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ORIGINAL ARTICLE

Mapping and characterization of a novel adult-plant leaf rust resistance gene *LrYang16G216* via bulked segregant analysis and conventional linkage method

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Abstract

Key message **A novel adult-plant leaf rust resistance gene *LrYang16G216* on wheat chromosome 6BL was identified and mapped to a 0.59 cM genetic interval by BSA and conventional linkage method.**

Abstract Leaf rust (*Puccinia triticina*) is one of the most devastating fungal diseases of wheat (*Triticum aestivum* L.). Discovery and identification of new resistance genes is essential to develop disease-resistant cultivars. An advanced breeding line Yang16G216 was previously identified to confer adult-plant resistance (APR) to leaf rust. In this research, a recombinant inbred line (RIL) population was constructed from the cross between Yang16G216 and a highly susceptible line Yang16M6393, and genotyped with exome capture sequencing and 55 K SNP array. Through bulked segregant analysis (BSA) and genetic linkage mapping, a stable APR gene, designated as *LrYang16G216*, was detected and mapped to the distal region of chromosome arm 6BL with a genetic interval of 2.8 cM. For further verification, another RIL population derived from the cross between Yang16G216 and a susceptible wheat variety Yangmai 29 was analyzed using the enriched markers in the target interval, and *LrYang16G216* was further narrowed to a 0.59 cM genetic interval flanked by the KASP markers *Ax109403980* and *Ax95083494*, corresponding to the physical position 712.34–713.94 Mb in the Chinese Spring reference genome, in which twenty-six disease resistance-related genes were annotated. Based on leaf rust resistance spectrum, mapping data and physical location, *LrYang16G216* was identified to be a novel and effective APR gene. The *LrYang16G216* with linked markers will be useful for marker-assisted selection in wheat resistance breeding.

Keywords *Triticum aestivum* · *Puccinia triticina* · Adult-plant resistance · Bulked segregant analysis · Linkage mapping

Introduction

Common wheat (*Triticum aestivum* L., $2n = 6x = 42$, AABBDD) is one of the most important cereal crops worldwide. Leaf rust caused by *Puccinia triticina* (*Pt*) is a prevalent and devastating fungal disease of wheat, which can cause 7–20% yield losses, even up to 50% under epidemic conditions (Bolton et al. 2008; Huerta-Espino et al. 2011). In China, leaf rust pandemics had been documented in 1969, 1973, 1975, 1979 and 2012, respectively, resulting in enormous losses of wheat production and economy (Zhou et al. 2013). In recent years, due to the changed climate, the incidence of leaf rust has increased in the major wheat growing regions (Huerta-Espino et al. 2011). The cultivation and utilization of resistant cultivars based on host resistance is widely accepted as the most effective, economical and environmental-friendly strategy to control this disease.

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Resistance to leaf rust is usually categorized into two types: all stage (or seedling) resistance (ASR) and adult-plant resistance (APR). ASR, also described as race-specific resistance, is effective throughout the life of the plant and exhibits a high level of resistance against specific avirulent pathotypes. This type of resistance is often conferred by a single major gene and interacted with the pathogen in a gene-for-gene manner (Flor 1956). However, such genes are prone to being overcome and no-longer provide protection when a new virulent isolate emerges and becomes predominant under strong selection pressure (Chen 2005). In contrast, APR is usually inherited quantitatively and conferred by several minor effect resistance genes. Wheat varieties showing APR are often susceptible at seedling stage, but ultimately present moderate or higher resistance against *Pt* pathotypes at post-seedling or adult-plant stages. APR genes can be race-specific and non-race-specific. Non-race-specific APR genes are effective against more *Pt* pathotypes and can retard the infection, growth and reproduction of the pathotypes, manifesting the characteristics of low infection frequency, longer latent period, smaller pustule size, less production of urediniospores and slow disease progression (Caldwell 1968). Thus, this type of resistance is also described as slow rusting resistance. Compared with the seedling resistance, APR has proven more durable and favored by breeders (Bjarko and Line 1988; Marone et al. 2009).

At present, 80 leaf rust (prefixed *Lr*) resistance genes have been permanently designated in wheat (Kumar et al. 2021; McIntosh et al. 2017; Qureshi et al. 2018), among which fifteen genes, including *Lr12*, *Lr13*, *Lr22* (alleles *Lr22a* and *Lr22b*), *Lr34*, *Lr35*, *Lr37*, *Lr46*, *Lr48*, *Lr49*, *Lr67*, *Lr68*, *Lr74*, *Lr75*, *Lr77* and *Lr78*, are considered as APR genes (Da Silva et al. 2018; Kolmer et al. 2018a, 2018b, 2018c; Kumar et al. 2021; McIntosh et al. 2014, 2017; Singla et al. 2017). Exploration and identification of new leaf rust resistance genes, especially APR genes, will be helpful for broadening the genetic basis of resistance and contributing to durable resistance breeding.

Conventional QTL linkage mapping is a common method for identifying the genes or QTL underlying the traits of interest and provide the basis for map-based cloning of the candidate genes. Generally, it requires assaying the genotypes and phenotypes of all the individuals for the target traits collected from a sample population (Win et al. 2017; Zou et al. 2016). With the great advances in wheat genome sequencing, the reference genomes of bread wheat cv. Chinese Spring, wild emmer (AABB) cv. Zavitan, the progenitor species cv. *Triticum Urartu* (AA) and *Aegilops tauschii* (DD), and 10 + wheat have been released during the past few years (Avni et al. 2017; Jia et al. 2013; Ling et al. 2018; Luo et al. 2017), which greatly accelerates the discovery of DNA variants and development of high-throughput of molecular markers (Kumar et al. 2016; Wu et al. 2018). Among them, single nucleotide polymorphisms (SNPs) are primary markers for genetic analyses based on chip hybrid. Various versions of SNP arrays, such as 9 K, 35 K, 55 K, 90 K, and 660 K SNP arrays, have been developed and widely applied in genotyping of wheat (Sun et al. 2020). In addition, with the significant reduction in sequencing cost and increased data quality of next-generation sequencing, exome capture technologies covering the majority of the gene coding regions based on the genome sequence data of Chinese Spring have been developed, with lower cost than the whole genome sequencing (Mo et al. 2018). Benefiting from these high-throughput genotyping technologies, recently, BSA has become a simple and high-efficiency method to dissect genes or QTL associated with target phenotypes, which works with bulked of DNA or RNA samples from sets of individuals exhibiting contrasting extreme phenotypes in the whole population (Wang et al. 2018; Zou et al. 2016). This approach has been a useful complement to conventional linkage mapping and widely adopted in wheat genomics (Cao et al. 2019; Dong et al. 2020; Trick et al. 2012; Wang et al. 2017; Wu et al. 2017).

The middle and lower reaches of the Yangtze River (MLRYR) in China are the second largest wheat growing region, also an epidemic area for multiple wheat diseases, such as *Fusarium* head blight, powdery mildew, wheat yellow mosaic virus, and stripe rust. Since the beginning of this century, the occurrence frequency of leaf rust also has increased significantly, which has become another major disease in this wheat region. Breeding wheat varieties pyramiding multiple resistance to the above diseases including leaf rust becomes very important.

Yang16G216 and Yang16M6393 are two elite wheat lines developed at Yangzhou Academy of Agricultural Sciences (YAAS), both conferring high resistance to powdery mildew controlled by *Pm21* (Bie et al. 2015; He et al. 2018) and wheat yellow mosaic virus controlled by *QYm.nau-2D* (Xiao et al. 2016), and moderate resistance to *Fusarium* head blight that was mainly contributed by the genetic background of Yangmai 158, the largest growing wheat variety in the 1990s in China. Yang16M6393 also carries the stripe rust resistance gene *Yr26* (Wang et al. 2008) that still displays high resistance in the MLRYR wheat region. Yang16G216 was observed to confer high and consistent resistance to leaf rust at adult-plant stage in the successive epidemic years 2014, 2015 and 2016, meanwhile Yang16M6393 was highly susceptible all along. For the less of commercial varieties and available germplasms with satisfied resistance to leaf rust in this wheat region, Yang16G216 had been a core parent for upgrading leaf rust resistance in pyramiding breeding work at YAAS. However, the genetic basis of APR to leaf rust in Yang16G216 was poorly understood. In previous work, a dual-role RIL population based on the cross "Yang16G216/Yang16M6393" had been constructed for both genetic

analysis of leaf rust resistance and resistance pyramiding breeding. Another RIL population from the cross between Yang16G216 and a susceptible variety Yangmai 29 was simultaneously constructed to validate candidate regions of the target APR gene(s). In this research, our objectives were to map the APR gene(s) in Yang16G216 and develop tightly linked markers for marker-assisted selection (MAS) in wheat resistance breeding.

Materials and methods

Plant materials

Yang16G216 and Yang16M6393 are two advanced wheat breeding lines developed at YAAS. Yangmai 18 and Yangmai 29 are two wheat commercial varieties bred and released by YAAS. All of them carry the powdery mildew resistance gene *Pm21* conferred by the wheat-*Dasypyrum villosum* T6V#2S.6AL translocation (Chen et al. 1995) and are immune to powdery mildew. Among them, Yang16G216 exhibits a high level of resistance to leaf rust at adult-plant stage, while Yang16M6393, Yangmai 18 and Yangmai 29 are highly susceptible. Two RIL populations were constructed: one mapping population consisting of 224 lines generated from the cross "Yang16G216/Yang16M6393" was used to identify the APR gene(s) to leaf rust in Yang16G216; the other comprising 240 lines generated from the cross "Yang16G216/Yangmai 29" was used to validate the target gene(s) identified in this study. The near isogenic lines (NILs) of Thatcher (Tc) carrying *Lr3*, *Lr3bg*, *Lr3ka*, respectively, were kindly provided by Prof. Zaifeng Li (Hebei Agriculture University) (Zhang et al. 2021).

Evaluation of leaf rust resistance of Yang16G216

The parents Yang16G216 and Yang16M6393 were tested with the mixed *Pt* pathotypes collected in the field of Yangzhou and the mixture of PHT and THT, most prevalently in China (Huerta-Espino et al. 2011), at both seedling and adult stages. Evaluation of the seedling reactions of Yang16G216, Yang16M6393, and the Tc NILs carrying independent *Lr3*, *Lr3bg*, and *Lr3ka*, was also conducted using six Chinese *Pt* pathotypes (Table S1) at the College of Plant Protection, Hebei Agriculture University, China.

In seedling test, the tested plants were grown in plastic pots in the greenhouse. When the first leaves were fully expanded, the plants were inoculated with *Pt* isolate or mixture. Inoculated plants were placed in plastic-covered cages and incubated at 18 °C and 100% relative humidity for 12–14 h in darkness, and then transferred to the greenhouse (22–25 °C) for further incubation.

In adult-plant test, the plants were inoculated at booting stage with a mixture of 1 mg/ml rust spores and Tween 20 (0.05%) using an atomizer. After inoculation, the plants were placed in a closed chamber with a humidifier filled with distilled water for 12 h in darkness and then transferred to the greenhouse for symptom development.

The infection types (ITs) were scored 10 to 14 days after inoculation using the 0–4 scale according to Roelfs et al. (1992), where 0 to 2 considered as resistant, while 3 and 4 considered as susceptible.

Field experiments and evaluation of APR to leaf rust of RIL populations

The Yang16G216/Yang16M6393 RIL population along with the two parental lines were grown and evaluated for leaf rust resistance at adult-plant stage during 2018–2021. In 2018–2019, the trial was conducted at Wantou experimental station of YAAS, designated as 2019WT. In 2019–2020, the trials were carried out both at Wantou experimental station of YAAS and Shatou experimental station of Yangzhou University, designated as 2020WT and 2020ST, respectively. In 2020–2021, the trial was conducted at Wantou experimental station, designated as 2021WT. The Yang16G216/Yangmai 29 RIL population was planted and evaluated for APR to leaf rust at 2019WT and 2020WT. All the trials were conducted in randomized complete blocks with two replicates. For each replication, approximately fifty seeds of the parents and each RIL were planted in 1.5-m rows with 0.25-m spacing between every two adjacent rows. Every 20 rows were planted with the susceptible parent Yang16M6393. The susceptible check, Yangmai 18, was also planted perpendicular and adjacent to the tested rows to produce an epidemic environment.

For the RIL populations, the evaluation of APR to leaf rust was relied on the natural occurrence. The infection types (ITs) for each RIL were visually evaluated according to Long and Kolmer (1989) with minor modification: 0 = neither uredinia nor hypersensitive flecks; 1 = necrosis surrounding small uredinia; 2 = necrosis surrounding small to moderate size uredinia; 3 = necrosis surrounding moderate size uredinia; 4 = large size uredinia without chlorosis. ITs of 0 and 1 were considered as highly resistant (HR), 2 as moderately resistant (MR), 3 as moderately susceptible (MS), and 4 as highly susceptible (HS), respectively. Each trial was scored twice at weekly intervals in later April to early May when acceptable levels of leaf rust appeared on the susceptible parent. The mean ITs of each line in each trial were used for QTL analysis. All the phenotypic data were calculated using the IBM SPSS Statistics 23.0 (IBM Corp., Armonk, NY).

Bulked segregant exome capture sequencing (BSE-seq) analysis

Based on the evaluation of leaf rust responses of the Yang16G216/Yang16M6393 RIL population in 2019WT, 2020WT and 2020ST, 50 RILs with consistently high resistance and 50 RILs highly susceptible were picked out, respectively. DNAs of the individual RIL from seeds were extracted separately using high-throughput DNA extraction kit and then mixed in equal amounts to form the resistant bulk (R bulk) and susceptible bulk (S bulk). DNAs of the two bulks and the parents were subjected to exome capture sequencing with deep coverage (90 ×) using the platform of MGISEQ-2000 from BGI by Molbreeding Biotechnology Co. Ltd. The GenoBaits Wheat Exon Capture Panel was developed based on the IWGSC RefSeq v1.0 sequence information (Appels et al. 2018) and IWGSC v1.1 annotation information, including 304,328 probes, covering more than 97% high confidence protein coding genes of the wheat genome.

Raw sequence reads were processed using software Fastp (v0.20.0) (Chen et al. 2018) to remove low-quality reads ($Q \leq 20$) and adapters. The clean reads were then aligned to IWGSC RefSeq v1.0 genome using software BWA (v0.7.16) (Li 2013) mem with default parameters. After that, the remaining clean reads were analyzed with GATK (v3.5–0-g36282e4) (Mckenna et al. 2010) module Unified Genotyper and Variant Filtration to call SNP variants. The resulting SNPs with sequencing depth less than 5 were abandoned, and the remaining ones were applied for BSA to detect the candidate regions associated with leaf rust resistance by the calculation of SNP-index (Fekih et al. 2013; Takagi et al. 2013) and Euclidean Distance (Hill et al. 2013). ANNOVAR (Wang et al. 2010) was used to annotate the identified SNPs, using IWGSC v1.0 high/low confidence genes as a reference.

The calculation of SNP-index (i.e., the proportion of a SNP from the reference to the total number in one bulk) was performed according to Takagi et al. (2013) with minor modification: a SNP index of 1 represents that all the SNPs of the loci in one bulk are the same as the reference parent Yang16G216; a SNP-index of 0 means all the SNPs completely different from the reference parent. Then, Δ(SNP-index) at each SNP was calculated as follows: Δ(SNP-index) = (SNP-index of R-bulk) − (SNP-index of S-bulk). Sliding window analysis was applied with 2 Mb window size and 100 kb increment in the process of Δ(SNP-index) plot. With 1000 replications of the simulation, the position intervals upper the significant threshold (99% confidence level) were considered as candidate regions. SNPs with Δ(SNP-index) value approximate to 1 in these candidate regions were identified as significantly associated with leaf rust resistance.

The Euclidean distance (ED) is calculated at each SNP using the software MMAPP according to Hill et al. (2013). In order to decrease the effects of low ED measurements/noise, a power of 5 of the original ED is taken as the substitution. Next, the data are fit using a Loess curve with a polynomial exponent of 1 and a span parameter determined by minimizing the corrected Akaike information criterion. Regions where the Loess fitted values are greater than three standard deviations above the genome-wide median were considered as peak regions. Within the identified peaks, the candidate SNPs were selected with the cutoff of a minimum distance of 0.5 and a minimum mutant pool allele frequency of 0.75.

BSA based on 55 K SNP array

Fifteen seeds of individual line of the Yang16G216/Yang16M6393 RIL population along with two parental lines were collected and sent to China Golden Marker (Beijing) Biotech Co. Ltd. Total genome DNA of each tested sample was extracted according to the procedure standardized by the company. DNA purity and integrity was then analyzed and confirmed. Genotyping was done with the Wheat 55 K SNP Array containing 53,063 markers.

For the trials of 2019WT, 2020WT and 2020ST, extremely resistant (HR) and susceptible (HS) phenotypes in the RIL population were separately collected to generate the R bulk and S bulk. Three pairs of R and S bulks were constructed, and each bulk contained 30 RILs. Firstly, the ambiguous SNP loci were filtered out. Then, the calculation of Δ(SNP-index) at each SNP followed the above mentioned. A graph of the distribution of Δ(SNP-index) on the whole genome was plotted. The genomic regions showed significant peaks exceeding the threshold (99% confidence) were considered as possible existence of leaf rust resistance loci.

Genetic map construction

For the obtained genotyping data of 55 K SNP array among the RILs, monomorphic and SNP loci with vague SNP calls, more than 10% missing values, minor allele frequency (MAF) below 5%, distorted segregation ratios confirmed by Chip-squared tests ($P < 0.05$) and individuals with more than 20% missing SNP calls were removed from the database. The software of JoinMap v4.0 was adopted to construct the genetic map using the remained markers. A minimum logarithm of the odds (LOD) score of 3.0 was used as the threshold, and recombination frequencies were transformed into the genetic distances in centimorgans (cM) based on the Kosambi mapping function (Kosambi 1944).

Genes or QTL were detected with additive tool (ICIM-ADD) in IciMapping v4.1 by inclusive composite interval

Fig. 1 Leaf rust responses of Yang16G216 (**a**) and Yang16M6393 (**b**) to *Pt* natural population at adult stage in field

mapping (ICIM) method based on the phenotype data. Likelihood LOD significance threshold was calculated by 1000 permutations at a p value ≤ 0.01. The walking speed genome scanning step was 1.0 cM. A LOD of 3.0 was set to declare QTL as significant. The phenotype variants explained (PVE) by individual QTL and additive effect at the LOD peaks were also calculated. The physical positions were determined by blasting the flanking marker sequences for QTL against the Chinese Spring reference sequence v1.0.

Development and analysis of Kompetitive Allele-Specific PCR (KASP) markers

SNPs located in the target genomic region based on BSE analysis and wheat 55 K SNP array were selected for conversion of KASP markers using the online platform polymarker website (http://polymarker.info/). Then, the standard FAM or VIC fluorescent sequence (FAM tail: 5′ GAAGGTGACCAA GTTCATGCT 3′; VIC tail: 5′GAAGGTCGGAGTCAACGG ATT 3′) was added at 5′ end of two forward primers of each designed KASP. The KASP primers were synthesized by Tsingke Biological Technology. The designed KASP primers were screened for polymorphisms between the parents, as well as the resistant and susceptible DNA bulks.

KASP assays were performed in a 10-μl volume reaction including 5 μl HiGeno 2 × probe mix (JasonGen Co. Ltd), 0.112 μl assay primer mix, 1 μl DNA (~ 20 ng/μl) and 3.89 μl ddH_2O. Amplification was conducted at 95 °C for 15 min, followed by 9 touchdown cycles of 95 °C for 20 s, touchdown starting at 65 °C for 1 min (decreasing 1 °C per cycle), and then followed by 32 cycles of 95 °C for 20 s, 57 °C for 1 min. End-point fluorescence data were read on an ABI Vii qPCR instrument (Applied Biosystems) and analyzed using the TaqMan Genotyper software v1.3.1. If the signature genotyping groups had not formed after the initial amplification, additional amplification cycles (usually 3–5) were applied, and the samples were read again.

Results

Evaluation of leaf rust resistance

In the successive leaf rust epidemic years 2014, 2015 and 2016, Yang16G216 showed high and consistent resistance (IT 0;), while Yang16M6393 showed high susceptibility (IT 3^{+}-4) at adult-plant stage in field (Fig. 1). The two parents were further challenged with the mixture of PHT and THT prevalently in China at adult stage in greenhouse. Yang16G216 and Yang16M6393 exhibited the same responses as in the field (Fig. S1).

Yang16G216 and Yang16M6393 were further tested at two-leaf stage with the *Pt* collection in Yangzhou and the mixture of PHT and THT in greenhouse, respectively. Results indicated that Yang16G216 and Yang16M6393 were both susceptible, displaying the infection types of 3 and 4, respectively (Fig. S1). Then, the two parents were further inoculated with six Chinese *Pt* pathotypes. Yang16M6393 was highly susceptible to all the pathotypes. Meanwhile, Yang16G216 performed susceptible scores (IT 3 or 4) against five pathotypes and only a resistant score (IT 0;) with the pathotype SKKT (Table S1). Hence, it was indicated that Yang16G216 confers APR to wheat leaf rust.

The phenotypes of the Yang16G216/Yang16M6393 RIL population and their parental lines under natural *Pt* infections were documented in four field environments. In the 2019WT environment, among the 224 RILs, except for 10 heterozygous lines, 105 RILs were scored as homozygous resistant (IT 0–2) while the remaining 109 lines were scored as homozygous susceptible (IT 3–4). Then the RIL population was continually identified in the 2020WT, 2020ST,

Table 1 Correlation coefficients for the leaf rust responses of the Yang16G216/Yang16M6393 RIL population at adult-plant stage among different field trials

Trials	2020WT	2020ST	2021WT
2019WT	0.80**	0.82**	0.75**
2020WT	–	0.89**	0.90**
2020ST	–	–	0.89**

**Indicates significance at $P=0.01$ level

and 2021WT environments. The Pearson's correlation coefficients for phenotype data collected in the four field environments were all positively significant at the $P=0.01$ level, ranging from 0.75 to 0.90 (Table 1). The results indicated that the leaf rust resistance responses were consistent across the environments.

Physical mapping of APR loci based on BSA of the exome capture sequencing

Using exome capture sequencing, a total of 304,368,052 and 237,021,098 raw reads were obtained from the R bulk and the S bulk, respectively. After quality control, more than 95% of the high-quality reads in both bulks were uniquely mapped to the Chinese Spring reference genome (Table S2). Through variations calling, 2,608,045 SNPs from the R bulk and 2,283,197 SNPs from the S bulk were identified for further analysis. A total of 122,965 high-quality SNP sites with polymorphic between the two parents were obtained to perform SNP-index analysis. Finally, 4,155 candidate SNPs were identified; among them, 3,856 SNPs were located on all 21 chromosomes, while 299 SNPs were not assigned on any chromosome. The significantly abundant enrichment of the disease resistance-associated SNPs was observed on chromosome 6B (3,485), and 94.09% of them were enriched in the physical interval 708.10–721.00 Mb on the long arm. The numbers of SNPs on chromosome 6D (135) were relatively high and most of the linked SNPs (117) were within the physical interval 468.00–470.40 Mb (Fig. 2).

According to the correlation threshold of ED calculation, three genomic intervals associated with leaf rust resistance were obtained, located on chromosomes 3D, 6B and 6D, respectively (Fig. S2). The physical intervals 710.55–720.99 Mb on chromosome 6B and 464.85–471.15 Mb on chromosome 6D clustered the most and more numbers of SNPs, respectively.

Physical mapping of APR loci based on BSA of the 55 K SNP array

According to the genotyping of DNA bulks with the 55 K SNP array, 5,432, 5,531 and 8,063 polymorphic SNPs between two bulks in 2019WT, 2020WT and 2020ST, respectively, were obtained to calculate the Δ(SNP-index) and then analyze for gene mapping of leaf rust resistance. Significant peaks were observed on chromosomes 3A, 5A and 6B, suggesting these were potential loci associated with leaf rust resistance (Fig. 3). Among them, the peak on chromosome 6B was the highest and greatly exceeded the threshold in all the three trials. The full intervals of the regions covered by threshold line on chromosome 6B ranged from 670.21 to 720.49 Mb, with the overall length of 50.28 Mb. In light of the QTL most probably located in the region covered by the highest Δ(SNP-index) peak, therefore the most probable interval of the locus was narrowed to the physical interval 712.24–720.49 Mb. Eight significantly associated SNPs were detected in the target interval. The SNPs with the highest values of Δ(SNP-index) were located at 712.39 Mb in 2019WT and 2020ST, and 714.46 Mb in 2020WT, respectively (Table 2). These results revealed that there was an effective gene or QTL involving leaf rust resistance on the distal region of chromosome arm 6BL.

The peak on chromosome 3A was detected in 2019WT and 2020ST, and the peak on chromosome 5A was only detected in 2020WT. These peaks were much smaller than that on chromosome 6B and instable among different trials. Two SNPs located on chromosome 6D were identified with higher Δ(SNP-index) value in the three pairs of bulks, one located at 468.81 Mb and the other one located at 472.73 Mb (Fig. 3). However, other SNPs physically spanning the two SNPs on the reference genome showed lower Δ(SNP-index) value than the threshold, indicating it is not a confidential locus.

Linkage map construction and mapping of APR loci in Yang16G216/Yang16M6393 RIL population

A total of 224 RILs from the cross Yang16G216/Yang16M6393 were genotyped with the 55 K SNP array. The results indicated that 4,016 of 53,063 SNPs showed polymorphism between the two parents. Additional 10 SSR primer pairs and 8 SNP markers were also developed and tested on the two parents. One SSR marker and one SNP marker showed polymorphisms between the parents (Table S3). Among all the polymorphisms markers, 1,025 SNPs showed severe segregation distortion ($P<0.05$) and were removed from the database. The remained 2993 SNPs were used to construct the linkage map. Finally, 2,401 markers were distributed in 21 linkage groups involving 20 chromosomes; except for chromosome 6A due to the two parents both contained the T6V#2S.6AL translocation. The entire map spanned a total length of 1000.52 cM with an average marker interval of 2.40 cM. The A, B and D genomes

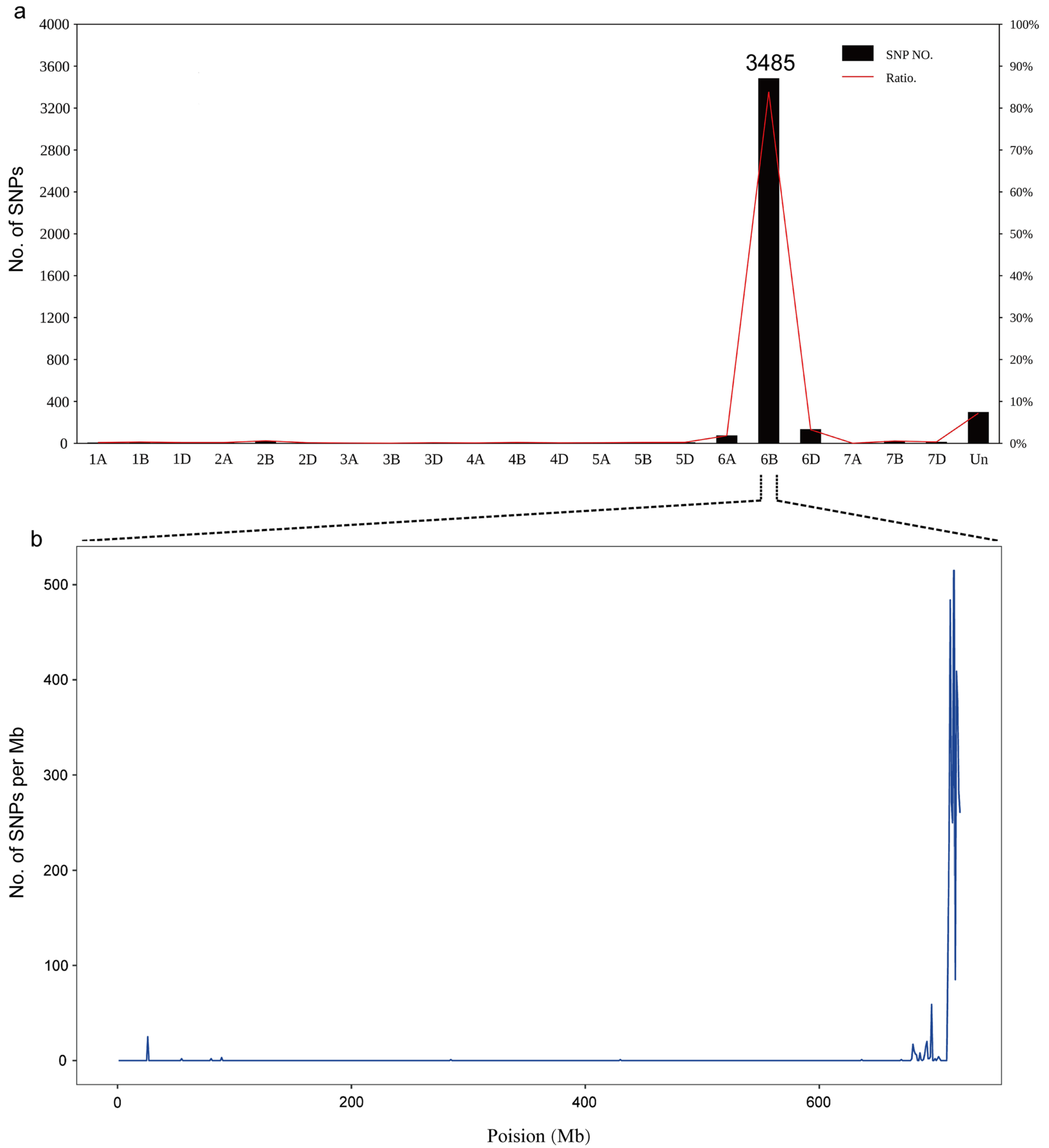

Fig. 2 Distribution of the candidate SNPs identified by BSE analysis on wheat chromosomes. **a** Distribution of SNPs on each wheat chromosome; **b** distribution of SNPs on chromosome 6B. Un indicates SNPs not anchored on any chromosomes

include 838, 789 and 774 markers covering lengths of 382.16, 300.55 and 317.81 cM with average marker intervals of 2.19, 2.63 and 2.44 cM, respectively. Except for two linkage groups on chromosome 4A, single linkage group was on the other 19 chromosomes each.

Based on the IT data collected, a major and staple gene, temporarily designated as *LrYang16G216*, was identified on chromosome 6B and significantly associated with resistance contributed by the parent Yang16G216. The phenotypic variation explained by the *LrYang16G216* locus ranged

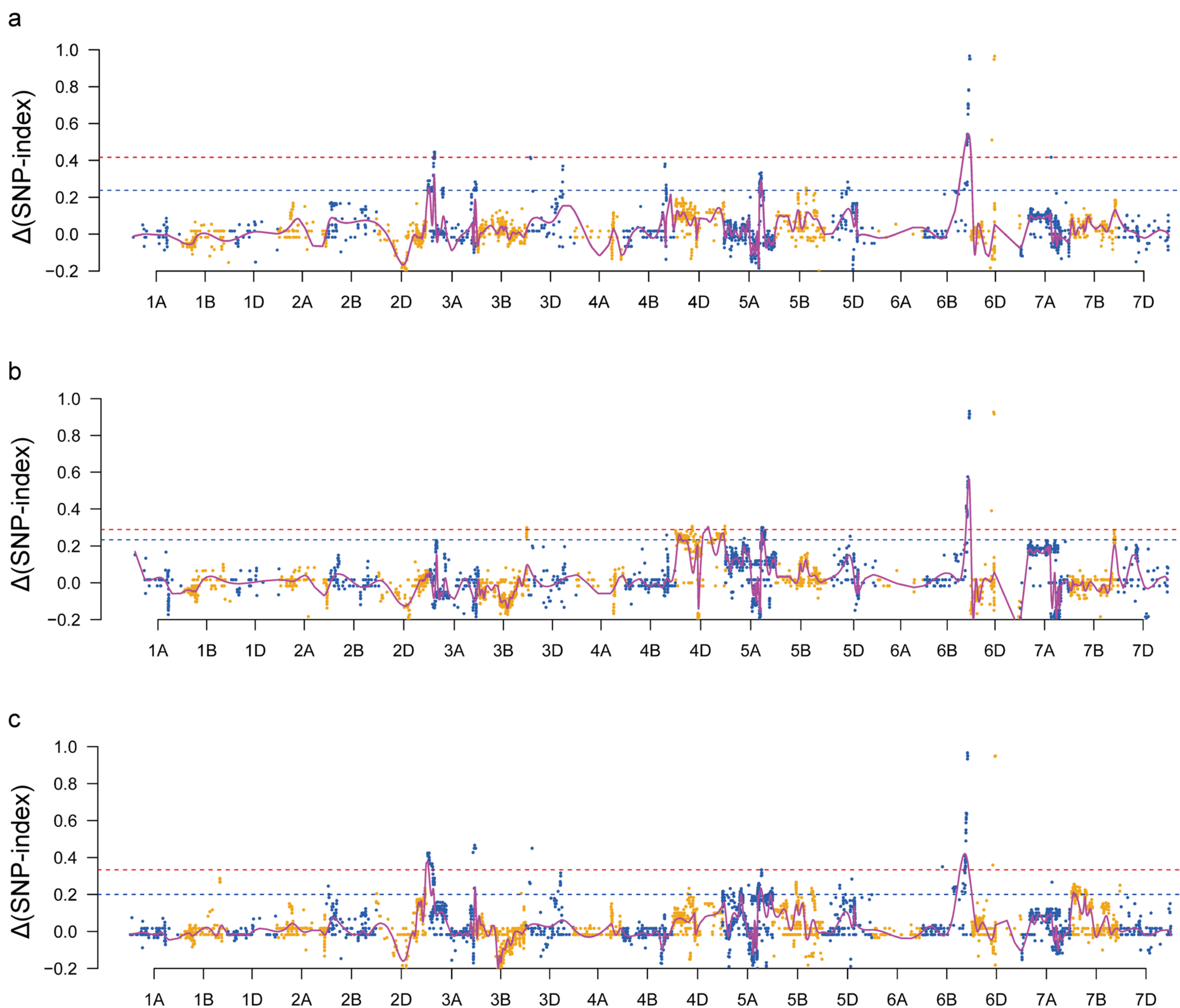

Fig. 3 Manhattan plot of the distribution of Δ(SNP-index) on each chromosome in 2019WT (**a**), 2020WT (**b**) and 2020ST (**c**). The blue line represents the 95%-confidence interval upper side; the red line represents the 99%-confidence interval upper side (colour figure online)

Table 2 Candidate intervals associated with leaf rust resistance identified by 55 K SNP array-BSA

Trials	Chromosome	Position interval (Mb)		Associated SNP No.	Summit Δ(SNP-index) value	Position of the summit value (Mb)
		Full	Most probable			
2019WT	3A	20.23–22.34	20.23–22.34	45	0.444	21.12
	6B	670.21–720.49	712.23–720.49	42	0.967	712.39
2020WT	5A	587.91–598.67	587.91–598.67	4	0.301	589.88
	6B	670.21–720.49	712.23–720.49	42	0.932	714.46
2020ST	3A	7.28–19.43	7.28–12.93	36	0.467	7.93
	6B	670.21–720.49	712.23–720.49	40	0.967	712.39

from 58.12 to 85.75% among different trials. *LrYang16G216* was positioned in a 2.8 cM interval between markers *Ax-109928834* and *SNP-8* (Fig. 4), with a corresponding physical interval of 711.34–714.62 Mb. However, the two candidate SNPs on chromosome 6D detected by 55 K SNP array-BSA failed to construct the genetic linkage map of 6D.

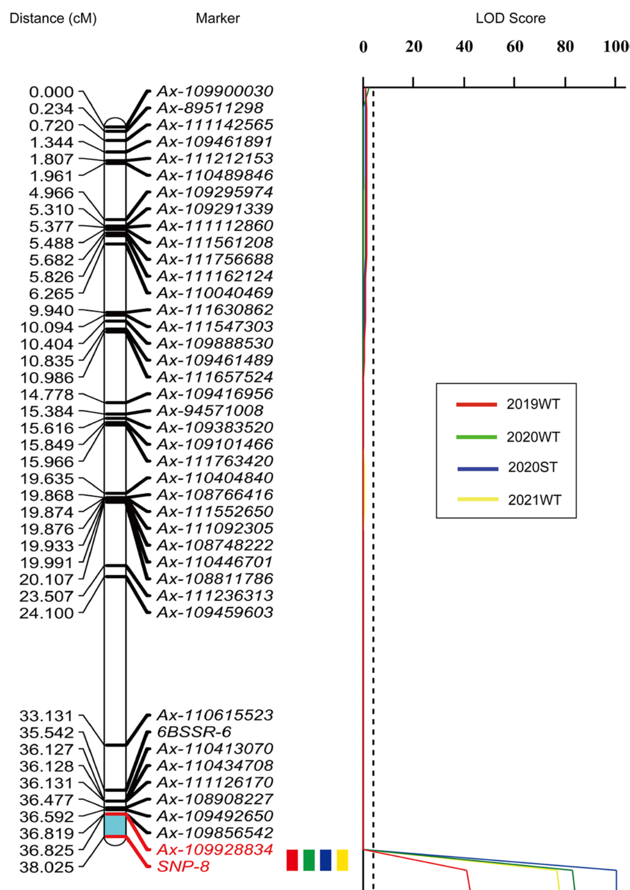

Fig. 4 Graphical display of the APR gene *LrYang16G216* on wheat chromosomes 6BL identified by ICIM in the Yang16G216/Yang16M6393 RIL population. The centimorgan (cM) distances between markers are shown to the left of the linkage group and the marker loci are shown along the right. The *vertical dotted line* represents the logarithm of the odds (LOD) significance threshold of 3.0. Red, green, blue, and yellow bars represent the *LrYang16G216* intervals identified in the 2019WT, 2020WT, 2020ST and 2021WT environments, respectively (colour figure online)

Furthermore, no gene/QTL was identified on chromosome 6D using the ICIM method. We concluded that the SNPs detected on chromosome 6D via BSA were unlikely associated with resistance.

Verification and further mapping of *LrYang16G216* in Yang16G216/Yangmai 29 RIL population

A set of 109 SNPs in the physical interval of 710–720 Mb on 6BL was selected for conversion to KASP markers. Then these markers were used to confirm polymorphism between the parents and bulks. Eight KASP markers showed polymorphisms between the parents and bulks (Table S3). Subsequently, these polymorphism markers on 6BL were used to genotype the Yang16G216/Yangmai 29 RIL population. Using ICIM with mean ITs of each line, *LrYang16G216* was further narrowed to a 0.59 cM interval flanked by the KASP markers *Ax109403980* and *Ax95083494* (Fig. 5a).

The sequence alignment of linkage markers around *LrYang16G216* in the genetic map with the Chinese Spring reference sequence showed that the relative physical positions of those markers were generally consistent with the genetic linkage map (Fig. 5b). The 0.59 cM genetic interval corresponds to the genomic interval 712.34–713.94 Mb (Fig. 5b). According to the Chinese Spring reference genome, 38 high confidence genes were annotated in the target physical interval. Among them, 26 genes encode proteins with motifs known to be related to plant disease resistance, including eight NBS-LRR-like resistance genes, eight disease resistance protein RPM1 genes, two receptor-like protein kinase genes, three protein kinase family protein genes, and one ABC transporter gene, sucrose transporter gene, serine/threonine-protein kinase gene, wall-associated receptor kinase-like protein gene and LRR receptor-like protein kinase gene (Fig. 5c, Table S4).

Discussion

Diverse leaf rust resistance loci (ASR and APR) have been identified in common wheat varieties and wild relatives of wheat (https://wheat.pw.usda.gov/GG3/WGC). Breeding experience has demonstrated that the resistance conferred by ASR genes is prone to loss due to virulent mutations of pathotypes, whereas the APR genes have proven more durable (Chen 2005; Marone et al. 2009). Up till now, fifteen genes conferring APR have been identified and mapped on wheat chromosomes (Da Silva et al. 2018; Kolmer et al. 2018a; Kolmer et al. 2018b; Kolmer et al. 2018c; Kumar et al. 2021; McIntosh et al. 2014; Singla et al. 2017). Only a few APR genes, such as *Lr34*, *Lr35*, *Lr46* and *Lr67*, remained effectiveness against current *Pt* isolates in China, however, when deployed individually, their resistance is usually inadequate especially under pandemic conditions (Li et al. 2014). Some APR loci were reported to be associated with adverse morphological trait, which partly limited their application in wheat high-yield breeding (Herrera-Foessel et al. 2011; Krattinger et al. 2009; Moore et al. 2015; Singh et al. 2011). Therefore, exploration and characterization of new leaf rust resistance genes, especially APR genes with none significantly adverse effects, is essential.

In this research, seedling test demonstrated that the advanced breeding line Yang16G216 showed susceptible responses to almost all tested *Pt* pathotypes except for SKKT, but exhibited high resistance at adult-plant stage even challenged with the same *Pt* collection or mixture, indicating that Yang16G216 confers effective APR to leaf rust. Through analysis of the Yang16G216/Yang16M6393 RIL population, we found the segregation ratio of resistance to

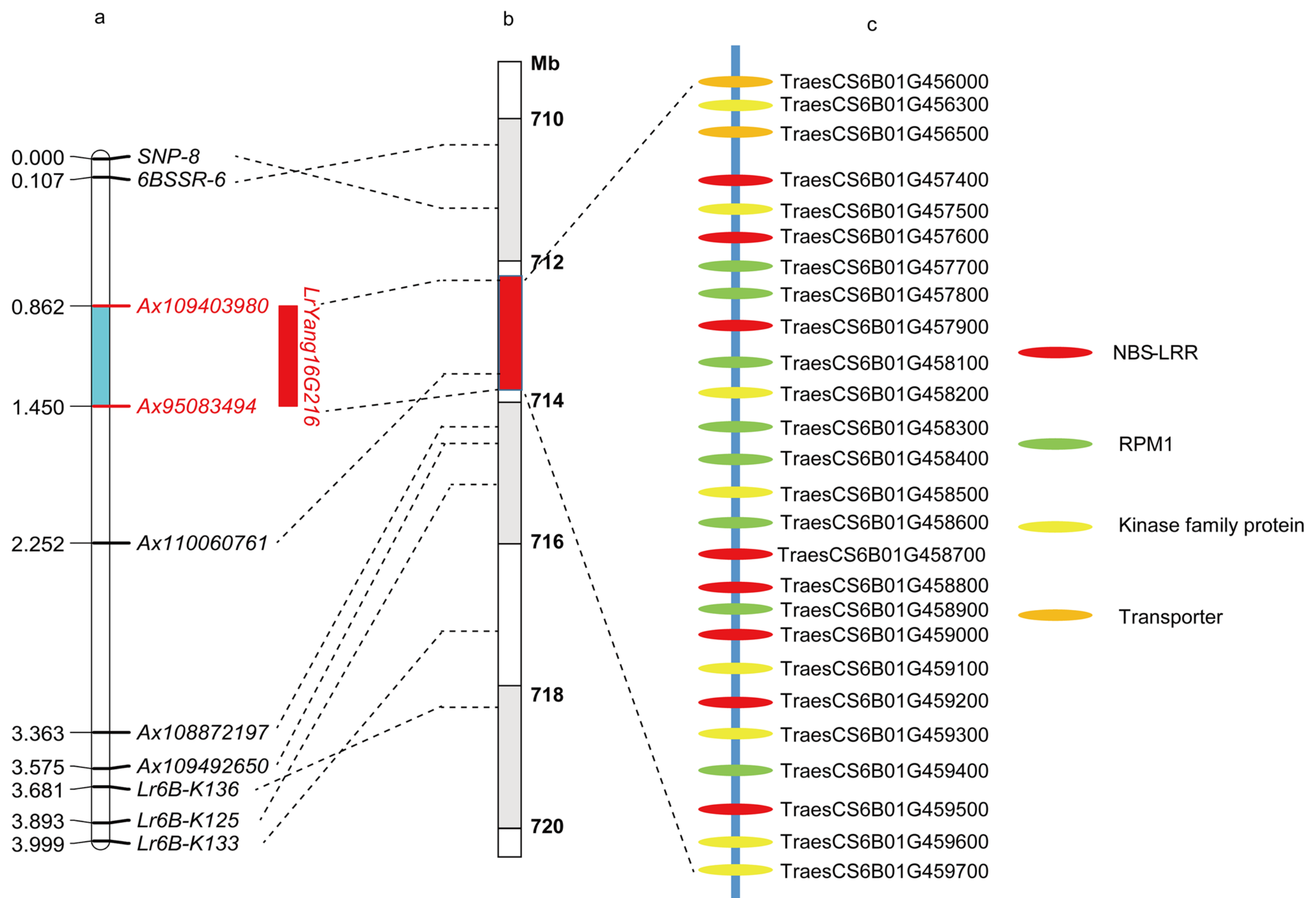

Fig. 5 The location of *LrYang16G216* and the annotated disease resistance-related genes in the *LrYang16G216* locus on wheat chromosome 6B. **a** Genetic linkage map of *LrYang16G216* using the Yang16G216/Yangmai 29 RIL population; red bar represents the location of *LrYang16G216*; Markers flanking the gene *LrYang16G216* are indicated with red font; **b** the corresponding physical location of the linked markers of *LrYang16G216*; **c** the disease resistance-related genes in the *LrYang16G216* locus according to the gene annotation of Chinese Spring IWGSC RefSeq v1.0 (colour figure online)

susceptibility was nearly 1:1 ($\chi^2_{1:1} = 0.04$, $P > 0.05$), suggesting the leaf rust resistance of Yang16G216 could be mainly controlled by a major gene. As expected, a stable and major APR gene *LrYang16G216* was mapped to the distal region of chromosome arm 6BL by BSA. Then, by genetic analyses of two independent RIL populations, *LrYang16G216* was further narrowed to a 0.59 cM genetic interval, corresponding to the physical region 712.34–713.94 Mb in the Chinese Spring reference genome.

So far, a total of six leaf rust resistance genes/QTL have been reported and mapped on chromosome arm 6BL, namely *QLr.fcu-6BL* (Chu et al. 2009), *QLr.cimmyt-6BL.1* (Rosewarne et al. 2012), *QLr.cimmyt-6BL.2* (William et al. 2006), *QLr.cim-6BL* (Lan et al. 2017) and *QLr-6BL* (Zhang et al. 2021) with APR, and *Lr3* (Haggag and Dyck 1973) with ASR. We comparatively analyzed the chromosome locations of the five reported APR QTL based on the IWSGC RefSeq v1.0 physical map (Table S5). *LrYang16G216* mapped at position 712.34–713.94 Mb was different from *QLr.fcu-6BL* (414.8–656.2 Mb), *QLr.cimmyt-6BL.2* (418.1 Mb), *QLr.cimmyt-6BL.1* (about 674.8 Mb) and *QLr-6BL* (688.3 Mb). *QLr.cim-6BL* (697.46–709.58 Mb) was mapped close to *LrYang16G216*, but which derived from tetraploid. The ASR gene *Lr3* (alleles *Lr3*, *Lr3ka* and *Lr3bg*), had been located at 716.3 Mb on the distal region of 6BL (Haggag and Dyck 1973; Zhang et al. 2021), at a similar position of *LrYang16G216*. In this research, *LrYang16G216*, *Lr3*, *Lr3ka*, *Lr3bg* were all tested by six *Pt* isolates in seedling stage and the result showed *LrYang16G216* has a similar resistance spectrum to *Lr3* and *Lr3bg*, but totally different from *Lr3ka* that is resistant to five of six isolates (Table S1). Interestingly, *LrYang16G216* got high resistance at adult-plant stage, but *Lr3*, *Lr3ka* and *Lr3bg* showed susceptibility with maximum disease severity (about 40%-50%) according to past reports, indicating it is different from *Lr3*, *Lr3ka* and *Lr3bg*. Taking all the above into account, *LrYang16G216* identified in this research could be a new APR gene resistant to leaf rust.

Until now, of all the reported *Lr* genes, nine genes have already been cloned, consisting of *Lr1* (Cloutier et al. 2007), *Lr10* (Feuillet et al. 2003), *Lr13* (Hewitt et al. 2021; Yan et al. 2021), *Lr14a* (Kolodziej et al. 2021), *Lr21* (Huang et al. 2003), *Lr22a* (Thind et al. 2017), *Lr34* (Krattinger et al. 2009), *Lr42* (Lin et al. 2022) and *Lr67* (Moore et al. 2015). The race-specific leaf rust resistance genes *Lr1*, *Lr10*, *Lr21*, *Lr42*, *Lr13* and *Lr22a* were all confirmed to encode proteins containing NBS-LRR motifs (Cloutier et al. 2007; Feuillet et al. 2003; Huang et al. 2003; Lin et al. 2022; Thind et al. 2017; Yan et al. 2021). *Lr14a* encodes a membrane-localized protein, whose structure is similar to Ca^{2+}-permeable non-selective cation channels (Kolodziej et al. 2021). Two slow-rusting resistance genes, *Lr34* and *Lr67*, encode proteins as ABC transporter and hexose transporter, respectively (Krattinger et al. 2009; Moore et al. 2015). In this research, based on the analysis of gene annotation in the target interval, a total of 26 high confidence genes encode proteins known to be associated with plant pathogen interactions and disease resistance, such as NBS-LRR-like resistance protein, RPM1, receptor-like kinase, ABC transporter, sucrose transporter, serine/threonine-protein kinase, wall-associated receptor kinase-like protein and LRR-receptor-like protein kinase. The genes annotated within the *LrYang16G216* locus will be helpful for candidate gene(s) prediction. More saturated molecular markers and a larger secondary population are required for fine mapping and cloning of *LrYang16G216*.

For a long time, leaf rust was not the main disease of wheat in the middle and lower reaches of the Yangtze River, China. Partly due to climate change, the incidence of leaf rust in this region increased sharply in recent years, and six leaf rust pandemic years have been documented since 2014, posing a great challenge to wheat production. However, due to lack of attention to this disease before, most of the commercial varieties in this area were susceptible. Moreover, there are few available leaf rust resistant resources in local germplasms, increasing difficulty in breeding of leaf rust resistant varieties. The emergence of the local elite line Yang16G216 with effective resistance to leaf rust and the mapping of the major APR gene *LrYang16G216* provide great help in wheat MAS work. In addition, due to its multi-disease resistance and high yield, Yang16G216 has been a core parent in YAAS.

What is more, the construction and application of the dual-role RIL population based on the cross "Yang16G216/Yang16M6393" not only helped to reveal the genetic basis of the APR conferred by Yang16G216, but also brought great breeding effectiveness. By MAS technique combining agronomic evaluation, several elite wheat lines pyramiding five disease resistance (*Fusarium* head blight, powdery mildew, wheat yellow mosaic virus, stripe and leaf rusts) have been selected from the Yang16G216/Yang16M6393 RIL population (Table S6), and directly introduced to the national wheat regional experiments in the middle and lower reaches of Yangtze River.

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Author contribution statement T.B. and R.Z. conceived and designed the experiments. R.Z., B.L., W.W. and Z.J. conducted genotyping of the RIL populations. R.Z., B.L., T.C. and L.W. conducted phenotyping of the RIL populations. R.Z, B.L. and W.W. performed genetic analysis. R.Z., B.L. and T.B. wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials All the data generated or analyzed in this study are available in the manuscript and the supplementary information.

Declarations

Conflict of interest The authors declare they have no conflict of interest.

Ethical approval The authors declare that the experiments comply with the current laws of the country in which they were performed.

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