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Genetic and Physical Mapping of a Putative *Leymus mollis*-Derived Stripe Rust Resistance Gene on Wheat Chromosome 4A

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Abstract

Wheat stripe rust is one of the most damaging diseases of wheat worldwide. The wheat-*Leymus mollis* introgression line M8664-3 exhibits all-stage resistance to Chinese stripe rust races. Genetic analysis of stripe rust resistance was performed by crossing M8664-3 with the susceptible line Mingxian169. Analysis of the disease resistance of F_2 and $F_{2:3}$ populations revealed that its resistance to Chinese stripe rust race 33 (CYR33) is controlled by a single dominant gene, temporarily designated as *YrM8664-3*. Genetic and physical mapping showed that *YrM8664-3* is located in bin 4AL13-0.59-0.66 close to 4AL12-0.43-0.59 on chromosome 4AL and is flanked by single-nucleotide polymorphism markers *AX111655681* and *AX109496237* with genetic distances of 5.3 and 2.3 centimorgans, respectively. Resistance spectrum and position analyses indicated that *YrM8664-3* may be a novel gene. Molecular detection using the markers linked to *YrM8664-3* with wheat varieties commonly cultivated and wheat-*L. mollis*-derived lines showed that *YrM8664-3* is also present in other wheat-*L. mollis* introgression lines but absent in commercial common wheat cultivars. Thus, *YrM8664-3* is a potentially valuable source of stripe rust resistance for breeding.

Wheat stripe (or yellow) rust, caused by *Puccinia striiformis* Westend. f. sp. *tritici* Erikss., is one of the most damaging diseases of wheat (*Triticum aestivum* L.) worldwide. Epidemics of stripe rust occur in most wheat-growing regions in China and cause more than 30% yield losses in a severe epidemic year (Chen 2005; Li and Zeng 2002). According to survey data, stripe rust affected 6.6, 4.9, and 4.1 million ha in China in 2002, 2003, and 2009, respectively (Han et al. 2010). Growing resistant cultivars is the most effective, economical, and environmentally friendly method to control the disease (Rosewarne et al. 2013). However, a new *P. striiformis* f. *tritici* race will overcome resistance in wheat cultivars with only a single stripe rust resistance gene, and spread rapidly within a few years (Wang et al. 1988). This phenomenon is responsible for the epidemic "boom-bust" cycles that have occurred in China in recent decades. For example, Chinese stripe rust race 29 (CYR29), with virulence to *Yr9*, and CYR32 and CYR33, with virulence to *Yr4b*, overcame resistance of the major wheat-resistant cultivars with *Yr9* or *Yr4b*. The emergence of these strains led to two nationwide stripe rust epidemics with estimated losses of 1.8 and 1.3 million tons of wheat in 1990 and 2002, respectively (Liu et al. 2012; Wan et al. 2004, 2007). Therefore, pyramiding of multiple genes in a cultivar may provide durable resistance (Singh et al. 2008).

Stripe rust resistance genes can be found in common wheat and closely related or distant relatives. Among the 76 officially named stripe rust resistance genes, more than 10 genes such as *Yr8* (Bansal et al. 2008), *Yr9* (Rizwan et al. 2007), *Yr17* (Jia et al. 2011), *Yr28* (Singh et al. 2000), *Yr37* (Marais et al. 2005), *Yr38* (Marais et al. 2010), *Yr40* (Kuraparthy et al. 2007a), *Yr50* (Liu et al. 2013), *Yr69* (Hou et al. 2016), and *Yr70* (Bansal et al. 2017), as well as some informally designated ones, are derived from closely-related relatives of wheat or more distant species: *Aegilops comosa*, *A. geniculata*, *A. kotschyi*, *A. neglecta*, *A. sharonensis*, *A. umbellulata*, *Haynaldia villosa*, *Secale cereal*, *Thinopyrum intermedium*, and *T. ponticum*. Wheat varieties containing alien resistance genes can be divided into three categories; namely, addition, substitution, and translocation lines (Du et al. 2013). Genomic in situ hybridization (GISH) and fluorescence in situ hybridization (FISH) are powerful techniques for visualizing alien chromatin in wheat-alien hybrids (Z. Li et al. 2016) when the resistance gene is derived from related species. However, GISH may not be able to detect all alien segments in wheat-alien introgression lines if alien segments in some varieties are too small (Liu et al. 2013). For instance, *Yr50*, *YrL693*, and *PmPB74* are present in introgression lines CH223, L693, and Pubing74, respectively. According to their breeding pedigrees and analyses of parental phenotypes, the stripe rust resistance in CH233 and L693 is inherited from *T. intermedium* and the powdery mildew resistance in Pubing74 is derived from *Agropyron cristatum*. Nevertheless, GISH analyses of CH223, L693, and Pubing74 did not detect any chromosomal segments of *T. intermedium* or *A. cristatum* (Huang et al. 2014; Liu et al. 2013; Lu et al. 2016). Thus, in some cases, only a small fragment from an alien species can change phenotypes in common wheat. Actually, this type of wheat-alien introgression line with a small alien fragment is desirable for the improvement of agronomic traits of wheat in breeding (Young and Tanksley 1989).

Leymus mollis (Trin.) Pilger ($2n = 4x = 28$, NsNsXmXm), a very important tetraploid species belonging to tribe Triticeae (Poaceae), is native to the northern coast and arid inland areas of China (Chen et al. 1985). The Ns genome of *L. mollis* is derived from *Psathyrostachys huashanica* Keng ($2n = 14$, NsNs), while the source of its Xm genome remains unclear (Wang and Jensen 1994; Zhou et al. 2010). *L. mollis* is a distant relative of wheat useful for wheat breeding because it is resistant to drought, salinity, and fungal diseases such as stripe rust, scab, and powdery mildew (Mohammed et al. 2013). Through embryo rescue and colchicine doubling, *L. mollis* has been hybridized with common wheat cultivar 7182 and then used to develop octoploid *Tritileymus* M842 ($2n = 8x = 56$, AABBDDNsNs or AABBDDXmXm) lines in China. A series of valuable wheat-*L. mollis* lines have been subsequently developed by crossing M842 with wheat varieties or nullisomic lines (Fu et al. 1993). For example, 05DM6 and 10DM57, both obtained from different *L. mollis*

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chromosome substitution and derived M842 lines, have been reported to possess resistance to leaf rust (Fu et al. 1997; Pang et al. 2014; Zhao et al. 2013). Shannong0096 and M8926-2, both *L. mollis* translocation lines, are highly resistant to stripe rust (Bao et al. 2012; Q. Li et al. 2016). Observations reported for all these materials indicated that disease resistance of wheat varieties can be enhanced by exploiting wheat-*L. mollis* lines.

M8664-3 is a wheat-*L. mollis* introgression line that has shown high resistance to Chinese stripe rust races in seedling tests. However, our preliminary study showed that GISH and FISH failed to detect the alien segments from *L. mollis* in M8664-3 (Supplementary Fig. S1) (Yang et al. 2015). The objectives of the present study were to characterize stripe rust resistance genes in M8664-3 using molecular markers to construct genetic and physical maps and to identify markers useful for molecular breeding and map-based cloning.

Materials and Methods

Plant materials. *L. mollis* accession BM01, wheat-*L. mollis* introgression line M8664-3, *P. huashanica* Keng, and common wheat cultivars Mingxian169 and 7182 used in this study were provided by Professor Jie Fu, Northwest Agriculture and Forestry University, Yangling, China. As the male parent, M8664-3 was crossed with the susceptible cultivar Mingxian169 to study the genetics of its stripe rust resistance and to develop mapping population. Two F_2 populations with 127 and 225 plants, their $F_{2:3}$ population, and the two parents were used in seedling tests in the greenhouse to identify the genes for all-stage resistance in M8664-3. Twenty-one Chinese Spring nulli-tetrasomic lines were used to verify the chromosomal location. In all, 14 wheat-*L. mollis*-derived lines and 108 wheat cultivars commonly cultivated in the Yangtze and Yellow Rivers region were used to evaluate the polymorphisms of the molecular markers flanking the resistance gene in M8664-3.

Seedling tests. Six *P. striiformis* f. *tritici* races (CYR29, CYR31, CYR32, CYR33, Sull-4, and Sull-7) with various combinations of virulence genes were chosen to test the resistance of M8664-3, 7182, Mingxian169, and *L. mollis* seedlings. CYR33 was used to test the seedlings of the 14 wheat-*L. mollis*-derived lines, 108 wheat cultivars, and F_1, $F_{2\text{-}1}$, and $F_{2\text{-}2}$ populations in 2013, and $F_{(2\text{-}1):3}$ and $F_{(2\text{-}2):3}$ populations in 2014.

Seedling tests were conducted under controlled greenhouse conditions, as described by Li and Zeng (2002). First, all of the seed were disinfected with 5% sodium hypochlorite solution for 0.5 h (Sauer and Burroughs 1986). Seed from the cultivars, parents, and F_1, F_2, and F_3 populations were sown uniformly in 10-cm pots filled with a mixture of farmyard manure and sandy loam soil, then incubated at 25°C to accelerate germination (Bansal et al. 2017). Throughout the experiment, the soil was kept moist. For the genetic analysis, 20 seeds for each parent were planted into four pots with 10 seeds/pot, 6 seeds of F_1 were planted into one pot, and 350 to 380 seeds of two F_2 generations were evenly planted into each pot with 10 seeds/pot. Seed of each F_2-derived F_3 family line were sown in the individual pot with 15 seeds/pot. For cultivars or lines, 10 seeds of each cultivar or line were sown in one pot, and the final seedling test result of each cultivar or line was derived from the highest frequency of infection types (IT). A few seed did not germinate normally but this had no influence for the seedling test. When the first leaves were fully expanded, about 10 days after sowing, all of the healthy seedlings were inoculated using the brushing method with fresh urediniospores of selected *P. striiformis* f. *tritici* isolates (Roelfs et al. 1992). The inoculated seedlings were placed in 10°C dew chambers without light for 24 h, then transferred into a controlled greenhouse maintained with photoperiod of 14 and 10 h of light and darkness, respectively, at 12 to 17°C (Li et al. 2009). When rust was fully developed on the susceptible control Mingxian169, 15 to 16 days after inoculation, IT were scored using scores of 0, 0;, 0;$^+$, 1, 1$^+$, 2, 2$^+$, 3$^-$, 3, 3$^+$, and 4 (Bariana and McIntosh 1993; Li et al. 2006). Plants with an IT of 0 to 2$^+$ were considered resistant and those with an IT of 3$^-$ to 4 were considered susceptible (Li et al. 2006; Wang et al. 1988).

Simple sequence repeat analysis and expressed sequence tag marker development. A bulk segregant analysis (BSA) strategy was used to identify markers linked with the stripe rust resistance gene (Michelmore et al. 1991). Resistant and susceptible bulks were composed of DNA separately extracted from fresh leaves of 10 resistant and 10 susceptible F_2 individuals, respectively, using the cetyltrimethylammonium bromide method as modified by Yan et al. (2003).

In total, 297 simple sequence repeat (SSR) primer pairs were chosen from a consensus genetic map using the GrainGenes website (http://www.graingenes.org). The strategy used for selection was to choose at least one primer pair every 6 centimorgans (cM) on each chromosome. When a marker linked to the resistance gene was found, more SSR markers were selected in the nearby region to enhance the marker density.

Based on the results of the SSR marker analysis, 200 expressed sequence tags (EST) in bins of 4AL12-0.43-0.59 and 4AL13-0.59-0.66 were downloaded from the GrainGenes website. Primer 5.0 software (http://www.primer-e.com/) was used to choose EST primers, and the MISA.PL (Thiel et al. 2003) software package was used to analyze microsatellites (SSR) in the EST.

Polymerase chain reaction (PCR) amplifications for SSR and EST-SSR (STS) analyses were performed on a S1000 Thermo Cycler (Bio-Rad Laboratories, Hercules, CA). The 15-μl reaction mixtures consisted of 1.5 μl each of forward and reverse primers (5 μM), 1.2 μl of $MgCl_2$ (25 mM), 1.5 μl of 10× PCR buffer, 2.1 μl of DNA template (50 ng/μl), 1.0 U of *Taq* DNA polymerase, and 0.3 μl of dNTP (10 mM). Sterile deionized distilled (dd) H_2O was used for a complement reaction system up to 15 μl. The PCR program consisted of an initial denaturation for 5 min at 94°C; followed by 35 cycles consisting of 1 min of denaturation at 94°C, 1 min at 50 to 65°C annealing temperature depending on primers, and 1 min of extension at 72°C; with a final extension at 72°C for 10 min (Xiang et al. 2016). Approximately 4 to 5 μl of PCR product and loading buffer mixture per sample was subjected to electrophoresis on a 6% polyacrylamide gel, as previously described (Chen et al. 1998). After electrophoresis, the gel was silver stained for visualization.

Single-nucleotide polymorphism genotyping. To explore more markers linked to the resistance gene, the resistant and susceptible DNA bulks of the $F_{2\text{-}2}$ population were analyzed on a wheat 660K single-nucleotide polymorphism (SNP) chip and genotyped by the CapitalBio Corporation (http://cn.capitalbio.com/). Physical locations of SNP markers on chromosomes were determined using the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq v1.0 (https://urgi.versailles.inra.fr/; http://www.wheatgenome.org/) (International Wheat Genome Sequencing Consortium 2014) as the reference database. The distribution frequencies of SNP locations on 21 chromosomes and the physical location of each marker on each chromosome were calculated using SPSS Statistics 17.0 software (https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software). Chromosome-specific SNP markers were then randomly selected from the most abundant represented chromosomes and chromosome regions. Those SNP markers were used to genotype individual $F_{2\text{-}2}$ plants for linkage analysis by the kompetitive allele-specific PCR (KASP) technique (Semagn et al. 2014). SNP-derived KASP primers were designed using the PolyMarker tool (polymarker.tgac.ac.uk). The KASP assay was conducted in 5-μl reaction volumes containing 50 to 100 ng of genomic DNA, 0.056 μl of assay primer mix (12 mM each allele-specific primer and 30 mM common primer), and 2.5 μl of 2× KASP V4.0 Mastermix (LGC Genomics, Beverly, MA). Sterile ddH_2O was used for a complement reaction system up to 5 μl (Chen et al. 2016). A complete KASP genotyping experiment for an SNP marker required a minimum of 22 samples, including at least two control checks that used ddH_2O instead of template DNA. Due to the small amount of assay primer mix required for the 5-μl reaction system, we usually mixed it with KASP V4.0 Mastermix multiple times, then added each reaction system separately. For example, we designed a KASP assay of 50 samples for an SNP marker. First, the stock DNA solution was diluted to 50 ng/μl with ddH_2O for use as template; then, a mixed liquid was constructed containing 2.8 μl (50 × 0.056 μl) of assay primer mix and 125 μl (50 × 2.5 μl) of Mastermix. Finally, 2.5 μl

of template DNA (slightly more than the minimum required for the reaction to proceed adequately) or ddH_2O which was considered as the control check and 2.5 μl of mixed solution was added to each reaction system (He et al. 2014). The PCR of KASP assays were performed in an S1000 Thermal Cycler with 384 wells (Bio-Rad Laboratories), programmed for 15 min of initial denaturation at 94°C, nine cycles of 20 s at 94°C and 1 min of touchdown starting at 65°C (decreasing by 0.8°C per cycle), followed by 30 to 40 cycles of amplification of 20 s at 94°C and 1 min at 57°C (Wu et al. 2017). Reaction fluorescent endpoint readings were carried out with a Biotek Synergy H1 Multi-Mode Reader (Bio-Tek Instruments Inc., Winooski, VT). The results were clustered and genotyped using KlusterCaller software (http://www.lgcgroup.com/).

Construction of genetic and physical reference maps. To determine goodness of fit of the observed numbers of plants or lines to predict progeny segregation ratios and to establish the number of stripe rust resistance genes, mode of inheritance, and genetic relationships of genes, χ^2 tests were conducted. Linkage analysis was conducted with JoinMap 4.0 (https://www.wur.nl/en/Expertise-Services/Research-Institutes/plant-research/Biometris-1.htm). Map distance in centimorgans was calculated according to the Kosambi mapping function (Kosambi 1944), and a logarithm of odds (LOD) score of 3.0 was used as the threshold for a declaration of linkage.

Construction of a physical map based on the IWGSC RefSeq v1.0 database allowed us to visually distinguish the chromosomal location of the different markers. To locate the physical position of each fragment on a chromosome, EST and SNP sequences were analyzed using Blast v2.5.0+ and Magic-BLAST v1.1.0. Because information about the original sequences was lacking, the amplified products of some SSR markers were sequenced using the DNA of Chinese Spring. MapChart v2.3 (Voorrips 2002) was used to draw the genetic and physical reference maps.

Molecular detection. *L. mollis*, *P. huashanica*, wheat cultivar 7182, and 14 wheat-*L. mollis*-derived lines were subjected to seedling tests and then used to verify identified linked markers. This type of test is mainly used to verify the origin and presence of a specific gene in the same set of materials. To detect the presence of *YrM8664-3* in commercial varieties, we also screened 108 wheat cultivars commonly cultivated in the Yangtze and Yellow Rivers region. These plant materials, together with M8664-3 and Mingxian169, were tested at the seedling stage under controlled conditions as described above with CYR33 avirulent to M8664-3. Genomic DNA of these wheat genotypes was extracted from the leaves of each seedling, then tested with two to five markers linked to the resistance gene locus (Peng et al. 2000; Xiang et al. 2016; Yan et al. 2003).

Results

The origin of the stripe rust resistance of M8664-3. As the donor parent, *L. mollis* was immune to all six *P. striiformis* f. *tritici* races, whereas its derived line, M8664-3, was resistant to all six races except CYR32. The receptor parent 7182 and the control cultivar Mingxian169 were susceptible to all *P. striiformis* f. *tritici* races (Table 1). *L. mollis* and M8664-3 also showed strong resistance in field, whereas 7182 and Mingxian169 exhibited the opposite reaction (Fig. 1). It is indicated that M8664-3 may also have other adult genes resistant to CYR32. Combined with the pedigree, these results demonstrate that the stripe rust resistance of M8664-3 is most likely derived from *L. mollis*.

Inheritance of seedling stripe rust resistance in M8664-3. IT data for parents, F_1 plants, two F_2 populations, and two $F_{2:3}$ populations is shown in Table 2. When inoculated with CYR33, all F_1 plants exhibited an IT of 0;, similar to the resistant parent M8664-3, indicating that the resistance of M8664-3 was dominant. Among all 127 $F_{2\text{-}1}$ and 225 $F_{2\text{-}2}$ plants, the ratio of resistant to susceptible plants was 97:30 and 160:65, respectively, corresponding to a 3:1 ratio ($\chi^2 < 3.84$, $P > 0.05$). $F_{(2\text{-}1):3}$ and $F_{(2\text{-}2):3}$ lines were derived from $F_{2\text{-}1}$ and $F_{2\text{-}2}$ populations separately. A few F_2 plants died during transplantation to the field. For all 119 $F_{(2\text{-}1):3}$ lines and 212 $F_{(2\text{-}2):3}$ plants, the ratios of resistant/segregating/susceptible lines were 32:58:29 and 48:102:62, respectively, corresponding to a 1:2:1 ratio ($\chi^2 < 5.99$,

Table 1. Infection types on wheat-*Leymus mollis* introgression line M8664-3, *L. mollis*, 7182, and Mingxian169 to six Chinese races of *Puccinia striiformis* f. *tritici* at the seedling stage

Races	Virulence formula[a]	*L. mollis*	M8664-3	Mingxian169	7182
CYR29	1,2,3,4,5,6,7,8,9,11,12,16	0	0;	4	4
CYR31	1,2,3,4,5,6,7,8,9,11,12,14,16,17	0	0;	4	4
CYR32	1,2,3,4,5,6,7,8,9,10,11,12,13,14,16,17	0	3⁻	4	4
CYR33	1,2,3,4,5,6,7,8,9,10,11,12,13,14,16	0	0;	4	4
Su11-4	1,2,3,4,6,7,8,9,10,11,13,14,16	0	0;	4	4
Su11-7	1,2,3,4,5,6,7,8,9,11,12,14,16	0	0;	4	4

[a] Chinese differential genotypes: 1, Trigo Eureka; 2, Fulhard; 3, Lutescens 128; 4, Mentana; 5, Virgilio; 6, Abbondanza; 7, Early Premium; 8, Funo; 9, Danish 1; 10, Jubilejina 2; 11, Fengchan 3; 12, Lovirn13; 13, Kangyin 655; 14, Shuiyuan 11; 15, Zhong 4; 16, Lovirn 10; 17, Hybrid 46; 18, *Triticum spelta album*; and 19, Guinong 22.

Fig. 1. Seedling test of **A,** M8664-3 and Mingxian169 to Chinese *Puccinia striiformis* f. *tritici* race CYR33; **B,** adult leaves of *Leymus mollis*, M8664-3, Mingxian169, and 7182 collected from the field under the same natural conditions; and **C** the adult plant of M8664-3.

$P > 0.05$). The results of these two independent tests indicated that a single dominant gene is involved in CYR33 resistance in M8664-3. We have tentatively designated this gene as *YrM8664-3*.

Identification of SSR and EST markers linked to *YrM8664-3*. The 127 F_{2-1} plants of Mingxian169/M8664-3 were used to map the resistance gene with microsatellite markers in 2014. Among the 297 SSR markers screened, only *Xbarc170* and *Xwmc698* were polymorphic between the contrasting parents and bulks. All plants in the F_{2-1} population were screened with the two markers. Genotypes of the two flanking markers and the phenotypes of all individuals were used for calculating the independence test LOD score (van Ooijen 2006) by Joinmap 4.0. *Xbarc170*, *Xwmc698*, and *YrM8664-3* (data set of individual phenotypes) were in the same group with the LOD score of 3.0. We thereby concluded that *Xbarc170* and *Xwmc698* are linked to *YrM8664-3*. Combined with known marker information, *YrM8664-3* was initially located on chromosome 4AL. *Xwmc262*, *Xgpw2331*, and *Xgpw3224* were then identified on 4AL and linked to *YrM8664-3*. The order of the linked markers was *Xbarc170-Xwmc262-Xwmc698-Xgpw2331-YrM8664-3-Xgpw3224*, with genetic distances of 9.0, 16.6, 7.7, 2.8, and 8.1 cM, respectively (Fig. 2D; Supplementary Table S1). These SSR markers were amplified in nulli-tetrasomic lines, which confirmed that *YrM8664-3* is located on chromosome 4A (Supplementary Fig. S2). *Xbarc170* was located in the breakpoint interval of 4AL13-0.59-0.66 (https://wheat.pw.usda.gov/) (Sourdille et al. 2004). Because extracted DNA from F_{2-1} plants was insufficient and the population was too small, the F_{2-2} population of Mingxian169/M8664-3 was used to identify EST sequences within the "4AL12-0.43-0.59" and "4AL13-0.59-0.66" interval. These EST, which included *BE446584*, *BE403251*, *BE403721*, *BE637642*, and *BE406959*, were thus linked to *YrM8664-3* (Fig. 2D). Based on the position information of these markers, *YrM8664-3* was located in the 4AL13-0.59-0.66 bin close to 4AL12-0.43-0.59.

Identification of SNP markers linked to *YrM8664-3*. The 225 F_{2-2} plants of Mingxian169/M8664-3 were used to map the resistance gene with SNP markers in 2016. Genotyping of SNP in the two parents and bulks using the 660K chip indicated that the resistance gene was most likely located on chromosome 4A, because it had the highest distribution frequency (Supplementary Fig. S3). By locating the SNP on chromosome 4A with reference to the IWGSC-RefSeq database, regions of 6×10^7, 50×10^7, and 60×10^7 bp were the most probable loci of *YrM8664-3* (Fig. 2A). Thirteen chromosome-specific SNP markers were selected from these three intervals and used to genotype all individual F_{2-2} plants using the KASP method. *AX111655681*, *AX109496237*, *AX109001562*, *AX86179210*, and *AX109895154* were found to be linked to *YrM8664-3* (Fig. 2D; Supplementary Fig. S4). The results of the two molecular marker

Table 2. Seedling responses of the parents and hybrid generations of cross Mingxian169/M8664-3 to CYR33 in the greenhouse

Parents and populations	Observed number of plants or lines[a]			Total	Expected ratio	χ^2	*P*
	Res	Seg	Sus				
M8664-3	18	–	0	18	…	…	…
Mingxian169	0	–	12	12	…	…	…
F_1	6	–	0	6	…	…	…
F_{2-1}	97	–	30	127	3:1	0.12	0.72
$F_{(2-1):3}$	32	58	29	119	1:2:1	0.23	0.89
F_{2-2}	160	–	65	225	3:1	1.81	0.18
$F_{(2-2):3}$	48	102	62	212	1:2:1	2.15	0.34

[a] Res = resistant, Seg = segregating, and Sus = susceptible.

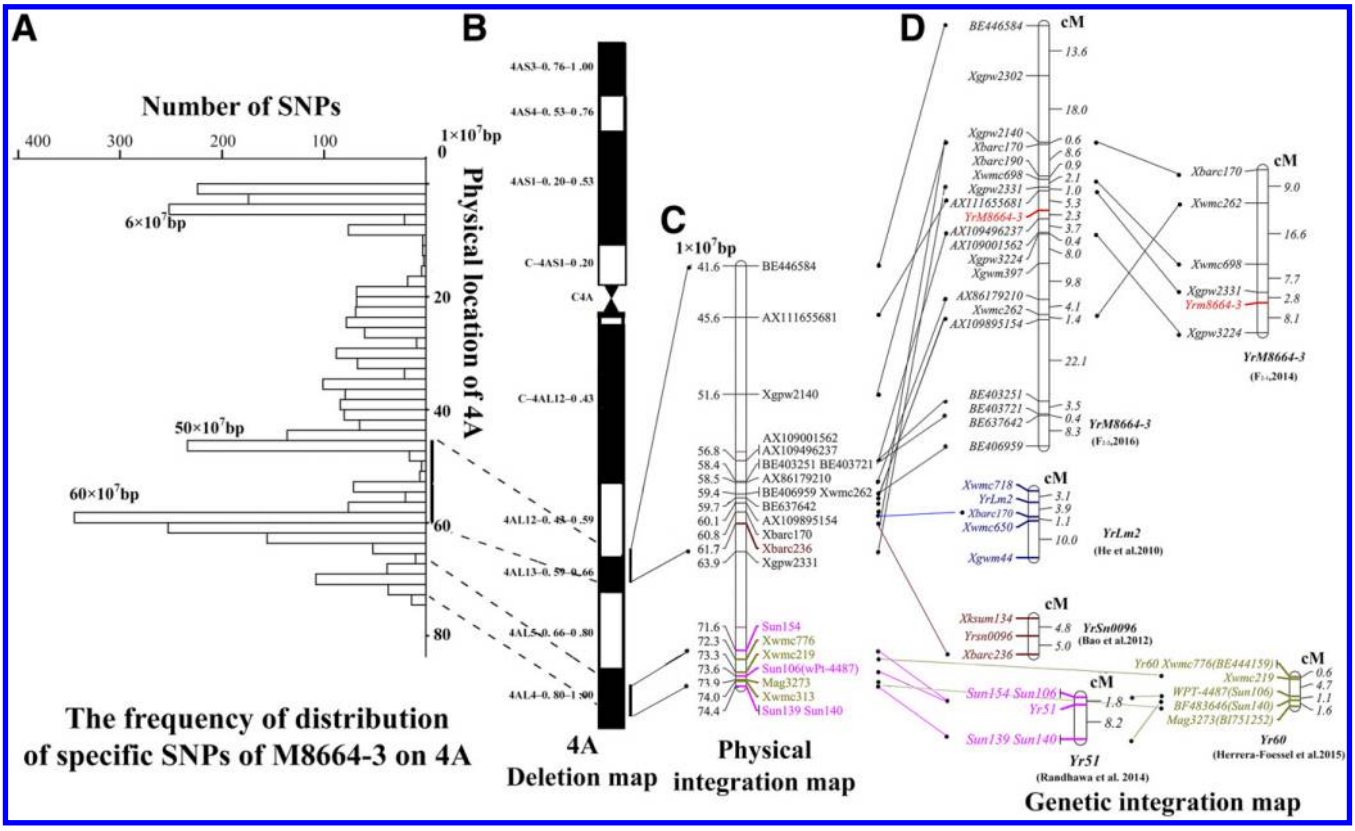

Fig. 2. Genetic and physical integration map of wheat stripe rust resistance genes on chromosome 4A. **A,** Frequency of distribution of specific single-nucleotide polymorphisms (SNP), which was based on bulk segregate analysis strategy on the physical map of M8664-3 on chromosome 4A. **B,** Deletion map of 4A (https://wheat.pw.usda.gov/NSF). **C,** Physical integration map of markers linked to resistance genes. Markers are shown on the right and their position in chromosome 4A of the IWGSC-RefSeq database are shown on the left. The numeric unit is 1×10^7 bp. **D,** Genetic maps of *YrM8664-3* (F_{2-2}, 2016), *YrM8664-3* (F_{2-1}, 2014), *YrSn0096* (Bao et al. 2012), *YrLm2* (He et al. 2010), *Yr51* (Randhawa et al. 2014), and *Yr60* (Herrera-Foessel et al. 2015) on chromosome 4AL. Map distances in centimorgans (cM) are shown on the right.

strategies were consistent, indicating that *YrM8664-3* is located on chromosome 4AL.

Construction of a complex genetic map. All markers amplified in the F_{2-2} population were used to construct an integrated genetic map (Fig. 2D). According to the map, the two closest flanking markers linked to *YrM8664-3* were *AX111655681* and *AX109496237*, with genetic distances from the gene of 5.3 and 2.3 cM, respectively. By comparing SSR genetic maps of two different populations, the positions of the two maps were consistent, with only the position of *Xwmc262* changed. This consistency indicated that the order of the markers linked to the gene did not vary greatly between the two populations.

Construction of a physical reference map. The IWGSC-RefSeq database was used as a reference to construct a physical map. The markers accordingly linked to *YrM8664-3* as well as markers linked to other resistance genes on 4AL are summarized in Figure 2 and Supplementary Table S2. The order of most markers was consistent between genetic and physical maps. Markers linked to *YrM8664-3* spanned a maximum range of 41.6×10^7 to 63.9×10^7 bp. This interval was based on the location of *BE446584* and *Xgpw2331*. The blast result of EST indicated that the range of 4AL12-0.43-0.59 is focused on 42×10^7 to 58×10^7 bp, and the range of 4AL13-0.59-0.66 is focused on 54×10^7 to 59×10^7 bp. Physical reference maps also showed that the interval of *YrM8664-3* was located in the 4AL13-0.59-0.66 bin close to 4AL12-0.43-0.59.

Molecular detection. All generated SSR markers were used to detect the presence of *YrM8664-3* in the donor parents of *L. mollis*, *P. huashanica*, and 7182. Specific bands present in M8664-3, *L. mollis*, and *P. huashanica* were amplified using *Xbarc190*, *Xwmc262*, and *Xwmc698* primers (Fig. 3). Most amplification results obtained for M8664-3 using other SSR markers were the same as in 7182. These results demonstrate that M8664-3 may carry introgressed fragments derived from the Ns genome of *L. mollis*.

Five markers (*Xwmc698*, *Xgpw2331*, *Xgpw3224*, *AX111655681*, and *AX109496237*) were used to detect the presence of *YrM8664-3* in the 14 wheat-*L. mollis*-derived lines (Supplementary Table S3). The three lines (M8725-3, M8724-2, and M8657-4) all had the same genotypes as M8664-3 and were resistant to CYR33. These three lines also have the same donor parents as M8664-3, which suggests that these three plant materials may carry *YrM8664-3*. Therefore, the transfer of *YrM8664-3* was not limited to only one wheat-*L. mollis*-derived line.

Xwmc698 and *Xgpw2331* were used to determine the absence of *YrM8664-3* in 108 wheat varieties commonly planted in the Yangtze and Yellow Rivers region (Supplementary Table S4). None of the wheat varieties had the M8664-3 alleles. These results indicated that *YrM8664-3* has not been used in these commercial wheat cultivars and can be incorporated into wheat cultivars via marker-assisted selection.

Discussion

***YrM8664-3* may be a novel stripe rust resistance gene.** *YrM8664-3*, a seedling resistance gene to CYR33, was identified and mapped in the bin of 4AL13-0.59-0.66 close to 4AL12-0.43-0.59 on chromosome 4AL. To date, four stripe rust resistance genes have been mapped to chromosome 4AL: two formally named genes, *Yr51* (Randhawa et al. 2014) and *Yr60* (Herrera-Foessel et al. 2015), and two informally designated genes, *Yrsn0096* (Bao et al. 2012) and *YrLm2* (He et al. 2010). To facilitate the analysis of these genes, a physical map was constructed with most of the markers linked to resistance genes using the IWGSC-RefSeq v1.0 database (Fig. 2). This map clearly showed the location of the four genes on chromosome 4A. *Yr51* and *Yr60* have been identified in common wheat cultivars AUS27858 and Almop, respectively. They were located in the bin 4AL4-0.80-1.00, at the end of chromosome 4A; they were within the 71.6×10^7- to 74.4×10^7-bp interval, at least 200 Mb away from *YrM8664-3*. SSR markers *Xgwm160*, *Xbarc78*, *Xwmc776*, and *Xwmc219* and EST markers *Sun154* (*BE444404.1*) and *Sun139* (*BF483646.1*) were validated in the Mingxian169/M8664-3 F_{2-2} population. These markers linked to *Yr51* or *Yr60* but exhibited no polymorphisms between parents and F_{2-2} population, indicating that they are not associated with *YrM8664-3*. In addition, AUS27858 was susceptible to CYR33, whereas M8664-3 was not (Supplementary Fig. S5). Both *Yrsn0096* and *YrLm2* are derived from *L. mollis* and may be close to *YrM8664-3* on the physical map. Markers *Xwmc718*, *Xksum134*, *Xbarc236*, *Xwmc650*, and *Xgwm44*, which are linked to *Yrsn0096* or *YrLm2*, were not linked to *YrM8664-3*. *Yrsn0096*, unlike *YrM8664-3*, was resistant to CYR32 in seedlings, whereas M854-3 (the carrier of *YrLm2*) was susceptible to CYR33. On the basis of this analysis, *YrM8664-3* is very likely a novel gene different from reported genes *Yr51*, *Yr60*, *Yrsn0096*, and *YrLm2*.

Cryptic alien introgression is possible in M8664-3. According to seedling tests, M8664-3 resistance is derived from *L. mollis* but no GISH signal was detected. However, detection of molecular markers linked to *YrM8664-3* revealed that M8664-3 has specific bands present only in *P. huashanica* ($2n = 14$, NsNs) and *L. mollis* ($2n = 4x = 28$, NsNsXmXm). These results can be explained by assuming that *YrM8664-3* is a resistance gene derived from the Ns genome of *L. mollis*. The translocation segments from *L. mollis* may be too small to be detected by GISH. This form of resistance transfer, called "cryptic alien introgression" (Kuraparthy et al. 2007a), has also been reported in materials containing *Lr57* (Kuraparthy et al. 2007a), *Lr58* (Kuraparthy et al. 2007b), *Yr50* (Liu et al. 2013), *YrL693* (Huang et al. 2014), and other resistance genes. Chromosomes of these materials have been shown to undergo normal bivalent pairing at meiosis and can recombine randomly in wheat (Caceres et al. 2012; Chen et al. 2012; Lu et al. 2016). One possible cause of cryptic alien introgression is the replacement of wheat homoeoloci by alien homoeoloci in a precise recombination-like manner during pairing and recombination between homologous chromosomes (Kuraparthy et al. 2007b).

The octoploid *Tritileymus* M842-12 ($2n = 8x = 56$, AABBDDNsNs) is a hybrid progeny of *L. mollis* and 7182. Backcrossing of *Tritileymus* with 7182 produced backcross descendants (BC_1F_1), which were inbred for many generations to ultimately yield some selected lines possessing stable agricultural traits. M8664-3 is one such line, and the small alien fragment introgression may have occurred during the

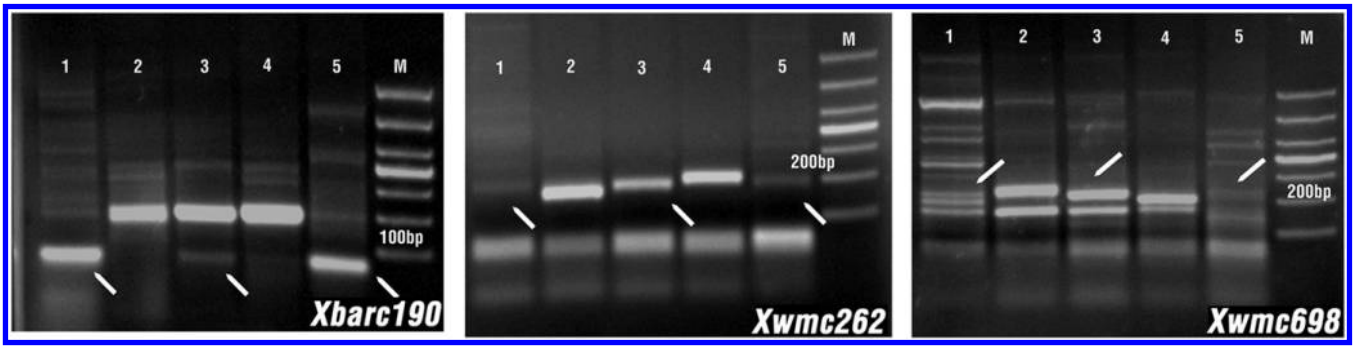

Fig. 3. Agarose gels showing the results of polymerase chain reaction amplification of the simple sequence repeat markers *Xbarc190*, *Xwmc262*, and *Xwmc698* in the donor parents. Lane 1, *Psathyrostachys huashanica*; lane 2, Mingxian169; lane 3, M8664-3; lane 4, 7182; and lane 5, *Leymus mollis*. Arrow icons indicate the specific bands which were present in *P. huashanica*, *L. mollis*, and M8664-3.

breeding process. Irregular pairing of homologous chromosomes may cause wheat homoeoloci to be replaced by alien homoeoloci in a precise recombination-like manner (Fig. 3). After years of inbreeding, these alien fragments may become genetically stable and able to recombine randomly in wheat. Small alien fragment introgression lines are valuable sources of resistance genes for common wheat, whereas large alien chromosomal fragments may carry additional genes for undesirable traits, resulting in "linkage drag" (Young and Tanksley 1989). Thus, the wheat-*L. mollis* introgression line M8664-3 can be used effectively as a donor of desirable genes responsible for excellent agronomic traits.

***YrM8664-3* is potentially valuable for resistance breeding.** Because CYR33 is one of the most damaging races of stripe rust in China, the introduction of wheat varieties with CYR33 resistance would be beneficial to grain production. *YrM8664-3*, a gene potentially derived from *L. mollis*, confers CYR33 resistance on seedlings. The detection rate of *YrM8664-3* in common wheat varieties was not very high, whereas multiple wheat-*L. mollis*-derived lines contained this gene. This observation indicates that *YrM8664-3* penetrated into the common wheat genome during the course of the creation of wheat-*L. mollis*-derived lines and expresses resistance to CYR33. This phenomenon is not surprising, because *YrM8664-3* has a certain heritability or an epistatic effect on other genes.

YrM8664-3 is located on 4AL, a chromosome harboring relatively few reported resistance genes, which makes it potentially valuable for resistance breeding. In order to get broad resistance, a pyramiding breeding strategy should be used to combine multiple race-specific resistance genes such as *YrM8664-3* and nonrace-specific genes. Using marker-assisted breeding, the resistance genes on different chromosomes can be pyramided to avoid competition between alleles and to complement the resistance of different genes. We also identified some markers linked to *YrM8664-3*. Use of these markers allowed *YrM8664-3* to be quickly identified in the hybrid progeny. This molecular detection strategy can shorten breeding and material selection times, reduce workloads, and improve the accuracy of the breeding process. Finally, further refinement of the physical interval of *YrM8664*-3 may provide a reference for future map-based cloning.

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