virulent (infection type 3G-5G) cultures segregated clearly in a 1:1 ratio (37:26, χ^2 [1:1] = 1.92, 0.1 < *P* < 0.5) (Fig. 2). Based on these reactions on Norin 4, the genotype of each culture was determined; the avirulent cultures were considered to be *PWT1* carriers (*PWT1;pwt2*) while the virulent cultures were considered to be *pwt1* carriers (*pwt1;pwt2*).

Reactions of wheat and barley lines to *PWT1*. The 56 wheat lines (28 hexaploid cultivars or accessions and 28 tetraploid

TABLE 2. Responses of barley cultivars (*Hordeum vulgare*) to BC₁F₁ cultures derived from the backcross, 3M2 × Br48^a

Cultivar	Infection type with			No. of BC ₁ F ₁ cultures			
				<i>PWT1</i> carriers		<i>pwt1</i> carriers	
	GFSII-7-2	3M2	Br48	Avirulent	Virulent	Avirulent	Virulent
H.E.S.4	0	2B	5G	37	0	0	26
Nakaizumi-zairai	0	2B	5G	37	0	0	26
Kobinkatagi	1-2B	1B	4-5G	37	0	0	26
Nepal	1-2B	2B	4-5G	37	0	0	26

^a Conidial suspension was adjusted to the low concentration (1×10^5 spores/ml).

TABLE 3. Primers used for mapping around the rDNA locus

Target gene	Template	Size of amplicon (bp)	Primers	
			Name	Sequence (5'-3')
MGG_11011	GFSII-7-2	585	7015Sc1_3	TCGACGAAGAAGGCCTG
			7015Sc1_4	CATTCATCCCAGCCTTCAT
MGG_11012	GFSII-7-2	489	7015Sc1_5	TGCTCATAAGTCAAAAGCGTC
			7015Sc1_6	GTCCACGGCATTGAAATAC
pex054	Ina168	506	Ina168-pex54-F ^a	TTAATTCAGCGAAAAAGT
			Ina168-pex54-R ^a	ATGGTACCGCAAATAGTT
pex150	Ina168	195	Ina168-pex150-F ^a	TGGCGACAAAGAGGTG
			Ina168-pex150-R ^a	GATGCACGGGGGAGAC
pex317	Ina168	175	Ina168-pex317-F ^a	CCTGGCCGTGACATCT
			Ina168-pex317-R ^a	GCGCGCAGGAAAGAC
rDNA in GFSII-7-2	–	516	ITS_rd5	CCGAGGTCAACCTTTAAAA
			ITS5 ^b	GGAAGTAAAAGTCGTAACAAGG
rDNA in Br48	–	516	ITS_rd6	CCGAGGTCAACCTTTAAAG
			ITS5 ^b	GGAAGTAAAAGTCGTAACAAGG

^a Yoshida et al. (28).

^b White et al. (26).

Fig. 1. Symptoms on seedlings of hexaploid cvs. Cheyenne (Cnn), Chinese Spring (CS), Norin 4 (N4), and Normandie (Nrm) and tetraploid accession KU108-1 (KU) inoculated with *Magnaporthe oryzae* Br48 and 3M2, 5 days after inoculation. Conidial suspension was adjusted to the low (1×10^5 spores/ml) or high (2×10^5 spores/ml) concentration.

accessions) were susceptible to the *Triticum* isolate Br48 and resistant to the *Setaria* isolate GFSI1-7-2 (Table 1). When they were inoculated with 3M2, the 28 hexaploid lines were all resistant whereas the 28 tetraploid lines were all susceptible (Table 1). Interestingly, there was a variation in the degree of resistance among the hexaploid lines (Fig. 1). When inoculated with 3M2 at the high concentration, the infection types of 'Cheyenne', Chinese Spring, Norin 4, and 'Normandie' were 1B, 4B, 5B, and 5B, respectively. When inoculated at the low concentration, the difference among the four cultivars became more distinct: 1B, 2B, 4B, and 5B, respectively.

To examine whether the avirulence of 3M2 on these hexaploid lines was conditioned by *PWT1*, 20 BC₁F₁ cultures were arbitrarily chosen from each of the *PWT1* and the *pwt1* carriers, and the hexaploid lines were inoculated with the 40 testers. The hexaploid lines were all resistant to all of the *PWT1* carriers and susceptible to all of the *pwt1* carriers (Table 1), suggesting that they recognize *PWT1*. On the other hand, the tetraploid lines were all susceptible to all of the BC₁F₁ testers (Table 1), indicating that they do not recognize *PWT1*.

When barley cultivars were inoculated with the 40 testers, they showed the same pattern of reactions as the hexaploid lines (data not shown), suggesting that the barley cultivars also recognize *PWT1*. To confirm it, they were inoculated with all of the 63 BC₁F₁ cultures. Again, they were resistant to all of the *PWT1* carriers and susceptible to all of the *pwt1* carriers (Table 2).

Mapping the *Pwt1* locus using the F₁ population from GFSI1-7-2 × Br48. The F₁ population produced by Murakami et al. (12) was composed of 10 tetrads or, in other words, was derived from only 10 meiotic events. For fine mapping, we crossed GFSI1-7-2 and Br48 again and produced a new F₁ population composed of 78 cultures. Each of these cultures was derived from a distinct ascus and, therefore, from an independent meiotic event.

Using this fresh F₁ population, a linkage map of the cosmid, plasmid, and AFLP markers was constructed. The chromosome number of each linkage group was determined based on the chromosome-specific markers (15). *Pwt1* was mapped to a small linkage group carrying the rDNA locus (Fig. 3), which was reported to be located on chromosome 2 (15). This result suggests that *Pwt1* is located on chromosome 2. Chromosome 2 in this map was composed of two linkage groups; the small one carrying rDNA and a large one carrying a telomere marker (Tel-B5.1s) at one end (Fig. 3). It was reasonable to speculate that the other

Fig. 2. Frequency distribution of infection types on hexaploid cv. Norin 4 in a BC₁F₁ population derived from the cross 3M2 × Br48. Conidial suspension was adjusted to the low concentration (1×10^5 spores/ml). Avirulence:virulence = 37:26, $\chi^2(1:1) = 1.92$ ($0.1 < P < 0.5$).

telomere should be on the small one but we could not find a telomere marker that was mapped to it.

The segregation of the rDNA locus was confirmed by PCR with both GFSII-7-2-specific and Br48-specific primer pairs. The segregation at the *Pwt1* locus was confirmed by repeating the infection assay four times. When these data were combined, there occurred one recombinant (*e-Xtra*), which produced 1.4 cM of distance between the two loci (Fig. 3). To locate more markers around the rDNA locus, the 70-15 genome sequence in the database was surveyed. Two genes (MGG_11011 and MGG_11012) were found in supercontig 1 in which rDNA was located (*e-Xtra*). These genes cosegregated completely with rDNA (Fig. 3). The nucleotide sequence in supercontig 1 other than the two genes and rDNA was occupied by a retrotransposon and solo-LTRs of other retrotransposons, and contained no genes suitable for mapping (*e-Xtra*).

The three *pex* genes (*pex054*, *pex150*, and *pex317*) selected in the preliminary screening were also subjected to segregation analysis. *Pex054* and *Pex150* cosegregated completely with rDNA and were located at the same locus as rDNA (Fig. 3). Between

pex317 and rDNA, however, one recombinant was observed. Consequently, *pex317* was located on the opposite side to *Pwt1* with a distance of 1.4 cM from rDNA. In other words, rDNA was flanked by *Pex317* and *Pwt1* (Fig. 3).

When A15C10, a marker located on the proximal side of rDNA, was analyzed together, this marker was located on the *Pex317* side of the *Pex317*-rDNA-*Pwt1* linkage group (Fig. 3). This result suggests that *Pwt1* is located on the telomeric side of rDNA.

Mapping the *Pwt1* locus using the BC₁F₁ population developed from 3M2 × Br48. Molecular mapping of *Pwt1* was further performed using the BC₁F₁ population derived from 3M2 × Br48. A Southern blot analysis with the rDNA probe is shown in Figure 4 as an example. The *Sac*I digestion liberated a single fragment (8.4 kb) corresponding to a single unit of the rDNA repeats in Br48 but two fragments (4.8 and 3.6 kb) in GFSII-7-2 and 3M2. In the BC₁F₁ population, these two types segregated in a 1:1 ratio. The segregation at the rDNA locus was perfectly concordant with the segregation at the *Pwt1* locus; all of the 37 cultures avirulent on Norin 4 had the 3M2 type signals whereas all of the 26 cultures virulent on Norin 4 had the Br48 type signal.

Fig. 3. Genetic mapping of the *Pwt1* locus using an F₁ population derived from GFSII-7-2 × Br48 and a BC₁F₁ population derived from 3M2 × Br48. Chromosome 2 in the F₁ map is shown with a relevant linkage group of chromosome 2 in the BC₁F₁ map. Asterisks indicate the chromosome 2-specific markers reported by Nitta et al. (15). Letters at the end of the markers (s and t) represent parents they are derived from. For example, MGG_11011-st represents cosegregating two fragments; that is, a fragment from GFSII-7-2 (*Setaria* isolate) and a fragment from Br48 (*Triticum* isolate), which were visualized by hybridization with the MGG_11011 probe. Tel-E2.5-t represents a 2.5-kb *Eco*RI fragment from Br48 which was visualized by hybridization with the telomere probe. In this case, its counterpart (a cosegregating fragment derived from GFSII-7-2) could not be detected. On the left is a standard map constructed from the segregation data in the Guy11 × 2539 F₁ (15) using the same computer program, parameters, and mapping function as those used for the GFSII-7-2 × Br48 and 3M2 × Br48 maps.

All of the other markers tightly linked to rDNA in the F₁ map (by 0 to 1.4 cM) cosegregated completely with rDNA (Fig. 3). Interestingly, a telomere signal (Tel-E2.5t) was found which cosegregated with the rDNA locus in this BC₁F₁ population (Fig. 3).

DISCUSSION

Results of infection assay with the blast fungus are affected by many factors (e.g., temperature, inoculum concentration, plant age, fertilizer, and so on). These factors are especially critical when we use young seedlings of winter crops such as wheat and barley. Takabayashi et al. (21) showed that the resistance of a wheat cultivar to an inappropriate pathotype of *M. oryzae* was temperature sensitive. Nga et al. (14) showed that the resistance of a wheat cultivar to an inappropriate species, *M. grisea*, was also temperature sensitive. Such temperature sensitivity was considered to be reasonable because wheat is a winter crop (14,21). In the present study, we showed that the inoculum concentration was also important; infection types were strongly affected even by reducing the inoculum concentration to a half (Fig. 1). However, with the knowledge that the critical marker for resistance conferred by *PWT1* is the brown color, it is not difficult to differentiate resistant and susceptible lesions even when seedlings are inoculated with a high concentration of conidia. This color-dependent determination of resistant and susceptible lesions may be applicable to *PWT3*, *PWT4*, and *PWT5*, other avirulence genes involved in the species specificity on wheat, because they are associated with hypersensitive reaction at the cytological level (unpublished data). The lesion size or damaged areas appear to be dependent on the number of germings that successfully penetrated cell walls. In aged or adult plants, the number of such germings should be much reduced by thick cell walls and the reaction induced by *PWT1* should be sufficient to protect the plants. The inoculum density-dependent fluctuation of lesion-size scores may be prominent only on young seedlings such as 7-day-old primary leaves used in our infection assay.

All of the hexaploid lines tested in the present study recognized *PWT1*. This result suggests that *PWT1* is a fundamental gene that universally conditions the avirulence of *Setaria* isolates on

hexaploid wheat. Furthermore, *PWT1* was recognized by barley cultivars. This fact seems reasonable because *PWT1* was identified as a gene controlling host species specificity. Interestingly, *PWT1* was not recognized by any of the tetraploid wheat lines. Further studies are needed to clarify why tetraploid wheat does not recognize *PWT1* although it shares A and B genomes with hexaploid wheat.

All of the hexaploid lines responded to *PWT1* but the degree of the resistance differed from line to line. The level of resistance of the representative cultivars was Cheyenne > Chinese Spring > Norin 4 > Normandie (Fig. 1). We propose two alternative hypotheses for explaining these differences. Let us assume that *PWT1* is recognized by *Rwt1*, a putative resistance gene corresponding to *PWT1* in a gene-for-gene manner. One hypothesis is that these cultivars have different alleles at the *Rwt1* locus that have suffered from various levels of degradation; Cheyenne has the strongest *Rwt1* allele while Normandie has the weakest *Rwt1* allele. The other is that these cultivars carry a strong, functional allele at the *Rwt1* locus but have various degrees of defects downstream of resistance mechanisms. To determine which is the case, *Rwt1* must be identified. Unfortunately, we could not find a hexaploid line that did not recognize *PWT1*, a prerequisite for detecting *Rwt1*. We are now trying to produce synthetic hexaploids that do not recognize *PWT1* from tetraploid cultivars and *Aegilops tauschii* accessions.

In the BC₁F₁ map, *PWT1* was completely linked to a telomere marker (Tel-E2.5t) (Fig. 3). This telomere signal was derived from Br48. Interestingly, this signal was absent in the F₁ population from GFSI1-7-2 × Br48 produced in the present study. Furthermore, this signal was again found in the F₁ population from GFSI1-7-2 × Br48 produced by Murakami et al. (12) (data not shown). We assume that mycelial cells of Br48, which contributed to the sexual hybridization with GFSI1-7-2 in the present study, may have happened to lose this telomere fragment. This is not surprising because telomere fingerprint profiles are known to change frequently during vegetative growth (3). The loss of the telomere signal observed in the new F₁ population may be evidence that the reorganization or changes of telomeric regions occurs during vegetative growth on artificial solid media.

Fig. 4. Cosegregation of the ribosomal DNA locus and *Pwt1* in the BC₁F₁ population derived from the cross 3M2 × Br48. Genomic DNA of the BC₁F₁ cultures was digested with *Sac*I and hybridized with a ribosomal DNA clone, pZS9D10-rDNA-S. Results of infection assay on 'Norin 4' are shown at the bottom of the panel. A, avirulent; V, virulent.

Avirulence genes involved in cultivar specificity tend to be located on subtelomeric regions (11,17,25). *PWL1*, a cloned avirulence gene involved in species specificity on weeping lovegrass, was suggested to be linked to a telomere on the basis of cosegregation of a *PWL1*-flanking sequence with a telomere signal (9). *PWL2*, another cloned avirulence gene involved in species specificity on weeping lovegrass, was not closely linked to a telomere but was located on an unstable region which was rich in both high-copy and low-copy repeated DNAs (20). The present study showed that *PWT1*, an avirulence gene involved in species specificity on wheat, is tightly linked to a telomere and probably located on a subtelomeric region. Based on these results, there seems to be no difference between avirulence genes involved in cultivar specificity and those involved in species specificity from the viewpoint of the instability of chromosomal regions in which they are located.

Are there differences between the two categories of avirulence genes from the viewpoint of their function or contribution to fitness? To answer this question, more avirulence genes must be cloned from both categories and compared. We started a project aiming at cloning *PWT1*. Clues to the position of *PWT1* were not obtained from the genome sequence of *M. oryzae* strain 70-15 in the database. The supercontig 1 carrying rDNA in the database did not harbor any candidate genes (*e*-Xtra). Moreover, there is a considerable possibility that *PWT1* may not be present in the genome of 70-15. Yoshida et al. (28) tried to find avirulence genes corresponding to rice resistance genes in the 70-15 database sequence, but failed. They finally found three novel avirulence genes in 1.68-Mb regions which were present in Ina168 but absent in the assembled sequence of 70-15. Chromosome walking is now underway from the rDNA locus toward *PWT1*.

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