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Rmg8 **and** ***Rmg7*****, wheat genes for resistance to the wheat blast fungus, recognize the same avirulence gene** ***AVR-Rmg8***

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Running title: Dual recognition of a wheat blast avirulence gene

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SUMMARY

Rmg8 and *Rmg7* are genes for resistance to the wheat blast fungus (*Pyricularia oryzae*) located on 2B chromosome in hexaploid wheat and 2A chromosome in tetraploid wheat, respectively. *AVR-Rmg8*, an avirulence gene corresponding to *Rmg8*, was isolated from a wheat blast isolate through map-based strategy. The cloned fragment encoded a small protein containing a putative signal peptide. *AVR-Rmg8* was recognized not only by *Rmg8* but also by *Rmg7*, suggesting that these two resistance genes are equivalent to a single gene from the viewpoint of resistance breeding.

Wheat blast, caused by a host genus-specific subgroup (*Triticum* pathotype or *Triticum* isolates) of *Pyricularia oryza*e (*Magnaporthe oryzae*), is a devastating disease of wheat. It first appeared in Brazil in 1985 and then spread to neighboring countries in South America (Cruz et al., 2012; Kohli et al., 2011; Urashima et al., 1993). In 2011 wheat blast was found in North America - at a University of Kentucky Research and Education Center research plot in U.S.A. (Pratt, 2012). Its causal agent (Kentucky strain) was different from *Triticum* isolates prevailing South America and similar to *Lolium* isolates indigenous to North America (Farman et al., 2017). In 2016 wheat blast suddenly appeared in Bangladesh and caused severe and extensive damage to wheat production (Callaway, 2016). This first outbreak of wheat blast in Eurasia was considered to be caused by *Triticum* isolates from South America (Islam et al., 2016; Malaker et al., 2016). In 2017 this devastating disease spread to India (Bhattacharya and Mondal, 2017), the second largest wheat producing country in the world. Currently, wheat blast is becoming a pandemic disease that may threaten wheat production all over the world.

The most effective and practical way for controlling wheat blast is breeding for resistance. Cruz et al. (2016) found that a chromosomal segment (2NS) from *Aegilops ventricosa* conferred resistance to the wheat blast fungus. However, gene(s) responsible for the 2NS resistance have not been identified. Zhan et al. (2008) identified *Rmg2* and *Rmg3*, first genes for resistance to the wheat blast fungus, in common wheat cultivar Thatcher. However, they were not effective at the heading stage (unpublished data), and therefore were not useful for resistance breeding because the wheat blast fungus mainly infects spikes in farmer's fields. Recently, Tagle et al. (2015) identified a new resistance gene, *Rmg7*, in tetraploid accessions. These accessions were resistant to the wheat blast fungus not only at the seedling stage but also at the heading stage. They also identified its corresponding avirulence gene, *AVR-Rmg7*. Anh et al. (2015) identified another

new gene, *Rmg8*, in common wheat cultivar S-615, which was resistant to the wheat blast fungus at both seedling and heading stages. They also identified its corresponding avirulence gene, *AVR-Rmg8*. Interestingly, segregation analysis suggested that *AVR-Rmg8* was closely linked to *AVR-Rmg7* or was located at the same locus as *AVR-Rmg7* (Anh et al. 2015). Here, we show that *AVR-Rmg8* and *AVR-Rmg7* are the same, single gene recognized by two resistance genes, *Rmg7* in tetraploid wheat and *Rmg8* in hexaploid wheat.

Anh et al. (2015) identified *AVR-Rmg8* using a BC_1F_1 population derived from a cross between Br48 (*Triticum* isolate of *P. oryzae* collected in Brazil) and 200R29 (F_1 culture derived from Br48 x MZ5-1-6, an *Eleusine* isolate of *P. oryzae* collected in Japan) (Fig. 1). Briefly, Br48 and 200R29 were avirulent and virulent, respectively, on *Triticum aestivum* cv. S-615 carrying *Rmg8* at 25^{o}C (Anh et al., 2015). When S-615 was inoculated with 73 BC_1F_1 cultures derived from Br48 x 200R29 and incubated at 25 ^{o}C, avirulent (*AVR-Rmg8*) and virulent (*avr-Rmg8*) cultures segregated in a 1:1 ratio (Anh et al., 2015). Five cultures were arbitrary chosen from each of the *AVR-Rmg8* carriers and *avr-Rmg8* carriers. Equal amounts of their genomic DNA were mixed, and resulting DNA mixtures (A-bulk and V-bulk) were subjected to bulked segregant analysis (Michelmore et al., 1991) for screening of ~200 SSR markers (Zheng at al., 2008) covering the entire *P. oryzae* genome. We found some markers polymorphic between the two bulks, all of which were located on chromosome 7. These markers were subjected to a linkage analysis using 67 cultures (excluding 6 unstable or attenuated cultures) derived from Br48 x 200R29. The linkage map showed that *AVR-Rmg8* was flanked by MAT (the mating type locus) and MGM301 on chromosome 7 (Fig. 2).

Next, we expanded the mapping population from 67 to 449 cultures (Fig. 1), screened them for recombinants with two flanking markers, MAT and MGM301, and finally found 58

recombinants. Whole genome sequences of *Triticum* isolate Br48 and *Eleusine* isolate Z2-1 (Yoshida et al., 2016) flanked by MAT and MGM301 were surveyed for SNPs, which were then converted to CAPs markers. Genotyping of the 58 recombinants with these new markers revealed that *AVR-Rmg8* is located on a 45kb fragment flanked by two markers, 1338.1.2 and 1106.3.1 (Fig. 3a). This fragment contained 5 genes encoding putative secreted proteins (755.4, 755.2, 2570.1, 4315.4, 1106.3), all of which co-segregated perfectly with *AVR-Rmg8* (Fig. 3b).

Fragments containing these candidate genes (2.1-2.4kb for each) were amplified by PCR using primers listed in Table 1 and ligated with pBluescript II SK (+) digested with *Eco*RV. Recombinant plasmids cloned in *E.coli* DH5α were introduced into protoplasts of 200R29 through PEG-mediated co-transformation with pSH75 (Kimura and Tsuge, 1993) carrying a hygromycin B phosphotransferase gene as described by Tosa et al. (2005). Ten stable transformants were obtained for each clone. When S-615 (carrying *Rmg8*) was inoculated with these transformants and incubated at 25ºC, all transformants containing the fragments with 755.4, 755.2, 2570.1, and 1106.3 were virulent whereas six of the ten transformants containing the fragment with 4315.4 were avirulent (Fig. 3b), suggesting that the 2.3-kb fragment with 4315.4 contain *AVR-Rmg8*.

200R29 showed moderate avirulence on S-615 at 22ºC (Anh et al., 2015), the standard temperature for infection assay of wheat with *P. oryzae*. To confirm the effect of the fragment at the standard temperature, we screened the F_1 population derived from Br48 x MZ5-1-6, and found 200R54 (Fig. 1) which were highly virulent on S615 even at 22ºC. Further analyses were performed using 200R54 as a recipient of transgenes. Three fragments (F2.2, F1.7, F1.1) were amplified from the 2.3kb fragment containing 4315.4 (Fig. 3c) using primers listed in Table 1, ligated with pBluescript II SK (+) digested with *Eco*RV, and cloned in *E.coli* DH5α. Cloned fragments were introduced into 200R54 through co-transformation with pSH75, resulting in 8-9

stable transformants for each clone. They were subjected to infection assay at 22°C with S-615 (*Rmg8*) together with *T. aestivum* cv. Shin-Chunaga (Sch) and cv. Hope as controls carrying *rmg8*. All results supported the assumption that the gene 4315.4 was *AVR-Rmg8* (Fig. 3c, Fig. 4). *AVR-Rmg8* encoded a small protein composed of 109 amino acids (Fig. 3d), which contained a putative signal peptide but showed no similarity to other known proteins in the database. Interestingly, *T. dicoccum* KU120 (St24) carrying *Rmg7* (Tagle et al., 2015) showed the same pattern of reactions to these transformants as S-615 (Fig. 4). This result suggests that *AVR-Rmg8* is recognized not only by *Rmg8* but also by *Rmg7*. Taken together, we conclude that *AVR-Rmg8* and *AVR-Rmg7* are the same, single gene recognized by two resistance genes, *Rmg8* in hexaploid wheat and *Rmg7* in tetraploid wheat. *Rmg8* and *Rmg7* are different not only in their chromosomal locations but also in their temperature sensitivity. At 21-24°C both genes were effective against all *Triticum* isolates collected in Brazil in 1990-1992 (Tagle et al. 2015; Anh et al. 2015). At 26 °C, however, *Rmg8* was still effective while *Rmg7* was noneffective (Supplementary Fig. 1). The relationship between *AVR-Rmg8* and *Rmg7/Rmg8* appears a gene-for-genes (plural) interaction (Feyter et al., 1993) in which one avirulence gene corresponds to more than one resistance gene.

Gene-for-genes interactions have also been reported in other host-parasite interactions (e.g., Ashfield et al., 2004; Dong et al., 2011; Dou et al., 2010; Feyter et al., 1993; Hall et al., 2009; Houterman et al., 2008; Lokossou et al., 2009; Parlange et al., 2009). It is an intriguing question how such interactions evolved. Ashfield et al. (2004) suggested that convergent evolution, rather than the conservation of an ancient specificity, was responsible for the generation of AvrB-specific resistance genes, *RPM1* in *Arabidopsis thaliana* and *Rpg1-b* in *Glycine max*. In our case the gene-for-genes interaction between *AVR-Rmg8* and *Rmg8/Rmg7* may be explained by conservation of an ancient specificity. *Rmg8* and *Rmg7* are located at the distal regions of long arms of

homoeologous chromosomes, 2B and 2A, respectively (Anh et al., 2015). They may be homoeologous genes derived from a single ancestral gene that was present in a common ancestor before the differentiation of A and B genomes.

The result reported here has an important implication in breeding for resistance to wheat blast. Once a mutation occurs in *AVR-Rmg8*, a wheat blast strain carrying the mutated *AVR-Rmg8* will defeat not only *Rmg8* but *Rmg7*. These two resistance genes appears to be equivalent to a single gene from the viewpoint of resistance breeding.

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Supporting information

Supplementary Fig. 1. Responses of S-615 and St24 at three different temperatures.

Figure legends

Figure 1. Pedigree of *P. oryzae* strains used in this study.

Figure 2. Genetic map of the *AVR-Rmg8* region constructed using 67 BC_1F_1 cultures derived from Br48 x 200R29. Original positions of markers reported by Zheng et al. (2008) are shown on the right. cM = centimorgans.

Figure 3. Cloning of *AVR-Rmg8*. (a) Fine genetic map of the *AVR-Rmg8* region constructed using 58 recombinants selected from 449 BC_1F_1 cultures derived from Br48 x 200R29 with two flanking markers, MAT and MGM301. Shown in square brackets are numbers of recombinants between markers. (b) Physical map of the 45kb region delimited by the fine mapping in (a). Black arrows indicate five candidate genes detected in this region. Shown in parentheses are number of transformants avirulent on S-615 / number of transformants tested. (c) Detailed analysis of the 2.3kb fragment selected in (b). White arrows indicate primers used for amplification of fragment F2.2, F1.7, and F1.1. Shown in parentheses are number of transformants avirulent on S-615 / number of transformants tested. (d) Nucleotide and amino acid sequences of gene 4315.4 (*AVR-Rmg8*) conferring the avirulence on S-615. The predicted signal peptide is shadowed.

Figure 4. Symptoms on primary leaves of S-615 (*rmg7*/*Rmg8*), St24 (*Rmg7*/*rmg8*), Sch (*rmg7*/*rmg8*), and Hope (*rmg7*/*rmg8*) inoculated with Br48 (*Triticum* isolate), 200R54 (F_1 culture derived from Br48 x MZ5-1-6), and transformants of 200R54 carrying an

empty vector (200R54+Vector), F2.2 (200R54+F2.2), F1.7 (200R54+F1.7), and F1.1 (200R54+F1.1), 6 days after inoculation. One representative transformant was arbitrarily chosen from each of the 8-9 transformants with F2.2, F1.7, and F1.1 (Fig. 3c).

Table 1. Primers used in this study

	Primer	
Target fragment	Name	Nucleotide sequence
2.4kb cotaining 755.4	755.4F	AACCAAAGGCCCAACTACTG
	755.4R	GCATTGTTGACTCGAAGTGC
2.1kb containing 755.2	755.2F	GTCCTGGCCTTTTCATTGCG
	755.2R	CAGAGCTGATAGGAAGGCGG
2.4kb containing 2570.1	2570.1F	GCGCCTACCAGAGCATGTAT
	2570.1R	CGCACCGTACAAGCAAAAGG
2.3kb containing 4315.4	4315.4F	TGCGGCTGTGTATTGTACCT
	4315.4R	TTGCACCATTTATCGCCTGC
2.1kb containing 1106.3	1106.3F	CGGCCTCATATCTTACCACG
	1106.3R	ATGGAGCTGGACGATTTCAC
F2.2	P7	GCCATTTGCAGGATTGGTAT
	P8	GATCGTTTCGAGCGTGATCT
F1.7	P1	GCTCTCCCAATCGTCGATAA
	P8	GATCGTTTCGAGCGTGATCT
F1.1	P2	CGGTCTCTTCAGTGGCTAGG
	P8	GATCGTTTCGAGCGTGATCT

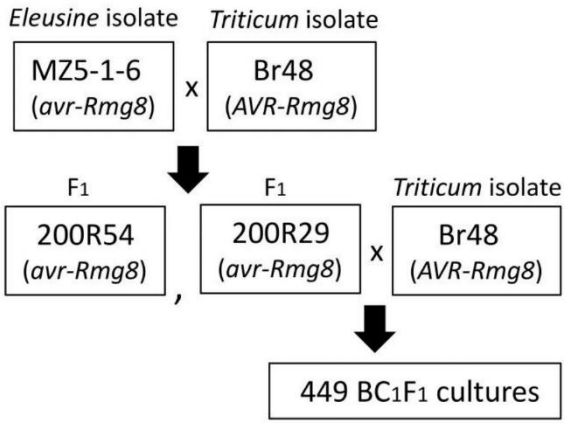


Fig. 1. Pedigree of *P. oryzae* strains used in this study.

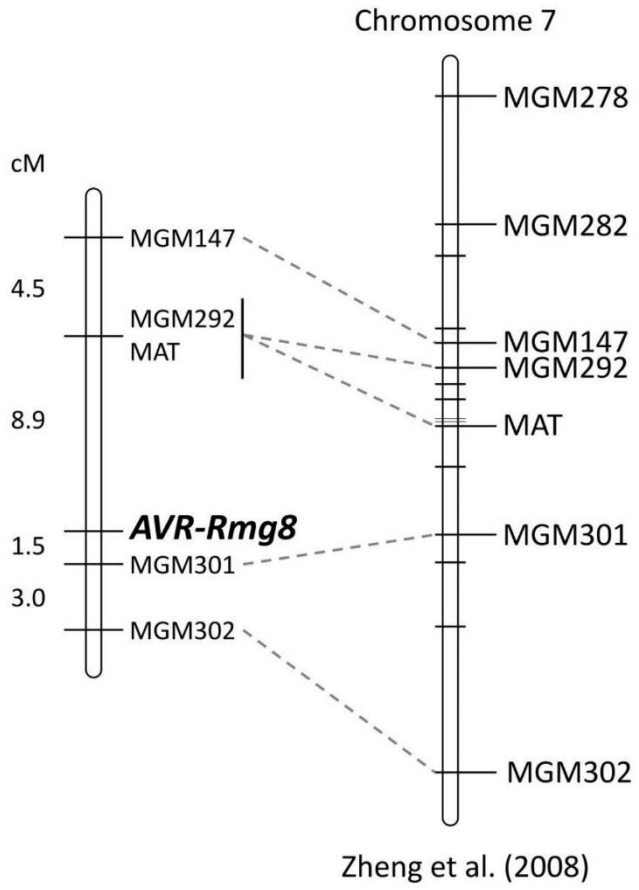
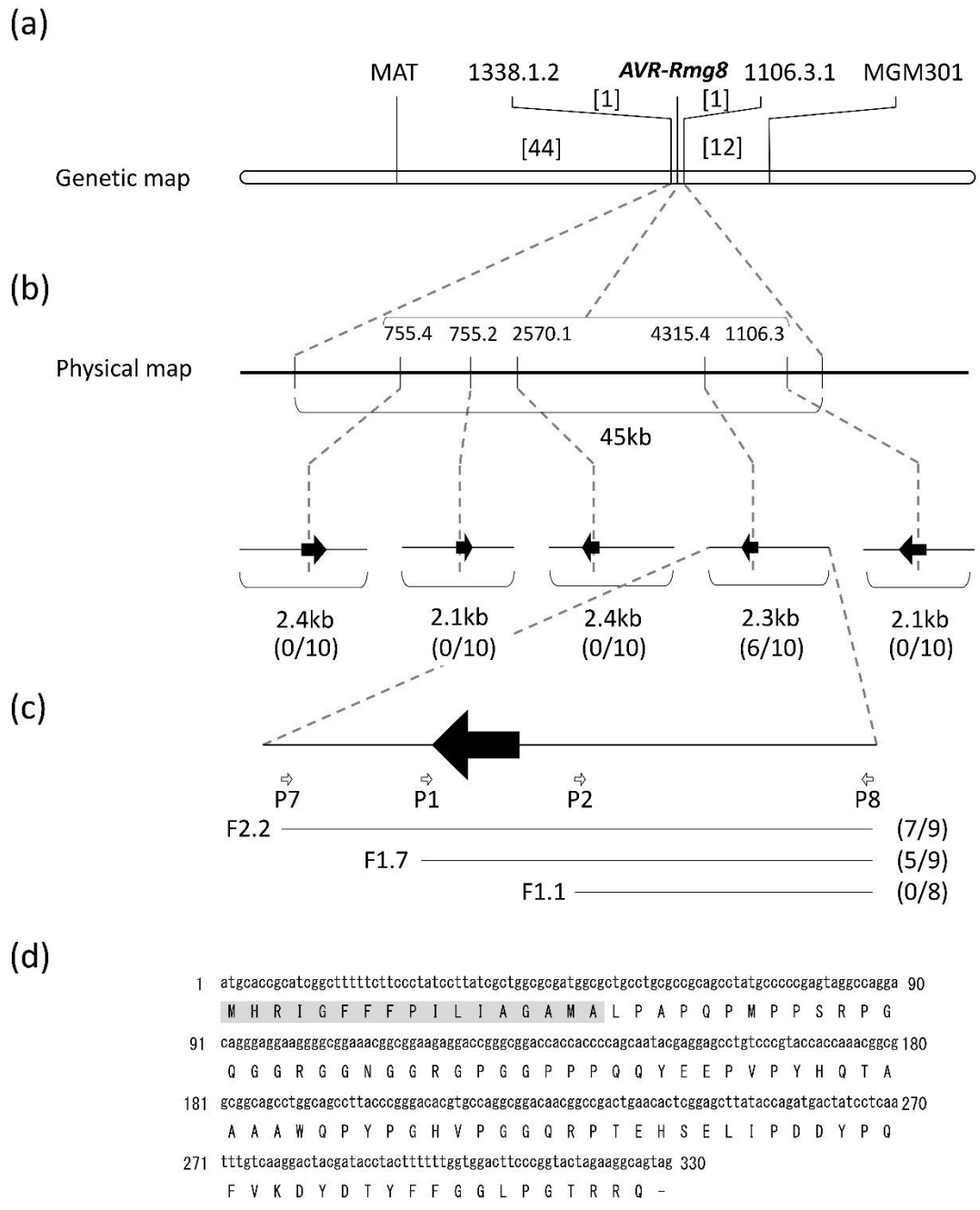


Fig. 2. Genetic map of the *AVR-Rmg8* region constructed using 67 BC_1F_1 cultures derived from Br48 x 200R29. Original positions of markers reported by Zheng et al. (2008) are shown on the right. cM = centimorgans.



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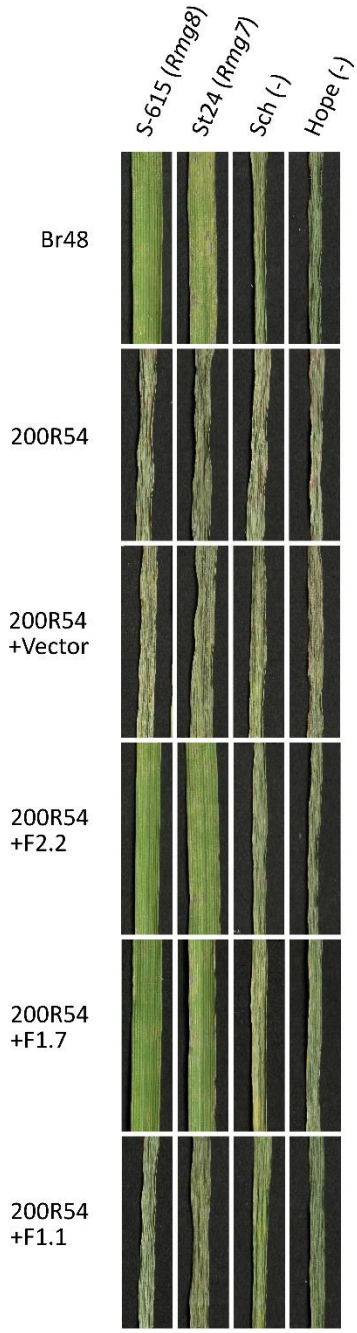
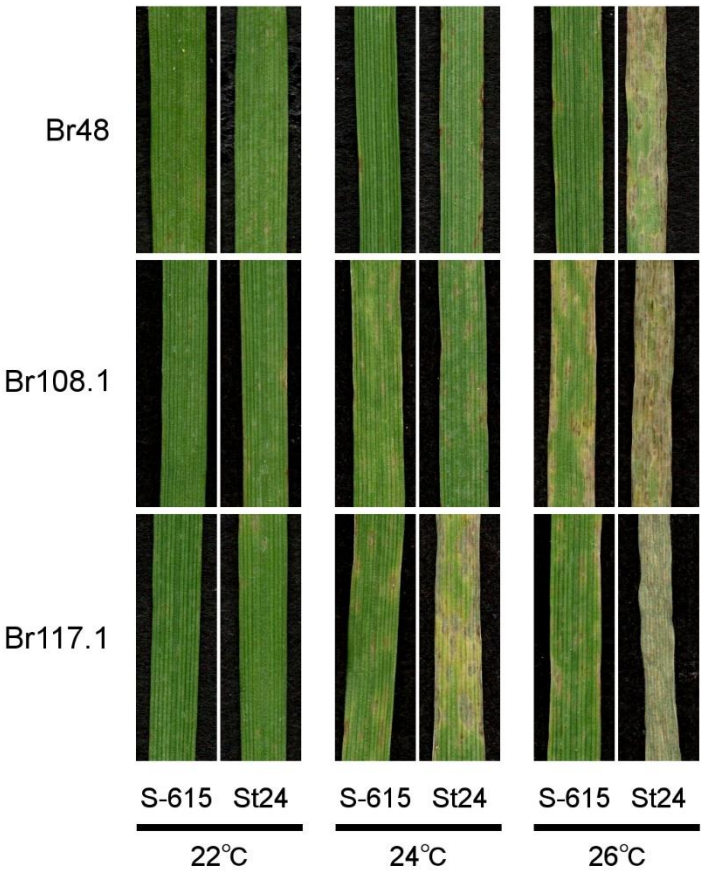


Fig. 4. Symptoms on primary leaves of S-615 (*rmg7/Rmg8*), St24 (*Rmg7/rmg8*), Sch (*rmg7/rmg8*), and Hope (*rmg7/rmg8*) inoculated with Br48 (*Triticum* isolate), 200R54 (F_1 culture derived from Br48 x MZ5-1-6), and transformants of 200R54 carrying an empty vector (200R54 +Vector), F2.2 (200R54+F2.2), F1.7 (200R54+F1.7), and F1.1 (200R54+F1.1), 6 days after inoculation. One representative transformant was arbitrarily chosen from each of the 8-9 transformants with F2.2, F1.7, and F1.1 (Fig. 3c).



Supplementary Fig. 1. Responses of S-615 (*rmg7*/*Rmg8*) and St24 (*Rmg7*/*rmg8*) to *Triticum* isolates (Br48, Br108.1, Br117.1) collected in Brazil in 1990-1992. Inoculated primary leaves were incubated at three different temperatures for 4 days.