

Fine mapping of a stripe rust resistance gene YrZM175 in bread wheat

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Research Article

Keywords: Candidate gene, Disease resistance, Molecular marker, Puccinia striiformis, T. aestivum

Posted Date: April 20th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-1537017/v1>

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Abstract

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (PST), is a worldwide wheat disease that causes large losses in production. Fine mapping and cloning of resistance genes are important for accurate marker-assisted breeding. Here, we report the fine mapping and candidate gene analysis of stripe rust resistance gene *YrZM175* in a Chinese wheat cultivar Zhongmai 175. Fifteen F₁, 7,325 F₂ plants and 117 F₂:3 lines derived from cross Avocet S/Zhongmai 175 were inoculated with PST race CYR32 at the seedling stage in a greenhouse, and F₂:3 lines were also evaluated for stripe rust reaction in the field using mixed PST races. BSR-seq analyses revealed 13 SNPs in the region 762.50–768.52 Mb on chromosome arm 2AL. By genome mining we identified SNPs and InDels between the parents and contrasting bulks, and mapped *YrZM175* to a 0.72-cM, 636.4-kb interval spanned by *YrZM175-InD1* and *YrZM175-InD2* (763,452,916–764,089,317 bp) including two putative disease resistance genes based on IWGSC RefSeq v1.0. Collinearity analysis indicated similar target genomic intervals in Chinese Spring, *Aegilops tauschii* (2D: 647.7–650.5 Mb), *Triticum urartu* (2A: 750.7–752.3 Mb), *Triticum dicoccoides* (2A: 771.0–774.5 Mb), *Triticum turgidum* (2B: 784.7–788.2 Mb), and *T. aestivum* cv. Aikang 58 (2A: 776.3–778.9 Mb) and Jagger (2A: 789.3–791.7 Mb). Through collinearity analysis, sequence alignments of resistant and susceptible parents and gene expression level analysis, we predicted *TRITD2Bv1G264480* from *Triticum turgidum* to be a candidate gene for map-based cloning of *YrZM175*. A gene-specific marker for *TRITD2Bv1G264480* co-segregated with the resistance gene. Molecular marker analysis and stripe rust response data revealed that *YrZM175* was different from genes *Yr1*, *Yr17*, *Yr32* and *YrJ22* located on chromosome 2A. Fine mapping of *YrZM175* lays a solid foundation for functional gene analysis and marker-assisted selection for improved stripe rust resistance in wheat.

Key Message

A stripe rust resistance gene *YrZM175* in Chinese wheat cultivar Zhongmai 175 was mapped to a genomic interval of 636.4 kb on chromosome arm 2AL, and a candidate gene was predicted.

Introduction

Wheat (*Triticum aestivum* L.) is a staple food crop worldwide, providing substantial amounts of nutrients for humans (Shewry 2009; Shewry and Hey 2015). The estimated global wheat production in 2021 was 770 million tonnes (FAO, <http://www.fao.org/worldfoodsituation/en/>). Wheat stripe rust (or yellow rust), caused by *Puccinia striiformis* f. sp. *tritici* (PST), is a widely distributed disease that causes millions of tonnes of yield losses every year (Chen and Kang 2017). Stripe rust occurred in 1.78 million ha of wheat in China during 2011–2016 and has since increased to about 5 million ha annually (Huang et al. 2020). Resistant cultivars are the most environment-friendly approach to control stripe rust (Xia et al. 2007). Although many resistance genes have been identified, relatively few of them, such as *Yr5*, *Yr15* and *Yr18*, continue to confer resistance in China. Therefore, it is necessary to mine new stripe rust resistance genes and to deploy them in wheat cultivars, preferably in multiple gene combinations.

More than 80 stripe rust resistance genes (*Yr1–Yr83*) have been formally catalogued (McIntosh et al. 2017; Feng et al. 2018; Nsabiya et al. 2018; Gessese et al. 2019; Pakeerathan et al. 2019; Li et al. 2020). Among formally catalogued and other named genes nine have been cloned (*Yr5/YrSP*, *Yr7*, *Yr10*, *Yr15*, *Yr18*, *Yr36*, *Yr46*, *YrAS2388* and *YrU1*). *Yr18*, *Yr36* and *Yr46* confer adult-plant resistance and encode a putative ATP-binding cassette (ABC) transporter (Krattinger et al. 2009), a protein with a kinase domain and a lipid binding domain (Fu et al. 2009), and a hexose transporter (Moore et al. 2015), respectively. *Yr5/YrSP* (alleles), *Yr7*, *Yr10*, *Yr15*, *YrAS2388* and *YrU1* are all-stage resistance genes. *Yr15* encodes a protein with kinase-pseudokinase domains, and the others encode various nucleotide-binding site and leucine-rich repeat (NBS-LRR) proteins. *Yr5/YrSP* and *Yr7* belong to a gene cluster originated from *Triticum spelta* (Marchal et al. 2018); *YrAS2388* was come from *Aegilops tauschii* (Liu et al. 2014, Zhang et al. 2019); *Yr15* was derived from *Triticum dicoccoides* (Klymiuk et al. 2018) and *YrU1* was from *Triticum urartu* (Wang et al. 2020).

Wheat cultivar Zhongmai 175 is widely grown in the North Winter Wheat Zone of China; it has high yield and good resistance to stripe rust. It also shows moderate to high resistance in Sichuan and Gansu provinces, the hotspots for stripe rust epidemics and year-round survival in China. One of the parents of Zhongmai 175 was the French line VPM1 which had high resistance to powdery mildew, stem rust, leaf rust and stripe rust, and was widely used as a disease resistant germplasm by breeders. Zhongmai 175 carries *Yr17* from VPM1 and a putatively new stripe rust resistance gene *YrZM175* that was located in chromosome 2A (Lu et al. 2016). The aims of this study were to fine-map *YrZM175*, predict a candidate gene for further analysis, and to develop gene-specific markers for research and breeding.

Materials And Methods

Plant materials

A total of 7,325 F₂ plants derived from the cross of Avocet S/Zhongmai 175 were used for fine mapping of *YrZM175*. One hundred and seventeen random F₂:₃ lines were evaluated for stripe rust reaction in the greenhouse and field to confirm the results from the F₂ population.

Evaluation of stripe rust reaction

Fifteen F₁, 7,325 F₂ plants and 117 F₂:₃ lines of Avocet S/Zhongmai 175 and the parents were inoculated at the seedling stage in controlled greenhouse conditions with PST race CYR32, kindly provided by Dr. Gangming Zhan (Northwest A & F University, Yangling, Shaanxi). CYR32 is avirulent to Zhongmai 175 and virulent to Avocet S. Ten to 15 seeds were planted in a 9 × 9 × 9 cm plastic pot, along with three seeds of susceptible cultivar Mingxian 169 as control. Seedlings were inoculated with CYR32 at 1.5-leaf stage (7–8 days old) by brushing fresh urediniospores from fully infected Mingxian 169 leaves. Inoculated plants were kept in plastic boxes with 100% humidity at 10 °C in darkness for 24 h, then transferred to 15 ± 2 °C conditions with 16 h of light (20,000 lux) daily. Infection types (ITs) were scored 14–18 days after inoculation based on a 0–4 scale (0–2 as resistant, 3–4 as susceptible) (Bariana and McIntosh 1993).

Fifteen F1 plants and 117 F2:3 lines along with both parents with 30–40 seedlings each were also evaluated for stripe rust reaction at Pidu Experimental Station, Sichuan Academy of Agricultural Sciences in Chengdu, in the 2017–2018 and 2018–2019 cropping seasons. F2:3 lines were sown in two replications. Avocet S was used as a susceptible control and spreader, inoculated with mixed PST races (CYR32, CYR33, and CYR34). Infection types (ITs) and disease severity (DS) were recorded twice at 4- to 5-day intervals when the DS of Avocet S reached 90–100%. DS was scored as the percentage of infected leaf area. ITs were scored similarly to those at seedling stage mentioned above.

DNA and RNA extraction

Leaf samples of plants were used to extract genomic DNA using the CTAB method (Saghai-Maroo et al. 1984). For F2:3 lines, equal amounts of leaf tissue from 8–10 plants of each line were mixed for DNA extraction. Because of large genome differences between Zhongmai 175 and Avocet S and the absence of some genes in the target region in Avocet S, we later chose Mingxian 169 as a susceptible control for RNA isolation. Fresh leaf tissues of Zhongmai 175 and Mingxian 169 inoculated with CYR32 were collected before inoculation (0 h) and at 2, 4, 6, 8, 10, 12, 24 h post inoculation (hpi), and at 10 a.m daily in 2 to 14 days post inoculation (dpi) with three biological replications for RNA extraction using the Plant RNA Kit (Omega Biotek, Inc. Norcross, Georgia USA).

BSR-seq assay

Based on phenotypic evaluations at 14 dpi, 50 resistant (IT = 0;) and 50 susceptible plants (IT = 4) were separately mixed to form resistant (R) and susceptible (S) pools for RNA extraction and sequencing. The pooled leaf samples and parents were sent to Novogene (Tianjin, China) for RNA-Seq. After quality testing, single RNA libraries were constructed for each sample and sequenced in an Illumina HiSeq4000 platform, generating 150 bp paired-end reads. BWA (Burrows-Wheeler Aligner) (Li et al. 2009) was used to align the clean reads of each sample against the reference genome of Chinese Spring, *Aegilops tauschii* ssp. *stragulata* accession AL8/78 (Luo et al. 2017), *Triticum urartu* accession G1812 (Ling et al. 2018), *Triticum turgidum* ssp. *dicoccoides* accession Zavitan (Avni et al. 2017), *Triticum turgidum* ssp. *durum* cv. Svevo (Maccaferri et al. 2019), and *Triticum aestivum* cv. Aikang 58 and Jagger (Walkowiak et al. 2020) (<https://www.ncbi.nlm.nih.gov/assembly/?term=triticeae>), respectively, after a series of quality control (QC) procedures. SNPs/InDels calling between the two pools was performed using the Unified Genotyper function in GATK (McKenna et al. 2010). The homozygous SNPs/InDels were extracted to calculate the SNP/InDel index. The sliding window method was used to present the SNP/InDel index of polymorphic sites in whole genome. The differences in SNP/InDel indices between the two pools were calculated as the delta SNP/InDel index to determine the target region of the resistance gene. SNPs/InDels with delta SNP/InDel index of 1 or -1 are considered highly reliable.

Marker development through BSR-seq and genome mining

Highly reliable SNPs from BSR-seq analyses were converted into cleaved amplified polymorphic sequence (CAPS) markers following Thiel et al. (2004) based on their 700–1,000 bp flanking sequences

identified in the Chinese Spring reference genome RefSeq v1.0 (IWGSC 2018) and the six other genome sequences mentioned above. Chromosome-specific primers for each SNP were designed with DNAMAN 8.0 software. Due to the limited number of SNPs from BSR-seq, more variations between the parents were identified from gene sequences in the target region in reference to the RefSeq v1.0 (IWGSC 2018). According to gene annotations from RefSeq v1.0 (IWGSC 2018) and the six other genome sequences, gene-specific primers were designed from putative disease resistance genes in the target scaffolds to isolate gene sequences in the parents. PCR sequencing results for the parents were aligned to identify the polymorphic SNPs/InDels using Geneious Prime 2019.0.3 software. InDels between the parents were converted to sequence-tagged site (STS) markers, whereas CAPS markers were developed from SNPs.

Genetic map construction

Chi-squared (χ^2) tests were conducted to determine the goodness-of-fit of the observed and expected ratios of segregation in the F_2 and $F_{2:3}$ populations using SAS 9.2 (SAS Institute, Cary, NC, USA). All susceptible F_2 plants were tested by molecular markers in the target region for linkage analysis with the resistance gene. Genetic distances between polymorphic SNP, STS and SSR markers linked to the stripe rust resistance gene were calculated using the Kosambi mapping function (Kosambi 1943) computed by Mapmaker 3.0 with an LOD threshold of 3.0 (Lincoln et al. 1993). The linkage map was graphically drawn with MapDraw V2.1 (Liu and Meng 2003).

Collinearity analysis of homologous sequences

Collinear alignment of genes and homoeologous intervals was performed using TGT website (<http://wheat.cau.edu.cn/TGT/>) by blasting markers flanking *YrZM175* to obtain physical positions of homologous sequences in related species in *Triticum* multi-omics center databases on WheatOmics 1.0 (<http://wheatomics.sdau.edu.cn/>) and to confirm corresponding homoeologous intervals. Gene annotations within homoeologous intervals were searched and downloaded in EnsemblPlants (<http://plants.ensembl.org/index.html>) and related disease resistance genes were checked.

Genome walking to get sequences of candidate genes

Genome walking is an effective technique to obtain unknown sequences adjacent to known sequences. To find the physical position of the start point with different sequences in the target region between Zhongmai 175 and Chinese Spring to perform genome walking, we designed chromosome-specific primers at 763.83 Mb upstream of *TraesCS2A02G562800* in Chinese Spring RefSeq v1.0 to perform PCR with Zhongmai 175 and Chinese Spring as templates. If sequences of PCR products were common between Zhongmai 175 and Chinese Spring we continued to design primers to amplify downstream sequences from 763.83 Mb until sequences of PCR products showed polymorphisms between Zhongmai 175 and Chinese Spring. Then the position of the polymorphic sequence was set as the start point for genome walking using the Genome Walking Kit (TaKaRa, Dalian). Walking primer designing and PCR amplifications were performed following the manufacturer's protocol. Purified PCR products were ligated with pEASY-T5 Blunt-Zero cloning vectors at 25°C for 2 h and transformed into Trans1-T1 competent

cells by the heat shock (TransGen Biotech Co., Ltd., Beijing). After coating the plate and incubating at 37°C for 24 h, 6–10 positive clones from each PCR product were randomly selected and sequenced by Shanghai Sangon Biotech Co., Ltd (<http://www.sangon.com/>).

Quantitative real-time PCR

First-strand cDNA was synthesized using the PrimeScript RT Reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan). The cDNA products of Zhongmai 175 and Mingxian 169 were used for quantitative real-time PCR (qPCR) assays in a BioRad CFX system with the iTaq Universal SYBR Green Supermix (BioRad). Gene-specific primers for candidate genes and the *ACTIN* gene as internal control were designed using DNAMAN 8.0 software. Each sample had three biological replicates. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ equation (Schmittgen and Livak 2008).

Results

Stripe rust resistance of Zhongmai 175 at seedling stage

Zhongmai 175 confers resistance to a broad spectrum of PST races (Lu et al. 2016) and displays high resistance at all growth stages (Fig. S1). Among 7,325 F_2 plants derived from Avocet S/Zhongmai 175, 5,532 and 1,793 plants were resistant and susceptible, respectively, conforming with a 3:1 ratio ($\chi^2 = 1.06$, $P = 0.30$). The $F_{2:3}$ lines segregated 33 homozygous resistant, 56 segregating and 28 homozygous susceptible, conforming with a 1:2:1 ratio ($\chi^2 = 0.35$, $P = 0.84$) (Table 1). Thus, the stripe rust resistance in Zhongmai 175 was conferred by a single dominant gene.

Table 1
Seedling reactions of Zhongmai 175, Avocet S, F₁, F₂ plants and F_{2:3} lines from the cross of Avocet S/
Zhongmai 175 to PST race CYR32

Material	Total no.	Infection type						Expected ratio	χ^2	P
		0	0;	1	2	3	4			
Zhongmai 175	15	11	4							
Avocet S	12					3	9			
F ₁	15	10	5							
F ₂	7,325	1,277	3,817	283	155	774	1,019	3:1	1.06	0.30
		RR ^a		Rr ^b		rr ^c				
F _{2:3}	117	33		56		28		1:2:1	0.64	0.73
^a homozygous resistant										
^b segregating										
^c homozygous susceptible										

Fine mapping of YrZM175

BSR-seq analysis revealed 13 highly reliable SNPs in the genomic region 762.50–768.52 Mb on chromosome arm 2AL based on the Chinese Spring RefSeq v1.0 (IWGSC, 2018) (Figs. 1A and S2). We initially screened 35 simple sequence repeat (SSR) markers on chromosome 2A and identified nine markers polymorphic between the parents; among them, *Xwmc658*, *Xgwm382* and *Xgwm311* on 2AL were linked to *YrZM175* with genetic distances of 6.51 cM, 5.12 cM and 5.12 cM, respectively (Figs. 1B and S3). The physical positions of *Xwmc658* and *Xgwm382* (*Xgwm311*) are at 771.16 Mb and 772.96 Mb, respectively, in agreement with the results of BSR-seq. The 13 polymorphic SNPs were mainly concentrated in two physical positions, i.e., four SNPs at 762.50–762.64 Mb and six around 768.52 Mb on 2AL (Table S1). We developed CAPS markers *YrZM175-CAPS1* and *YrZM175-CAPS2* (Table S2), based on a SNP at 762.50 Mb and another at 768.52 Mb, with genetic distances of 0.81 cM and 2.57 cM from *YrZM175*, respectively.

Many polymorphic SNPs/InDels between parents were identified by sequencing genes in the target region and were converted to CAPS or STS markers (Table S2). *YrZM175* was then mapped in a genetic interval of 1.41 cM between *YrZM175-CAPS1* and *YrZM175-InD1*, spanning a 1.58 Mb (762.50–764.08 Mb) genomic region containing 35 high-confidence genes, including six putative disease resistance genes (Table S3) based on IWGSC RefSeq v1.0. After designing gene-specific PCR primers (Table S4) and sequencing six putative resistance genes in Zhongmai 175, Avocet S and Mingxian 169,

TraesCS2A02G560600 showed a SNP between Zhongmai 175 and Mingxian 169 in the CDS domain, but the gene was absent in Avocet S. During genome walking and mining, we identified two polymorphic sites and converted them to markers *YrZM175-CAPS5* and *YrZM175-InD2* (Fig. S3) with genetic distances of 0.27 cM and 0.12 cM from the resistance gene, respectively. Finally, we mapped *YrZM175* in a genetic interval of 0.72 cM between *YrZM175-InD1* and *YrZM175-InD2*, spanning a 636.4 kb (763,452,916–764,089,317 bp) on chromosome arm 2AL (Figs. 1B and 2A). There were nine high-confidence genes in this genomic region based on IWGSC RefSeq v1.0, including putative disease resistance genes *TraesCS2A02G562800* and *TraesCS2A02G563200*.

Analysis of genes in the 636.4 kb interval of Chinese Spring

The NBS-LRR gene *TraesCS2A02G563200* showed no difference between Zhongmai 175, Avocet S and Mingxian 169, indicating that it was not a gene candidate. No PCR product was obtained following amplification of kinase gene *TraesCS2A02G562800* in all three genotypes, exhibiting that this gene also was not a candidate. The genome sequence was quite different or absent in the three cultivars compared with Chinese Spring (Fig. 2B). After two cycles of genome walking from 763.83 Mb upstream of *TraesCS2A02G562800* we obtained a 3,860-bp genome sequence from Zhongmai 175. Blasting of this sequence against the *Triticum* genome databases in WheatOmics 1.0 we found that this sequence was identical with a receptor-like kinase gene *TRITD2Bv1G265970* on chromosome arm 2BL in *Triticum turgidum* ssp. *durum* cv. Svevo (Maccaferri et al. 2019). Isolation of the entire 7,739-bp open-reading frame (ORF) of *TRITD2Bv1G265970* in Zhongmai 175, Mingxian 169 and Avocet S with gene-specific primers (Table S5) according to the reference genome of Svevo found no sequence difference between Zhongmai 175 and Avocet S in the ORF domain, whereas the sequence of the ORF in Mingxian 169 was different from that in Zhongmai 175. Sequencing following amplification of a 3,000-bp promoter sequence upstream of the ATG start codon by genomic-specific primers (Table S6) referring to Svevo revealed three SNPs, single 1-bp and 11-bp insertions, and single 9-bp, 11-bp and 23-bp deletions in Avocet S compared with Zhongmai 175 (Fig. S4). Annotation of the promoter sequence of Zhongmai 175 on database PLACE (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) indicated that the SNPs/InDels could change several important cis-acting regulators such as ABA responsive element (ABRE), carbohydrate metabolite signal responsive element 1 (CMSRE-1) and salicylic acid regulatory element GT-1 motif. However, tests of all susceptible F₂ plants with a gene-specific marker developed from a polymorphic site between Zhongmai 175 and Avocet S in the promoter, and the marker located 0.21 cM from the resistance gene failed to show an association. We concluded that *TRITD2Bv1G265970* was not the gene candidate for *YrZM175*.

Collinearity analysis of the target genomic interval

Based on *Triticum* genome databases (*Aegilops tauschii*, *Triticum urartu*, *Triticum dicoccoides*, *Triticum turgidum* and 10+ Genomes) at TGT website, we performed collinearity analysis of the closest marker *YrZM175-InD2* and the targeted genomic region according to the genome sequence of Chinese Spring RefSeq v1.0 (IWGSC, 2018). The 636.4-kb mapping interval showed a good collinearity with 10+ Genome varieties, whereas most of the wild relatives had inversions, duplications and deletions of genes

compared with Chinese Spring (Fig. S5). The genomic interval flanked by *YrZM175-CAPS5* and *YrZM175-InD1* in Chinese Spring RefSeq v1.0 was similar to corresponding intervals in *Ae. tauschii* ssp. *stragulata* accession AL8/78 (2D: 647.7–650.5 Mb), *T. urartu* accession G1812 (2A: 750.7–752.3 Mb), *T. turgidum* ssp. *dicoccoides* accession Zavitan (2A: 771.0–774.5 Mb), *T. turgidum* ssp. *durum* cv. Svevo (2A: 773.2–775.1 Mb), *T. turgidum* ssp. *durum* cv. Svevo (2B: 784.7–788.2 Mb), and *T. aestivum* cv. Aikang 58 (2A: 776.3–778.9 Mb) and Jagger (2A: 789.3–791.7 Mb), including eight, five, twelve, eight, eleven, three and four putative disease resistance genes in the target intervals, respectively (Fig. S6).

Analysis of genes in the collinear interval of other wheat varieties and related species

Based on BSR-seq analysis using genome sequence information from five species (*Ae. tauschii*, *T. urartu*, *T. dicoccoides*, *T. turgidum*, and *T. aestivum* cv. Aikang 58 and Jagger), there was an obvious single peak on homoeologous group 2 chromosomes in all species (Fig. S7). Based on two polymorphic SNPs between R and S pools from BSR-seq analysis in the collinear interval on chromosome 2AL referring *Triticum dicoccoides* and *Triticum turgidum* genome sequences, respectively, molecular markers were developed and tested on susceptible F₂ plants from cross Avocet S/Zhongmai 175, and the linkage distances of two SNPs with *YrZM175* were 0.12 cM. The results confirmed sequence similarity between Chinese Spring, *T. dicoccoides* and *T. turgidum*. Based on collinearity and homology analysis of genes in the collinear interval at website TGT, there were ten putative disease resistance genes in the region upstream of marker *YrZM175-InD2* on chromosomes 2A or 2D based on four *Triticum* reference genomes (*T. turgidum*, *T. dicoccoides*, *T. urartu*, and *Ae. tauschii*), whereas these genes were absent in Chinese Spring (Fig. 3). Amplifying and sequencing all ten genes (*TRITD2Av1G295090*, *TRITD2Av1G295160*, *TRITD2Av1G295250*, *TRIDC2AG082340*, *TRIDC2AG082360*, *TRIDC2AG082430*, *TuG1812G0200006348*, *TuG1812G0200006350*, *AET2Gv21292200*, *AET2Gv21292500*) in Zhongmai 175 and Avocet S indicated that these genes were also not present in Zhongmai 175 and Avocet S. Therefore, we inferred that Zhongmai 175 does not contain these gene sequences on chromosome arm 2AL.

A putative disease-resistance-like gene isolated in the target interval of chromosome arm 2AL in Zhongmai 175 by genome walking was almost the same (with two SNPs in the intron) as a kinase gene *TRITD2Bv1G265970* on chromosome arm 2BL in *T. turgidum*. This indicated that chromosome arm 2AL in Zhongmai 175 might carry genes originating from chromosome arm 2BL in *T. turgidum*. Analysis of 11 putative disease resistance genes (Table S7) in the collinear interval of 2BL in *T. turgidum* in Zhongmai 175 and Mingxian 169 detected differential expression of *TRITD2Bv1G264470* and *TRITD2Bv1G264480*. The expression levels of *TRITD2Bv1G264470* were significantly higher at 5–14 dpi than at 0 dpi in both cultivars, with higher expression in Mingxian 169 (Fig. 4A). The expression of *TRITD2Bv1G264480* was first observed in Zhongmai 175 at 2 hpi, then significantly increased at 1–14 dpi, with constantly higher expression level than in Mingxian 169 (Fig. 4B). *TRITD2Bv1G264470* and *TRITD2Bv1G264480* are NBS-LRR genes. Amplifying parental lines with gene-specific primers (Table S8) indicated no sequence difference at CDS domain of *TRITD2Bv1G264470* between Zhongmai 175 and Mingxian 169 or Avocet S. The ORF sequence of *TRITD2Bv1G264480* was 23,559 bp with a 5,661-bp CDS domain in *T. turgidum*.

The homoeologous CDS sequences of *TRITD2Bv1G264480* were amplified in the target interval on chromosome arm 2AL in Zhongmai 175, Mingxian 169 and Avocet S (Fig. S8). Based on one SNP at CDS domain between Zhongmai 175 and Avocet S, we developed a molecular marker *CAPS6* (Table S2) and tested all susceptible F₂ plants. *CAPS6* co-segregated with the resistance gene. Therefore, we considered that *TRITD2Bv1G264480* was a gene candidate for *YrZM175*.

Discussion

Identification of candidate genes for *YrZM175*

After anchoring the genomic interval of *YrZM175* according to the Chinese Spring genome sequence we found that the target region contained a cluster of putative resistance genes. We cloned six putative disease resistance genes in this interval; four had no sequence difference between Zhongmai 175 and Avocet S or Mingxian 169; NLR gene *TraesCS2A02G560600* had a SNP in exon 2 between Zhongmai 175 and Mingxian 169, and no PCR product for kinase gene *TraesCS2A02G562800* was obtained from Zhongmai 175 and Avocet S or Mingxian 169. We performed transgenic assays of *TraesCS2A02G560600* using two transgenic vectors; one was the CDS domain driven by Ubi promoter and another was the 6,251-bp ORF (including coding and intron region) with a 2,770-bp presumed native promoter and 1,500-bp terminator (3'-UTR). All the positive T₁ plants were susceptible to race CYR32 in seedling stage tests, indicating that *TraesCS2A02G560600* was not a gene candidate for *YrZM175*. We amplified five and two genes upstream and downstream, respectively, of *TraesCS2A02G562800* according to the Chinese Spring genome and again failed to obtain PCR products, indicating great sequence differences of this genomic region between Zhongmai 175 and the Chinese Spring reference genome. With the release of reference genomes such as *T. turgidum* ssp. *durum* cv. Svevo (Maccaferri et al. 2019), *T. aestivum* cv. Zang1817 (Guo et al. 2020), 10 + Genome (Walkowiak et al. 2020), and *T. aestivum* cv. Fielder (Sato et al. 2021) we were able to extend our investigation. Since *TraesCS2A02G562800* was the only undetermined putative resistance gene in the 636.4-kb target interval on chromosome arm 2AL of Zhongmai 175 according to the Chinese Spring genome, we performed genome walking to obtain the unknown sequence corresponding to *TraesCS2A02G562800* in Zhongmai 175. After sequencing and blasting, we found that products corresponding to *TraesCS2A02G562800* on 2AL in Zhongmai 175 were almost the same as a genome sequence (*TRITD2Bv1G265970*) on chromosome arm 2BL of durum wheat Svevo. Tests of relative *TRITD2Bv1G265970* expression in infected leaf samples of Zhongmai 175 and Mingxian 169 at 0–7 dpi showed that relative expression of *TRITD2Bv1G265970* in Zhongmai 175 was 3.8-fold higher than in Mingxian 169 at 1 dpi. However, the linkage distance between *TRITD2Bv1G265970* and the resistance gene was 0.21 cM, indicating that *TRITD2Bv1G265970* was not the gene candidate for *YrZM175*.

We amplified and sequenced ten putative disease resistance genes in the collinear chromosome arm 2AL interval in five *Triticum* genomes in Zhongmai 175 and obtained no candidate gene sequences. Finally, we assessed *TRITD2Bv1G264480* on chromosome arm 2BL in *T. turgidum* as a candidate gene based on a co-segregating marker, sequence alignment of resistant and susceptible parents and gene expression

patterns. Our research indicated that Zhongmai 175 has complicated genetic origins that caused difficulties in mapping the target genes according to single reference genome. In addition to the Chinese Spring genome sequence, it is also very necessary to refer other available genome sequences of wheat and related species for fine mapping and cloning of disease resistance genes in bread wheat.

Candidate gene TRITD2Bv1G264480

The putative resistance gene *TRITD2Bv1G264480* encodes an NBS-LRR protein that belongs to two overlapping homologous superfamilies: P-loop containing nucleoside triphosphate hydrolase, and apoptotic protease-activating factors (helical domain) (<http://www.ebi.ac.uk/interpro/entry/InterPro/IPR002182/>). The predicted protein of *TRITD2Bv1G264480* is closest to *TRIDC2BG090350* on chromosome 2B from *T. dicoccoides* (Fig. S9). The paralogous genes on chromosome 2A in genomes of seven bread wheat varieties clustered together phylogenetically (Fig. S9), with 89% and 66% sequence similarities in the CDS domain to *TRITD2Bv1G264480* on chromosome 2B and the paralogous gene *TRITD2Av1G294930* on chromosome 2A in *T. turgidum*, respectively, indicating much closer phylogenetic relationship with *TRITD2Bv1G264480* from chromosome 2B in *T. turgidum*, whereas the orthologous genes on chromosome 2B in *T. aestivum* cv. CDC Landmark, cv. CDC Stanley, cv. Fielder and cv. Julius showed 67.4% similarity with *TRITD2Bv1G264480*. In addition, paralogous genes on chromosomes 2A in *T. Urartu* and 2D in *Ae. tauschii* and orthologous genes on chromosome 2B in *T. spelta* also had quite distant phylogenetic relationships with *TRITD2Bv1G264480* (Fig. S9). Therefore, we inferred *TRITD2Bv1G264480* was transferred from chromosome 2B to 2A during evolution from an old crossover event of bread wheat. The CDS domain of candidate gene in Zhongmai 175 showed 97.8% and 96.8% similarities with *TRITD2Bv1G264480* (*T. turgidum*) and *TRIDC2BG090350* (*T. dicoccoides*), respectively, whereas the sequence similarities with homologues genes on chromosome 2A in other bread wheat varieties (Zang1817, Jagger, Fielder, Mace, CDC Landmark and Norin 61) were relatively low (all below 88.3%), indicating multiple allelic variations or haplotypes of the candidate gene present in bread wheat during evolution.

The relationship between YrZM175 and other Yr genes on chromosome 2A

Zhongmai 175 has been widely grown in the North Winter Wheat Zone of China since 2008. It was derived from the cross between Jing 411 and BPM27 (Fig. S10). BPM27 is a disease-resistant line developed by Prof. Zuomin Yang at China Agricultural University in the 1980s; one of its parents was VPM1, a French line derived from crosses of *Ae. ventricosa*, *T. turgidum* L. var. *carthlicum* (*T. persicum*) and common wheat cv. Marne (Fig. S10). The 2NS translocation fragment with *Yr17* in Zhongmai 175 came from *Ae. ventricosa*.

Lu et al. (2016) mapped *YrZM175* on chromosome arm 2AS using 344 F₂ plants and 147 F_{2:3} lines derived from cross Lunxuan 987/Zhongmai 175 tested with CYR29. The present study used many more F₂ plants from a cross of Avocet S and Zhongmai 175 but employed race CYR32. The discrepancy of gene locations can be attributed to different mapping populations, molecular markers and PST races

used in the two studies. Unfortunately, the PST race CYR29 used previously (Lu et al. 2016) was no longer available to test the present population, thus we are not very sure whether the resistance genes are the same or not. The race CYR32 is virulent to VPM1 (*Yr17*) (Table S9). Screening of the 1,793 susceptible F₂ plants in the Avocet S/Zhongmai 175 population with the marker URIC/LN2 (Helguera et al. 2003) as proxy for *Yr17* indicated linkage of 55.2 cM. *YrZM175* is located at the distal end (763,452,916–764,089,317 bp) of chromosome arm 2AL, whereas *Yr17* is located at the distal (25.0–38.0 cM linkage interval) of chromosome arm 2AS (Cruz et al. 2016) (Fig. S11). Lu et al. (2016) performed multi-pathotype test on VPM1 (*Yr17*) and Zhongmai 175 (*Yr17* and *YrZM175*), the two lines showed different reactions to 7 PST races at the seedling stage (Table S9). A test with the marker URIC/LN2 in 92 susceptible F₂ plants indicated that the linkage distance between *Yr17* and *YrZM175* was 39.0 cM (Lu et al. 2016), being similar to the present study.

To date, three *Yr* genes have been mapped on chromosome arm 2AL, i.e., *Yr1*, *Yr32* and *YrJ22* (Lupton and Macer 1962; Eriksen et al. 2004; Chen et al. 2016). *Yr1* is linked to *Xgwm382* (Fig. 5B), with a genetic distance of 5.6 cM (Zheng et al. 2017; Bansal et al. 2009). *Xgwm382* (at 772.96 Mb) was tested on all 1,793 susceptible F₂ plants from Avocet S/Zhongmai 175; it was linked to *YrZM175* (763,452,916–764,089,317 bp) with a genetic distance of 5.1 cM (Fig. 5C). The EST-SSR marker *BU099658* (763.69 Mb) may accurately show the presence of *Yr1* (Hasancebi et al. 2014), which locates at almost the same physical position as *YrZM175*. Because Zhongmai 175 and Chinese 166 have different pedigrees and different responses to PST races (Lu et al. 2016; Chen et al. 2016) (Table S9), we believe *YrZM175* and *Yr1* are different. Based on the linkage maps and physical positions of linked markers, the physical position of *YrZM175* is close to *Yr1*, possibly indicating allelism. To clarify their relationship, tests of allelism and gene cloning should be carried out. *Xgwm382* is also closely linked to *YrJ22* with a genetic distance of 2.5 cM (Fig. 5D) (Chen et al. 2016). The physical position of *YrJ22* is at 768.0–769.0 Mb on 2AL (Personal communication with Dr. Can Chen), a 5 Mb physical distance from *YrZM175*. As Jimai 22 and Zhongmai 175 have different pedigrees and different arrays of reactions to PST races (Table S9) we assume that *YrZM175* and *YrJ22* are different genes or alleles.

Eriksen et al. (2004) found that the marker *Xcdo678* linked with *Yr32* in a genetic distance of 35 cM, while *Xcdo678* is co-segregating with *Pm4* (Ma et al. 1994). *Pm4* and *Yr1* are closely linked to each other with a genetic distance of 2.0 cM (McIntosh and Arts 1996). As a result, the genetic distance between *Yr32* and *Yr1* is about 33 cM (Fig. 5A) (Yang et al. 2019), indicating that *Yr32* is far away from *YrZM175* and *Yr1*. Based on linkage maps and molecular marker analyses we conclude that *YrZM175* is different from *Yr1*, *Yr17*, *Yr32* and *YrJ22*.

Complexity Of The Distal Genome Region Of Chromosome 2al

Structural genomic variation such as presence-absence variations (PAVs) and copy number variations (CNVs) have been recognized to have the potential to generate phenotypic variation in maize (Springer et

al. 2009) and barley (Muñoz-Amatriaín et al. 2013). Megabase-scale PAVs of *Tripsacum* origin were confirmed to be under selection during maize domestication and adaptation (Huang et al. 2021). Rimbart et al. (2018) also found high frequencies of PAVs in the distal regions of wheat chromosomes. Akhunov et al. (2003) found that duplicated loci were most frequently located in the distal regions of chromosomes, and their distribution was positively correlated with recombination rate. The higher recombination rate indicates that these regions are fast-evolving in adapting to biotic and abiotic stresses. Besides, there are higher polymorphisms in the distal regions of chromosomes with the decreased levels of synteny between homoeologous chromosomes as distance from centromeres increase (Akhunov et al. 2003). Our experimental results on chromosome arm 2AL in Zhongmai 175 were in agreement with these related reports mentioned above; this is reflected by the result that the candidate gene *TRITD2Bv1G264480* for *YrZM175* likely originated from chromosome arm 2BL of *Triticum turgidum*. The distal region of chromosome has great structural genomic variations, therefore is easier to evolve new alleles that trigger phenotypic variation.

In conclusion, *YrZM175* is a stripe rust resistance gene that confers moderate to high resistance to stripe rust in the field. We fine mapped *YrZM175* and identified a candidate gene based on a high-resolution linkage map, collinearity analysis and gene expression analysis. Transgenic assays are ongoing to validate the functions of gene candidate for *YrZM175*. The identification and fine mapping of *YrZM175* provide options for deployment in combination with other effective resistance genes.

Declarations

Authors' contribution statement:

J. C. Wu performed the experiments and data analysis and wrote the paper.

D. A. Xu and L. P. Fu contributed to data analysis.

L. Wu participated in field trials.

J. H. Li participated in the transgenic assay.

W. H. Hao, Y. Dong, Y. Y. Wu contributed to scoring stripe rust in greenhouse.

F. J. Wang helped the population construction.

Z. H. He, H. Q. Si, C. X. Ma, and X. C. Xia designed the experiment and assisted in writing the paper.

All authors read and approved the final manuscript.

Acknowledgments

We thank Prof. R. A. McIntosh, Plant Breeding Institute, University of Sydney, for critical review of this manuscript. This work was funded by the National Natural Science Foundation of China (31971929,

31961143007), the National Key Research and Development Program of China (2016YFD0101802, 2016YFE0108600), and CAAS Science and Technology Innovation Program.

Conflict of interest:

We declare no conflicts of interest in regard to this manuscript.

Ethical Standards:

These experiments complied with the ethical standards in China.

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Figures

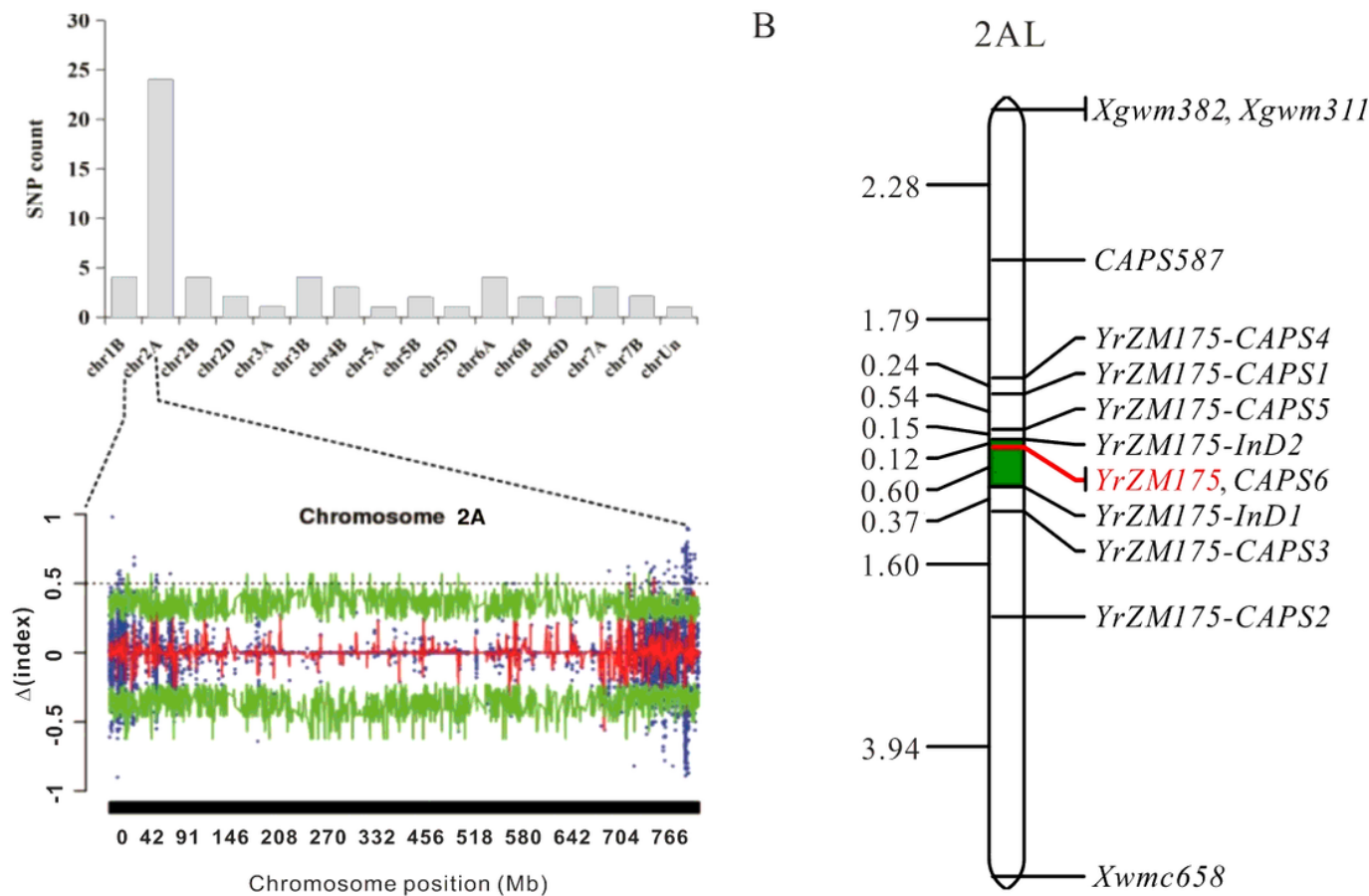


Figure 1

Distribution of SNPs and linkage map of *YrZM175*

A. Distribution of SNPs in each chromosome based on BSR-seq analysis and physical positions of SNPs on chromosome 2A. **B.** Linkage map of *YrZM175* determined from data of the Avocet S/Zhongmai 175 F_2 population. Marker names are displayed at the right. Genetic distance intervals (cM) are shown at the left. *CAPS6* co-segregated with *YrZM175*.

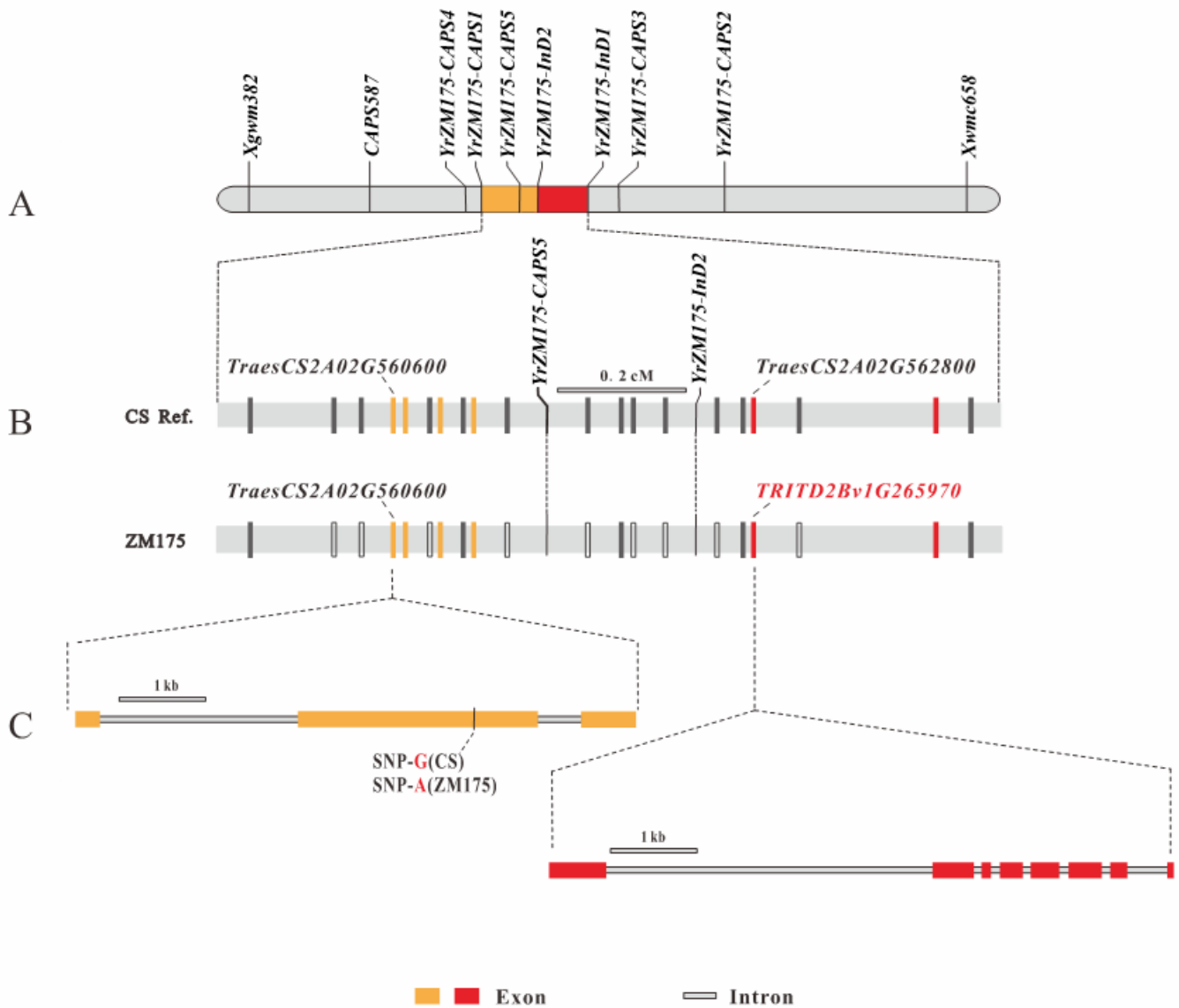


Figure 2

Physical map of chromosome arm 2AL segments harboring *YrZM175* in Zhongmai 175 and Chinese Spring

A. Linkage map of *YrZM175*. The 0.72 cM genetic interval containing *YrZM175* is highlighted in red and the adjacent 0.69 cM genetic interval is highlighted in orange. **B.** Schematic of genes within the genomic region of ZM175 and CS. Orange rectangles represent four putative disease resistance genes *TraesCS2A02G560600*, *TraesCS2A02G560700*, *TraesCS2A02G560900* and *TraesCS2A02G561100* in the adjacent 0.69 cM genetic interval. Only *TraesCS2A02G560600* showed difference; a SNP in the CDS domain between Zhongmai 175 and Mingxian 169. The red rectangles represent two putative disease resistance genes *TraesCS2A02G562800* and *TraesCS2A02G563200* in the 0.72 cM (636.4 kb region,

763.45–764.08 Mb) genetic interval. The gene in red corresponds to *TRITD2Bv1G265970* in *Triticum turgidum*. Hollow rectangles indicate genes that are absent in Zhongmai 175 relative to CS. **C.** Gene structures of *TraesCS2A02G560600* and *TRITD2Bv1G265970*. *TraesCS2A02G560600* has three exons and two introns. *TRITD2Bv1G265970* has eight exons and seven introns. The black bar in *TraesCS2A02G560600* represents the SNP between Zhongmai 175 (SNP-A) and Mingxian 169 or Chinese Spring (SNP-G). ZM175: Zhongmai 175; CS: Chinese Spring.

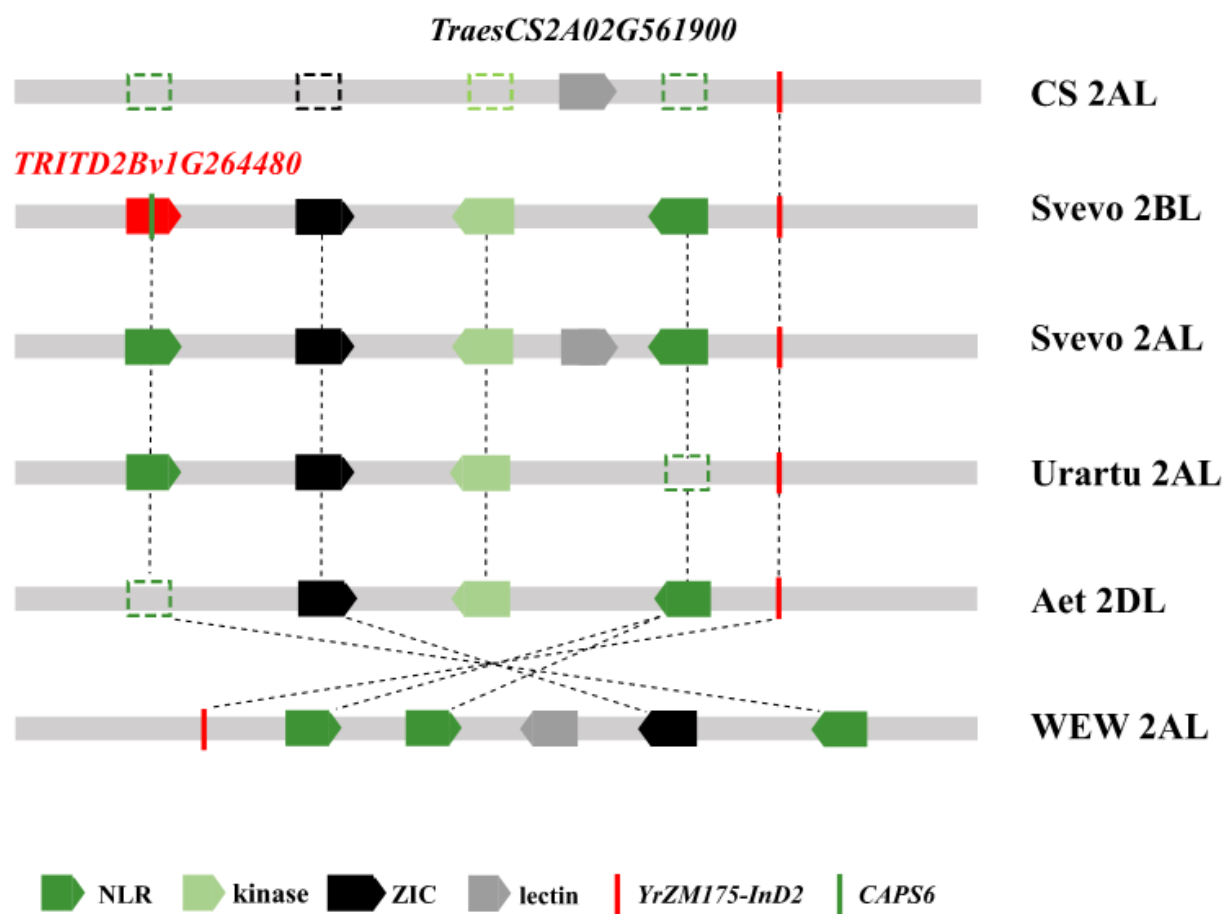


Figure 3

Collinearity of genes in the target genomic interval

Genes upstream of marker *YrZM175-InD2* (red bar) in genomic regions collinear with chromosome arm 2AL of Chinese Spring, 2AL of Svevo, 2BL of Svevo, 2AL of *T. urartu*, 2DL of *Ae. tauschii* and 2AL of *T. dicoccoides*. Dotted boxes represent genes that are absent in Chinese Spring, *T. urartu* and *Ae. tauschii*. *YrZM175-InD2* is close to gene *TraesCS2A02G561900* in Chinese Spring. The red box indicates candidate gene *TRITD2Bv1G264480* on chromosome 2BL of *T. turgidum*, and the green bar within red box indicates the co-segregating marker *CAPS6*. CS: Chinese Spring; Aet: *Ae. tauschii*; WEW: *T. dicoccoides*

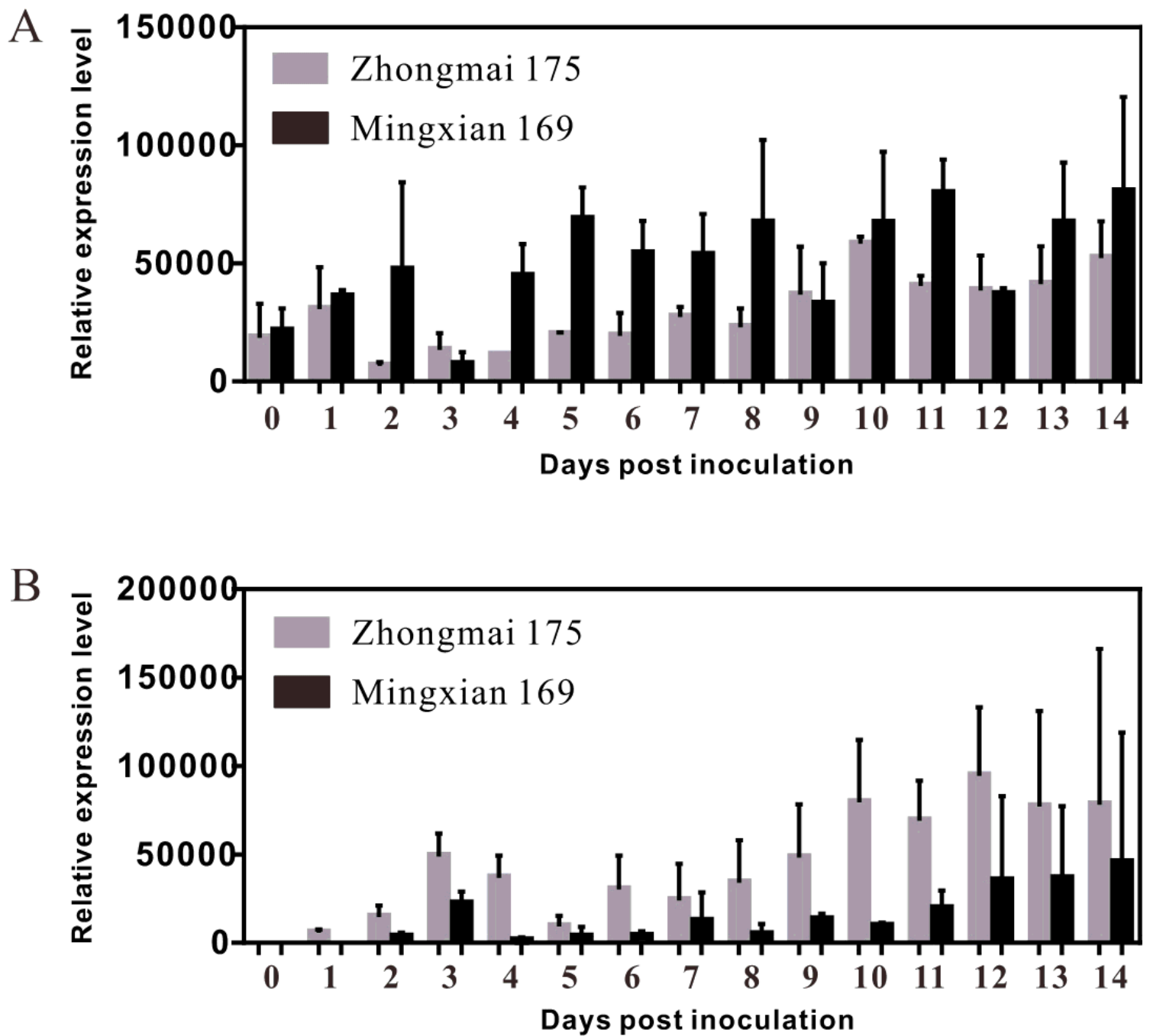


Figure 4

Relative expression levels of *TRITD2Bv1G264470* and *TRITD2Bv1G264480* in Zhongmai 175 and Mingxian 169 at 0–14 days post inoculation

A. Relative expression level of *TRITD2Bv1G264470* in Zhongmai 175 and Mingxian 169 at 0–14 dpi. **B.** Relative expression level of *TRITD2Bv1G264480* in Zhongmai 175 and Mingxian 169 at 0–14 dpi. dpi: days post inoculation.

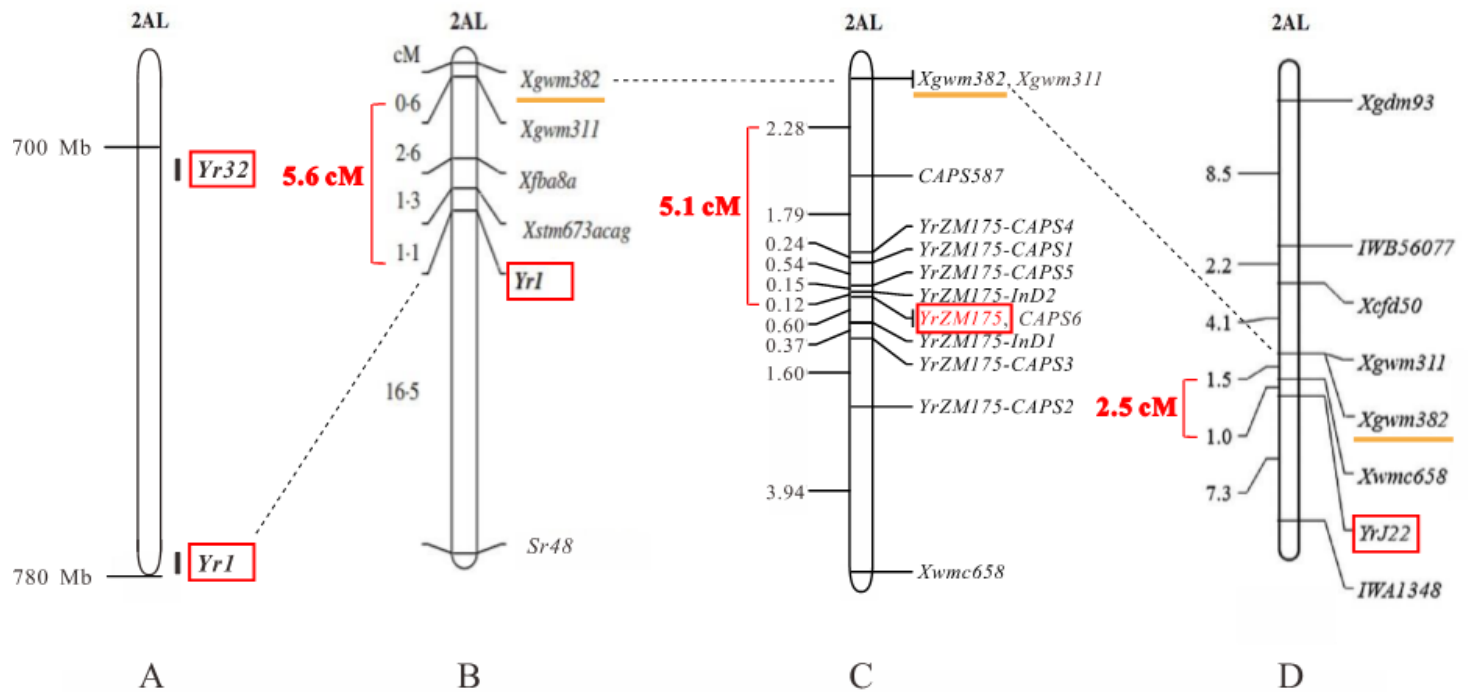


Figure 5

Relationship between *YrZM175* and *Yr1*, *YrJ22* or *Yr32*

A. Physical positions of *Yr32* and *Yr1* (Yang et al. 2019). **B.** Linkage map of *Yr1* (Bansal et al. 2009). **C.** Linkage map of *YrZM175* in the present study. **D.** Linkage map of *YrJ22* (Chen et al. 2016). The SSR marker *Xgwm382* linked to three genes *Yr1*, *YrZM175* and *YrJ22* is underlined in orange. The red lines and numbers indicate genetic distances between *Xgwm382* and *Yr1*, *YrZM175*, or *YrJ22*.

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