Theoretical and Applied Genetics (2024) 137:43
https://doi.org/10.1007/s00122-024-04553-9

ORIGINAL ARTICLE

# Dissecting the genetic basis of Fusarium crown rot resistance in wheat by genome wide association study

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Received: 1 November 2023 / Accepted: 10 January 2024 / Published online: 7 February 2024


## Abstract

***Key message*** **A locus conferring Fusarium crown rot resistance was identified on chromosome arm 3DL through genome wide association study and further validated in two recombinant inbred lines populations.**

**Abstract** Fusarium crown rot (FCR) is a severe soil borne disease in many wheat growing regions of the world. In this study, we attempted to detect loci conferring FCR resistance through a new seedling inoculation assay. A total of 223 wheat accessions from different geography origins were used to assemble an association panel for GWAS analysis. Four genotypes including Heng 4332, Luwanmai, Pingan 998 and Yannong 24 showed stable resistance to FCR. A total of 54 SNPs associated with FCR resistance were identified. Among the 10 putative QTLs represented by these SNPs, seven QTLs on chromosome 2B, 3A, 3D, 4A, 7A and 7B were novel and were consistently detected in at least two of the three trials conducted. *Qfcr.cau.3D-3*, which was targeted by 38 SNPs clustered within a genomic region of approximately 5.57 Mb (609.12–614.69 Mb) on chromosome arm 3DL, was consistently detected in all the three trials. The effects of *Qfcr.cau.3D-3* were further validated in two recombinant inbred line populations. The presence of this locus reduced FCR severity up to 21.55%. Interestingly, the collinear positions of sequences containing the four SNPs associated with two FCR loci (*Qfcr.cau.3A* and *Qfcr.cau.3B*) were within the regions of *Qfcr.cau.3D-3*, suggesting that genes underlying these three loci may be homologous. Our results provide useful information for improving FCR resistance in wheat.

## Introduction

Fusarium crown rot (FCR) is a soil-borne fungal disease which resulted in significant yield losses for wheat worldwide (Kazan and Gardiner 2018). It can be caused by various *Fusarium* species, but mostly by *Fusarium pseudograminearum* and *Fusarium culmorum* (Burgess et al. 2001; Smiley et al. 2005; Nicol et al. 2007; Zhang et al. 2015). The pathogens can survive in the crop residues for several years, making FCR a chronic problem once it occurs in the fields (Burgess 2005; Jansen et al. 2005). FCR was firstly identified in Australia and then was reported in other regions of the world, including USA, Canada, South Africa, the Middle East and China (Burgess et al. 1975; Kazan and Gardiner 2018). The fungi could infect root and stem base of wheat, resulting in brown discoloration on these organs and lead to the formation of white heads at mature stage (Kazan and Gardiner 2018; Gao et al. 2023). The mycotoxin produced during infection is also a concern for human and animal health (Chakraborty et al. 2006; Mudge et al. 2006).

One of the economical and environmentally friendly approaches to control the spread and damage of FCR is growing disease-resistant wheat varieties. Although immune germplasm is not available, those with partial resistance, such as 2–49, Sunco, CSCR6 and EGA Wylie, were identified (Wildermuth et al. 2001; Wallwork et al.2004; Ma et al. 2010; Zheng et al. 2014; Shi et al. 2020). Bi-parental mapping with these genotypes identified many quantitative trait loci (QTL) conferring FCR resistance (Wallwork et al. 2004; Collard et al. 2005, 2006; Bovill et al. 2006, 2010; Ma et al. 2010; Poole et al. 2012; Zheng et al. 2014; Martin et al. 2015; Li et al. 2023a). Some loci were consistently detected in multiple genetic backgrounds. For example, a

Communicated by Susanne Dreisigacker.

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QTL on the chromosome 3BL was repeatedly detected in wheat germplasm CSCR6, W21MMT70, Ernie, IRN497, Macon and Otis, respectively (Bovill et al. 2010; Li et al. 2010; Ma et al. 2010; Poole et al. 2012; Martin et al. 2015). QTL on chromosomes 4B, 2D and 5D were also detected in multiple genetic backgrounds (Wallwork et al. 2004; Bovill et al. 2006, 2010; Ma et al. 2010; Zheng et al. 2014).

As a powerful complementary approach to bi-parental mapping for traits of interest, genome-wide association study (GWAS) is widely employed to identify QTL associated with complex traits in major crops. Compared with bi-parental mapping, GWAS is based on linkage disequilibrium (LD) and usually uses natural populations as QTL mapping resources (Yu et al. 2006; Zhang et al. 2010; Zhou and Stephens 2012). In recent years, many novel and major loci for FCR were identified via this approach (Erginbas-Orakci et al. 2018; Yang et al. 2019; Alahmad et al. 2020; Jin et al. 2020; Pariyar et al. 2020; Rahman et al. 2020, 2021; Malosetti et al. 2021; Lin et al. 2022; Sohail et al. 2022; Hou et al. 2023). For example, Yang et al. (2019) identified a novel FCR QTL on chromosome 6A using a panel of 234 Chinese wheat cultivars released in the Yellow and Huai River wheat region. In the study of Jin et al. (2020), a novel locus on chromosome arm 5DL for FCR resistance was reported. A total of 23 significant and unique marker-trait associations were identified by Rahman et al. (2020).

Although significant efforts have been made, there is still urgent need to identify additional resistant germplasms and loci for FCR resistance since most of current wheat varieties are susceptible to this disease (Mitter et al. 2006; Bhatta et al. 2019; Yang et al. 2019; Jin et al. 2020; Laasli et al. 2022). In this study, we evaluated the FCR resistance level of 223 hexaploid wheat germplasms using a recently published seedling inoculation method (Li et al. 2022). A GWAS analysis was performed using 90 K SNP array. The aims of our study were to: (1) identify genomic regions associated with FCR resistance; and (2) validate the effects of GWAS-identified putative loci in different genetic backgrounds.

## Materials and methods

### Plant materials

A total of 223 wheat germplasms consisting of 153 Chinese cultivars, 36 advanced lines, 25 abroad cultivars, and nine Chinese landraces were used to assemble the association panel for the GWAS analysis (Table S1). Three FCR resistant varieties including 04zhong36, CSCR6, Sunco and one FCR susceptible variety Xinmai 26 were used as control (Jin et al. 2020). Plant height (PH) and heading date (HD) of the 223 accessions were recorded in the field station of China Agricultural University in Jize county, Handan city, Hebei province during seed increasing. Two recombinant inbred line populations, consisting of 113 and 118 $F_6$ recombinant inbred lines (RILs) that were derived from crosses of Chinese wheat varieties Doumai with Shi 4185 and Linmai 2 with Zhongmai 892, were used to validate the effects of putative QTL identified from the GWAS analysis.

### Seeding disease assessment

The *Fusarium pseudograminearum* strain NL5, provided by Professor Haiyan Hu at Henan Institute of Science and Technology, was used for FCR inoculation (Yang et al. 2015). The methods used for inoculum preparation, inoculation and FCR assessment were based on that described by Li et al. (2022). Briefly, NL5 was incubated on half-strength potato dextrose agar plates and grow at room temperatures (25 °C) for five to seven days. When the mycelium grew over the plate and showed peach-red to dark-red pigmentation, ten pieces of 6 × 4 mm culture medium with mycelium were taken out from the plate. The medium was then transferred into a conical flask containing 400 ml carboxymethy1 cellulose (CMC) liquid medium. The conical flask was put into a shaking incubator at 160 rpm and 25 °C for approximately 72 h. The spores were harvested and the concentration of spore suspension was adjusted to $1 \times 10^7$ spores/ml. Tween 20 was added to the spore suspension to a final concentration of 0.5% v/v before for inoculation.

FCR reactions of the 223 accessions were assessed in three trials. Each trial contained two replicates with ten seedlings in each replicate. At 7 days after planting (when most seedlings reached to one leaf and one heart stage), the spore suspensions were injected into the seedling stems using a 10 ml syringe with a needle. The injection stopped when the suspension flowed out from the top of the seedlings. FCR severity was assessed at about 15 days post inoculation by observing the disease symptoms on the leaf sheaths of each plant after inoculation, using a 0 (no obvious symptom) to 6 (the whole plant severely to completely necrotic) scale as described by Li et al. (2022). A disease index (DI) was then calculated for each line following the formula of $DI = (\sum nX/6N) \times 100$, where X is the scale value of each plant, n is the number of plants in the category and N is the total number of plants assessed for each line. Statistical analyses of the phenotype were conducted using IBM SPSS 22.0 software. The broad-sense heritability for each trait was estimated by the formula $H^2 = VG/(VG + VE)$, where VG and VE represent estimates of genetic and environmental variance, respectively.

### Genome wide association analysis

The genotypic data from the wheat iSelect 90 K SNP array for the 223 accessions was obtained from previous studies

(Lou et al. 2021; Wang et al. 2021). After filtering out SNPs with minor allele frequency (MAF) of < 5% and SNPs with > 10% missing data, 19, 496 SNPs remained and were used for following analysis. STRUCTURE 2.3.4 software (Evanno et al. 2005) was used to estimate the genetic population structure of panel based on polymorphic SNPs. A model-based Bayesian clustering approach was performed, where the numbers of hypothetical groups ranged from $K = 1$ to 10, using an admixture model with ten independent runs of 10, 000 burn-in time and 10, 000 MCMC (Markov chain Monte Carlo) replication number. The output from STRU CTURE was analyzed for the delta *K* value (DK) in STRU CTURE HARVESTER (Earl and vonHoldt 2011). The optimal *K* value was determined by the log probability of LnP(*K*) and delta K based on the rate of change of LnP(*K*) between successive *K*. A pairwise distance matrix derived from the simple matching distance for all SNP sites was calculated using TASSEL v5.2.57 (Bradbury et al. 2007) and neighbor-joining tree was visualized with MEGA 7.0 (Kumar et al. 2016).

GWAS was conducted using the mixed linear model by GAPIT packages in R version 4.0.2, and the variance–covariance kinship matrix (*K*) was calculated using the VanRaden method (VanRaden 2008; Zhang et al. 2010; Lipka et al. 2012). A threshold $-\log_{10}(P)$ value > 3.0 was set to detect significant associations between markers. LD analysis for the whole genome and the three sub genomes (A, B and D) of bread wheat was performed using the filtered marker data in TASSEL v5.2.57. Best linear unbiased predictors (BLUP) were estimated for each line using the lme4 package in R version 4.0.2. Collinearity analysis for selected loci among homologous chromosomes was performed on Triticeae-Gene Tribe (TGT) (http://wheat.cau.edu.cn/TGT/; Chen et al. 2020).

### Validation in two recombinant inbred line populations

Kompetitive allele-specific PCR (KASP) markers, which were designed for selected SNPs loci significantly associated with FCR resistance in the GWAS analysis, were used to genotype the two RILs populations of Doumai/Shi 4185 and Linmai 2/Zhongmai 892 to verify the effects of the selected locus in multiple genetic backgrounds. The genomic DNA of these two populations were extracted using a modified cetyltrimethylammonium bromide (CTAB) method (Maguire et al. 1994). Based on screening results, the individuals of the two validation populations were divided into three groups: group 1 carried resistant alleles of the SNP loci; group 2 carried susceptible alleles of the SNP loci, and group 3 were heterozygous and carried both resistant and susceptible alleles, respectively.

Twenty seedlings of each line in the two RILs populations were evaluated for FCR severity. The methods for inoculation and disease assessment were described as above. The average difference in DI between the two groups of homozygous plants was used to validate the effects of the GWAS-identified SNP loci. Student *t*-test was used to determine if there was significant difference between the group 1 and 2.

### Candidate gene analysis

Chinese Spring IWGSC RefSeq v 1.1 (Ma et al. 2021) was used to retrieve high-confidence annotated genes located within the SNP loci that we are interested in. Four parental lines of the two RILs populations, including Doumai, Shi 4185, Linmai 2 and Zhongmai 892, were used to investigate the expressions of selected genes of interest. Samples from these four varieties were harvested by cutting the shoot bases 2 cm at 72 h post inoculation (hpi). RNA was isolated with an RNA isolation reagent TRIzon PalTM from Cowin Biotech Co., Ltd., Jiangsu, Nanjing, China (www.cwbio.com). The qRT-PCR analysis was performed as described by Li et al. (2023b). A total of 30 seedlings from three biological replicates were pooled together to correct for variation between plants during infection. The average values from three technical replications were used in gene expression analysis. Relative expression levels were evaluated according to the relative quantification method ($2^{-\Delta\Delta C}{}_T$) (Livak and Schmittgen 2001). The wheat Actin gene (IPR: *IPR004000*) was used as an internal control. qRT-PCR primer sequences are listed in Table S2.

## Results

### Phenotypic data analysis

In the three trials conducted, the DI values of 223 accessions ranged from 29.17 to 85.94, with a mean value of 58.57–59.20 (Table 1). The correlation coefficients among the three trials and BLUP values varied from 0.86 and 0.97 (Table 2). The broad-sense heritability ($H^2$) of FCR resistance was 0.98, suggesting high level of genetic variations for this trait in the association panel (Table 1). Frequency distributions of FCR severity were approximately normally distributed (Fig. 1). Based on BLUP datasets, a total of seven accessions showed DI values less than 40.01, 21 accessions showed less DI than 50.01, and the majority of the accessions showed DI above 50.01 (195). The DI of resistance controls genotypes CSCR6, 04zhong36 and Sunco were 30.83, 33.33 and 56.67, whereas that of the susceptible control genotype Xinmai 26 was 74.17 (Table S1). Among the seven genotypes with DI values less than 40.01, Heng 4332,

**Table 1** Fusarium crown rot severity of the 223 germplasms in the association population

| Trials | Minimum | Maximum | Mean | SD | CV (%) | $H^2$ |
|---|---|---|---|---|---|---|
| CRS1 | 31.06 | 85.94 | 59.20 | 8.99 | 15.19 | |
| CRS2 | 30.24 | 84.51 | 58.57 | 10.19 | 17.40 | |
| CRS3 | 29.17 | 83.75 | 59.04 | 9.85 | 16.68 | |
| BLUP | 31.67 | 84.35 | 58.90 | 9.02 | 15.31 | 0.98 |

CRS1, average DI values in the first trial; CRS2, average DI values in the second trial; CRS3, average DI values in the third trial; BLUP, best linear unbiased predictions

SD, Standard deviation; CV, coefficient of variation; $H^2$, broad-sense heritability

**Table 2** Pearson correlations of Fusarium crown rot reactions in three trials and BLUP

| Trials | CRS1 | CRS2 | CRS3 | BLUP |
|---|---|---|---|---|
| CRS1 | 1 | | | |
| CRS2 | 0.86 | 1 | | |
| CRS3 | 0.91 | 0.92 | 1 | |
| BLUP | 0.96 | 0.97 | 0.96 | 1 |

CRS1, average DI values in the first trial; CRS2, average DI values in the second trial; CRS3, average DI values in the third trial; BLUP, best linear unbiased predictions

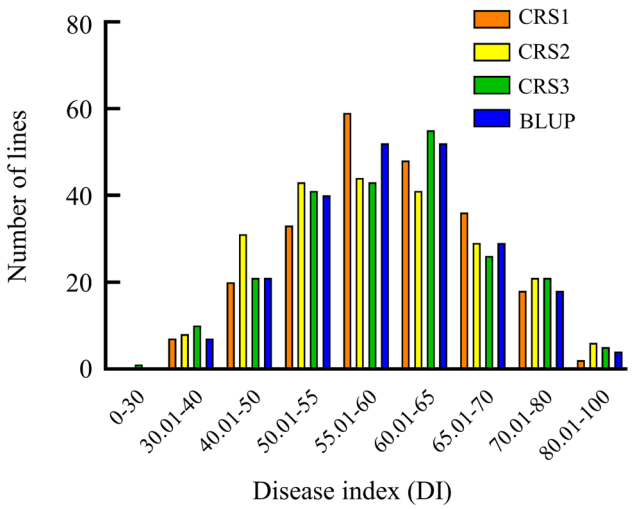


**Fig. 1** Phenotypic distributions of Fusarium crown rot resistance based on three trials and BLUP. CRS1, average DI values in the first trial; CRS2, average DI values in the second trial; CRS3, average DI values in the third trial; BLUP, best linear unbiased predictions

Luwanmai, Pingan 998 and Yannong 24 showed comparable resistance level to 04zhong36 and CSCR6 (Table S3).

## SNP marker statistics

After filtering, 19, 496 SNPs were used for GWAS analysis. A total of 7, 527, 10, 008 and 1, 961 SNPs were located on the A, B, and D genomes, respectively. The average marker densities of the three genomes were 0.65, 0.44, and 2.00 Mb per marker in each of the genomes, respectively. Among the 21 chromosomes, chromosome 1B had the largest number of markers (1959) and highest density (one marker per 0.35 Mb). The number of markers on chromosome 4D was lowest (56), with an average distance of 8.91 Mb between markers (Table 3). We investigated the population structure of the association panel using STRUCTURE software. The delta $K$ value reached a sharp peak at $K=2$. Therefore, this association population was clustered into two subpopulations (Fig. S1a and Table S4). While 106 accessions (47.53%) were assigned into the Q1 group, 117 accessions (52.47%) were assigned into the Q2 group (Table S4). The LD decay distances of A, B and whole genome were similar (2–3 Mb), whereas that of D genome was about 7 Mb (Fig. S1b).

## Loci associated with FCR resistance, PH and HD

A total of 54 SNPs on chromosomes arms 2BL (3), 3AL (2), 3BL (2), 3DS (1), 3DL (39), 4AL (1), 5DL (4), 7AS (1) and 7BS (1) were significantly ($P<0.001$) associated with FCR resistance based on BLUP dataset (Fig. 2; Table 4). Of the 40 SNPs that were consistently detected in all the three trials and BLUP dataset, the SNP *BS00003119_51* on chromosome arm 3DL had the lowest $-\log_{10}(P)$ value (3.06) (Table 4). The $P$ values of the other four SNPs on chromosomes 2BL and 7BS also just reached the significant level (Table 4). In contrast, *wsnp_Ex_c12963_20529964* on chromosome arm 3DL was the most significant SNP associated with FCR resistance ($-\log_{10}(P)=5.67$). Two SNPs including *TA006354-0937* and *Excalibur_c6906_804* which explained the largest phenotypic variations (8.45%) were also located on chromosome 3DL.

Based on LD decay distance, the 54 SNPs represented 10 putative QTLs (Table 4). Of these QTLs, three QTLs located on chromosome 3D were consistently detected in three trials and BLUP dataset. They were designated as *Qfcr.cau.3D-1, Qfcr.cau.3D-2* and *Qfcr.cau.3D-3* based on the orders of their physical positions. The first QTL, namely *Qfcr.cau.3D-1*, was associated with *RFL_Contig5322_219* at 37.11 Mb. The second QTL *Qfcr.cau.3D-2* was associated with SNP *BS00062806_51* at 517.38 Mb.

**Table 3** Molecular marker distribution in the A, B, D and whole genomes

| Chromosome | Number of markers | | | | Map length (Mb) | | | | Density of Marker (Mb/marker) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | A | B | D | Total | A | B | D | Total | A | B | D | Total |
| 1 | 1185 | 1959 | 517 | 3661 | 593.55 | 688.76 | 495.23 | 1777.54 | 0.50 | 0.35 | 0.96 | 0.49 |
| 2 | 1113 | 1584 | 660 | 3357 | 780.71 | 801.25 | 650.74 | 2232.70 | 0.70 | 0.51 | 0.99 | 0.67 |
| 3 | 921 | 1311 | 242 | 2474 | 749.34 | 830.78 | 614.69 | 2194.81 | 0.81 | 0.63 | 2.54 | 0.89 |
| 4 | 868 | 692 | 56 | 1616 | 744.27 | 672.23 | 499.16 | 1915.66 | 0.86 | 0.97 | 8.91 | 1.19 |
| 5 | 1086 | 1878 | 160 | 3124 | 708.44 | 712.69 | 562.83 | 1983.96 | 0.65 | 0.38 | 3.52 | 0.64 |
| 6 | 1151 | 1435 | 192 | 2778 | 615.82 | 720.98 | 472.89 | 1809.69 | 0.54 | 0.50 | 2.46 | 0.65 |
| 7 | 1203 | 1149 | 134 | 2486 | 736.69 | 750.60 | 634.59 | 1371.28 | 0.61 | 0.65 | 4.74 | 0.55 |
| All | 7527 | 10,008 | 1961 | 19,496 | 4928.82 | 4426.69 | 3930.13 | 13,285.64 | 0.65 | 0.44 | 2.00 | 0.68 |

The third QTL *Qfcr.cau.3D-3* was targeted by 38 SNPs clustered within a genomic region of approximately 5.57 Mb (609.12–614.69 Mb) on chromosome 3DL. In addition to these three loci on chromosome 3D, the other seven loci on chromosome 2B, 3A, 3B, 4A, 5D, 7A and 7B were detected in at least two trials and BLUP data set. Of these QTLs, two loci including *Qfcr.cau.3A* and *Qfcr.cau.3B* on chromosome 3AL and 3BL were associated with two SNPs, respectively. Collinearity analysis indicated that the collinear positions of sequences containing four SNPs associated with these two loci were within the regions of *Qfcr.cau.3D-3* (Fig. S2).

Seven SNPs were associated with PH in the association panel, representing five QTLs on chromosome 1B, 2D, 3A, 4D and 5B. Among these putative QTLs, *Qph.cau.2D* associated with two SNPs including *tplb0053n05_793* and *tplb0057n10_689* were located near *Rht8* (Table S5). For HD, a total of 13 significant SNPs representing eight putative QTLs were identified. Chromosome 5AL, which harbored *Vrn1*, had the largest number of SNPs for HD. Similar to PH, no HD-related SNP was on chromosome 3D (Table S5). The correlation analysis indicated that the correlation values between PH, HD and FCR severity were 0.03 and 0.08, respectively (Table S6).

### Validation of *Qfcr.cau.3D-3*

Due to the large number of SNPs associated with *Qfcr.cau.3D-3*, the effect of this locus was further validated in multiple genetic backgrounds. A KASP marker *KASP3D61373* was developed for the significant SNP *Kukri_c19514_1602* associated with this locus (Table S2 and Table 4). Among the four parental lines of the validation populations, Doumai and Linmai 2 carried the resistance alleles of *KASP3D61373* and had DI values of 58.33 and 59.26, respectively. Shi 4185 and Zhongmai 892, which had the susceptible alleles, had DI values of 67.59 and 75.00, respectively (Table S7).

Based on the marker profile of the population of Doumai/Shi 4185, group 1 contained 53 lines with the DI values ranging from 41.30 to 70.83, whereas group 2 contained 52 lines with the DI values varying from 52.50 to 85.19 (Fig. 3). The difference between the two groups was 21.55% (Fig. 3 and Table S7). A similar trend was observed in the population of Linmai 2/Zhongmai 892. The lines with resistant alleles of *Qfcr.cau.3D-3* had an averaged DI value of 56.80, compared to that of lines with the susceptible alleles (DI = 72.26) with the average difference of 21.39% (Fig. 3 and Table S7).

### Putative candidate genes for *Qfcr.cau.3D-3* on chromosome 3DL

Based on LD analysis, genes located within 7 Mb regions on each side of *Qfcr.cau.3D-3* were treated as candidates and a total of 264 high confidence genes were identified. Of these genes, 30 genes may be related to plant disease resistance based on their functions (Table S8). Eight genes were selected for qRT-PCR analysis. Of these genes, the fold changes of five genes failed to reach the significant level (Fig. 4b, c, d, g and h). In contrast, the expression of *TraesCS3D02G524700* encoding pathogenesis-related protein PR-4 significantly increased in the four parental genotypes at 72 hpi (Fig. 4a). Compared with that of Zhongmai 892 and Shi 4185 which carried susceptible allele of *Qfcr.cau.3D-3*, the fold changes of this gene in Linmai 2 and Doumai with resistance alleles were much higher (Fig. 4a). Nearly 18-fold and 15-fold changes of this gene were observed in Doumai and Linmai 2 at 72 hpi (Fig. 4a). Similarly, *TraesCS3D02G535700* and *TraesCS3D02G541500*, which encoded protein kinase (receptor-like cytoplasmic kinase 176) and disease resistance protein RPP13, were also significantly induced by FCR infection in four varieties at 72 hpi (Fig. 4e and f).

## Discussion

A reliable inoculation method which could generate reproducible results is essential for the genetic analysis of FCR resistance in wheat (Liu and Ogbonnaya 2015). Due to the

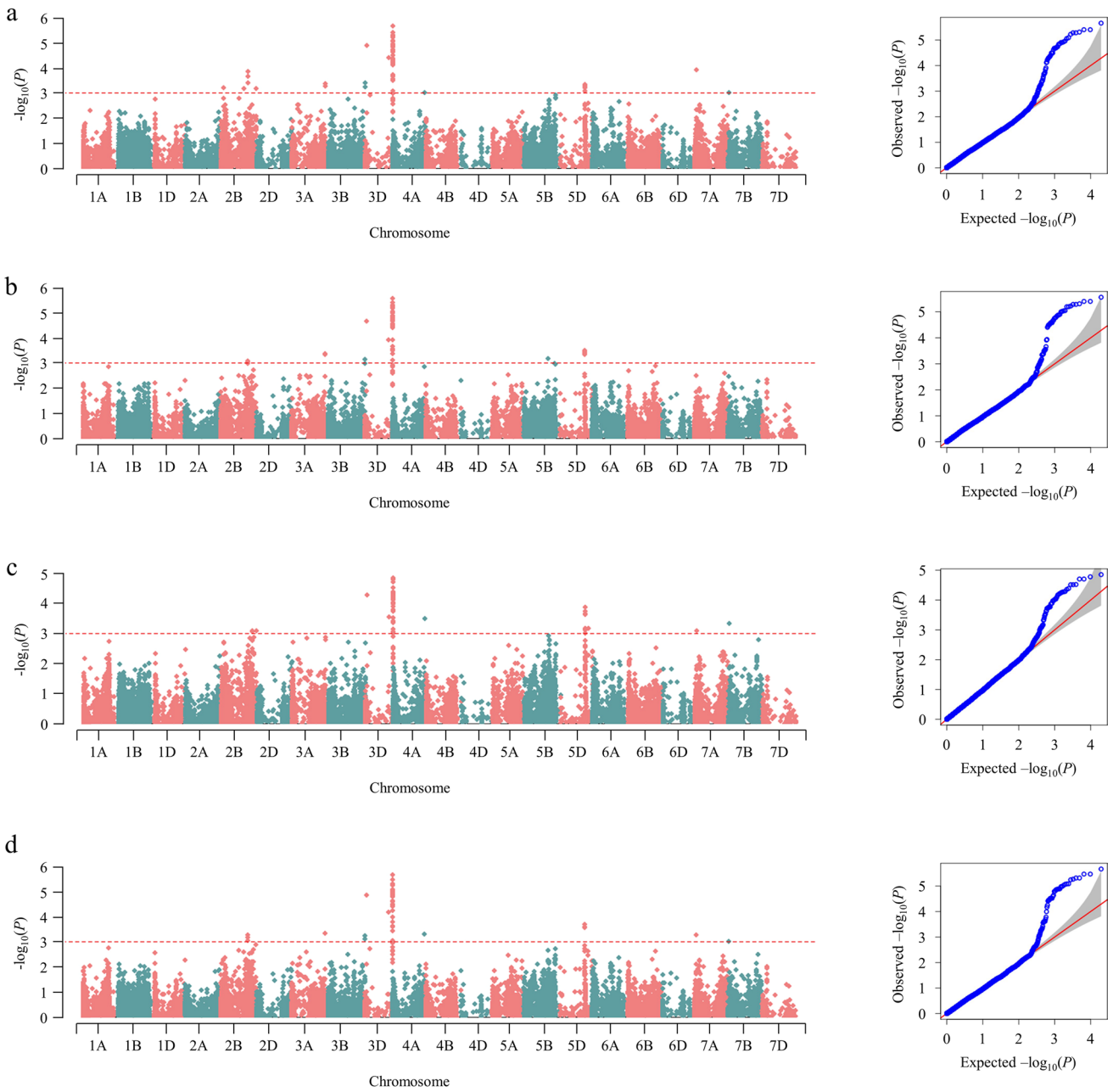


**Fig. 2** Manhattan and Q-Q plots for Fusarium crown rot resistance. The dashed line represents the significance threshold $-\log_{10}(P)$ value of 3.0. **a** Average DI values in the first trial; **b** average DI values in the second trial; **c** average DI values in the third trial; **d** best linear unbiased predictions

long cycle and strong environmental influences in field assays, most of the current FCR-related studies was conducted using wheat seedlings grown in controlled environments (Wildermuth and McNamara 1994; Mitter et al. 2006; Li et al. 2008; Yang et al. 2019; Shi et al. 2020). In those studies, wheat plants were inoculated through various assays such as placing conidia on seedling stem (stem droplet), soaking 1- to 4-day old seedlings in fungal spore suspension (seedling dip) or placing colonized grains above the seed (colonized grain) (Wildermuth et al. 2001; Mitter et al. 2006; Li et al. 2008; Erginbas-Orakci et al. 2018). Previously, we developed a spore suspension injection method to screen a total of 139 wheat and barley germplasms by injecting spore suspensions of *Fusarium pseudograminearum* into their seedling stems (Li et al. 2022). Compared with other inoculation methods, this assay was able to consistently establish a 100% infection and finish disease assessment within a short time frame of approximately 22 days (Li

**Table 4** QTLs for Fusarium crown rot resistance identified by genome-wide association analysis

| QTL | SNP | Chr | Position (Mb) | -log$_{10}$(*P*) | | | | PVE(%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | CRS1 | CRS2 | CRS3 | BLUP | CRS1 | CRS2 | CRS3 | BLUP |
| *Qfcr.cau.2B* | *Ex_c55735_1012* | 2B | 605.00 | 3.42 | 3.05 | – | 3.06 | 5.76 | 6.16 | – | 5.62 |
| | *Kukri_rep_c112675_676* | 2B | 605.00 | 3.85 | – | – | 3.26 | 3.17 | – | – | 3.00 |
| | *wsnp_Ex_c55735_58127324* | 2B | 605.00 | 3.67 | 3.08 | – | 3.17 | 3.14 | 3.23 | – | 3.00 |
| *Qfcr.cau.3A* | *BobWhite_c5246_196* | 3A | 746.63 | 3.27 | 3.35 | – | 3.33 | 5.18 | 6.02 | – | 5.47 |
| | *RAC875_c4841_753* | 3A | 747.61 | 3.38 | 3.37 | – | 3.35 | 4.94 | 5.63 | – | 5.12 |
| *Qfcr.cau.3B* | *CAP7_c3438_196* | 3B | 821.08 | 3.40 | 3.16 | – | 3.24 | 3.09 | 3.38 | – | 3.13 |
| | *IAAV686* | 3B | 822.11 | 3.24 | – | – | 3.12 | 6.00 | – | – | 6.11 |
| *Qfcr.cau.3D-1* | *RFL_Contig5322_219* | 3D | 37.11 | 4.90 | 4.68 | 4.28 | 4.88 | 7.12 | 7.93 | 7.11 | 7.40 |
| *Qfcr.cau.3D-2* | *BS00062806_51* | 3D | 517.38 | 4.41 | 3.94 | 3.54 | 4.17 | 6.49 | 6.95 | 6.16 | 6.55 |
| *Qfcr.cau.3D-3* | *wsnp_Ex_c12369_19730765* | 3D | 609.12 | 3.66 | 3.54 | – | 3.59 | 7.09 | 7.95 | – | 7.31 |
| | *Excalibur_c3821_1355* | 3D | 609.62 | 3.07 | 3.11 | – | 3.06 | 5.62 | 6.48 | – | 5.85 |
| | *RAC875_c5222_59* | 3D | 609.62 | 3.07 | 3.11 | – | 3.06 | 5.62 | 6.48 | – | 5.85 |
| | *Excalibur_c4302_2208* | 3D | 610.35 | 3.56 | 3.51 | 3.15 | 3.60 | 5.94 | 6.73 | 5.98 | 6.23 |
| | *BS00024422_51* | 3D | 611.00 | 3.74 | 3.66 | 3.40 | 3.78 | 5.69 | 6.43 | 5.83 | 5.98 |
| | *D_GDEEGVY02F0MHM_250* | 3D | 611.11 | 4.68 | 4.87 | 4.22 | 4.85 | 6.23 | 7.30 | 6.35 | 6.64 |
| | *wsnp_Ku_c2249_4335279* | 3D | 611.25 | 4.69 | 4.49 | 4.03 | 4.64 | 3.90 | 4.35 | 3.86 | 4.04 |
| | *TA006354-0937* | 3D | 611.25 | 5.40 | 5.40 | 4.70 | 5.47 | 8.05 | 9.20 | 8.02 | 8.45 |
| | *Excalibur_c17654_1090* | 3D | 611.29 | 5.06 | 4.83 | 4.25 | 4.98 | 4.16 | 4.64 | 4.06 | 4.30 |
| | *BS00105800_51* | 3D | 611.55 | 5.22 | 4.88 | 4.36 | 5.09 | 4.28 | 4.70 | 4.15 | 4.39 |
| | *D_GDEEGVY01CO81T_81* | 3D | 612.06 | 3.49 | 3.36 | – | 3.42 | 3.05 | 3.42 | – | 3.15 |
| | *BS00003119_51* | 3D | 612.27 | 3.69 | 3.56 | 3.06 | 3.62 | 3.13 | 3.51 | 3.02 | 3.23 |
| | *Excalibur_c6906_804* | 3D | 612.88 | 5.40 | 5.40 | 4.70 | 5.47 | 8.05 | 9.20 | 8.02 | 8.45 |
| | *wsnp_Ku_c7264_12545135* | 3D | 612.90 | 4.85 | 5.00 | 4.29 | 4.99 | 7.33 | 8.54 | 7.37 | 7.77 |
| | *wsnp_Ex_c12963_20529964* | 3D | 612.90 | 5.66 | 5.56 | 4.85 | 5.67 | 4.18 | 4.74 | 4.13 | 4.36 |
| | *IAAV5582* | 3D | 613.12 | 4.96 | 4.75 | 4.20 | 4.89 | 7.94 | 8.86 | 7.77 | 8.21 |
| | *CAP11_c1051_121* | 3D | 613.39 | 4.74 | 4.90 | 4.01 | 4.81 | 3.78 | 4.42 | 3.07 | 3.98 |
| | *Ku_c9895_1750* | 3D | 613.48 | 5.28 | 5.29 | 4.52 | 5.31 | 7.86 | 9.00 | 7.76 | 8.23 |
| | *Excalibur_c46790_502* | 3D | 613.61 | 4.35 | 4.56 | 3.72 | 4.44 | 6.64 | 7.80 | 6.55 | 7.01 |
| | *BS00087693_51* | 3D | 613.62 | 4.30 | 4.50 | 3.74 | 4.41 | 6.37 | 7.49 | 6.34 | 6.75 |
| | *BS00066691_51* | 3D | 613.69 | 4.12 | 3.91 | 3.34 | 3.99 | 6.88 | 7.63 | 6.58 | 7.04 |
| | *wsnp_Ex_c5061_8986366* | 3D | 613.69 | 4.49 | 4.58 | 3.77 | 4.52 | 7.32 | 8.48 | 7.14 | 7.66 |
| | *Kukri_rep_c87658_1436* | 3D | 613.69 | 4.49 | 4.58 | 3.77 | 4.52 | 7.32 | 8.48 | 7.14 | 7.66 |
| | *Excalibur_c54670_110* | 3D | 613.70 | 4.37 | 4.77 | 3.90 | 4.60 | 6.51 | 7.85 | 6.59 | 7.00 |
| | *RAC875_c51595_177* | 3D | 613.70 | 4.91 | 5.31 | 4.17 | 5.07 | 7.39 | 8.86 | 7.27 | 7.86 |
| | *Kukri_c19514_1602* | 3D | 613.73 | 4.49 | 4.58 | 3.77 | 4.52 | 7.32 | 8.48 | 7.14 | 7.66 |
| | *Excalibur_c15009_1082* | 3D | 613.73 | 4.62 | 5.20 | 3.98 | 4.87 | 6.67 | 8.16 | 6.63 | 7.17 |
| | *RAC875_c19233_256* | 3D | 613.78 | 4.26 | 4.45 | 3.88 | 4.44 | 6.74 | 7.91 | 6.92 | 7.21 |
| | *RAC875_c43838_146* | 3D | 613.78 | 4.93 | 5.22 | 4.27 | 5.08 | 7.46 | 8.83 | 7.42 | 7.92 |
| | *Excalibur_c56069_128* | 3D | 613.78 | 4.32 | 4.66 | 3.74 | 4.48 | 6.67 | 7.98 | 6.63 | 7.11 |
| | *RFL_Contig2432_953* | 3D | 614.20 | 4.13 | 4.40 | 3.51 | 4.25 | 6.77 | 8.04 | 6.64 | 7.17 |
| | *Kukri_c1458_1705* | 3D | 614.20 | 5.31 | 5.20 | 4.39 | 5.24 | 7.98 | 9.01 | 7.71 | 8.26 |
| | *BS00042131_51* | 3D | 614.36 | 4.72 | 5.04 | 4.10 | 4.89 | 7.11 | 8.46 | 7.08 | 7.57 |
| | *BS00078098_51* | 3D | 614.36 | 5.28 | 5.29 | 4.52 | 5.31 | 3.93 | 4.50 | 3.88 | 4.12 |
| | *D_GBF1XID02HLMWB_65* | 3D | 614.63 | 4.84 | 4.79 | 3.97 | 4.79 | 3.89 | 4.43 | 3.74 | 4.03 |
| | *Excalibur_c21265_461* | 3D | 614.67 | 4.68 | 5.02 | 4.52 | 5.03 | 6.98 | 8.32 | 7.40 | 7.59 |
| | *BS00070059_51* | 3D | 614.69 | 4.23 | 4.52 | 4.03 | 4.51 | 6.41 | 7.63 | 6.75 | 6.95 |
| | *BS00070060_51* | 3D | 614.69 | 5.09 | 5.05 | 4.78 | 5.27 | 7.54 | 8.58 | 7.86 | 8.02 |
| *Qfcr.cau.4A* | *Tdurum_contig75819_559* | 4A | 712.86 | 3.02 | – | 3.48 | 3.30 | 4.62 | – | 5.48 | 5.08 |

**Table 4** (continued)

| QTL | SNP | Chr | Position (Mb) | -log$_{10}$(*P*) | | | | PVE(%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | CRS1 | CRS2 | CRS3 | BLUP | CRS1 | CRS2 | CRS3 | BLUP |
| *Qfcr.cau.5D* | *D_GCE8AKX02IXEFJ_281* | 5D | 548.86 | 3.35 | 3.39 | 3.62 | 3.61 | 5.20 | 5.98 | 5.90 | 5.52 |
| | *BS00022688_51* | 5D | 548.87 | 3.27 | 3.35 | 3.73 | 3.70 | 4.98 | 5.78 | 5.85 | 5.67 |
| | *RFL_Contig4176_605* | 5D | 548.88 | 3.07 | 3.48 | 3.64 | 3.56 | 4.86 | 6.00 | 5.84 | 5.55 |
| | *D_GDS7LZN02I3554_251* | 5D | 548.94 | 3.20 | 3.51 | 3.87 | 3.61 | 4.98 | 6.02 | 6.04 | 5.67 |
| *Qfcr.cau.7A* | *BS00069242_51* | 7A | 32.62 | 3.91 | – | 3.08 | 3.28 | 5.72 | – | 5.43 | 5.37 |
| *Qfcr.cau.7B* | *IAAV5530* | 7B | 15.30 | 3.01 | – | 3.32 | 3.02 | 4.91 | – | 5.52 | 5.00 |

PVE, phenotypic variation explained; CRS1, average DI values in the first trial; CRS2, average DI values in the second trial; CRS3, average DI values in the third trial; BLUP, best linear unbiased predictions

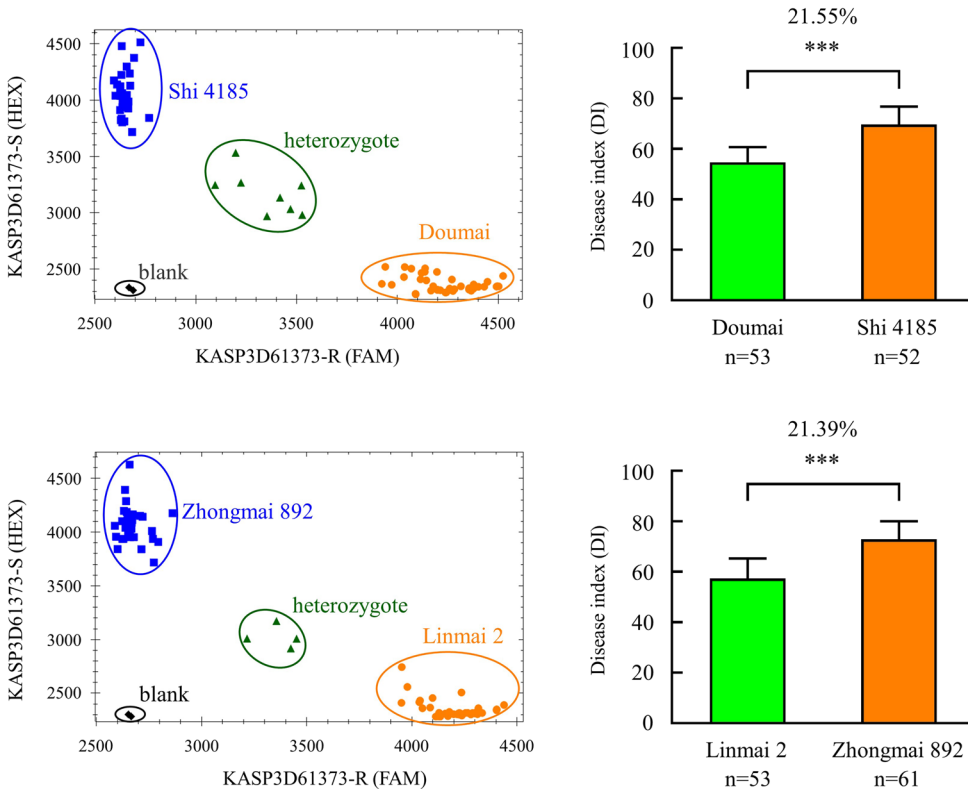


**Fig. 3** Effects of *Qfcr.cau.3D-3* in the 'Doumai/Shi 4185' and 'Linmai 2/Zhongmai 892' populations. 'Red' and 'blue' represent groups with and without the resistant allele of the corresponding QTL. ***, significant at $P < 0.001$

et al. 2022). In this study, we further investigated the appliance of injection assay in GWAS analysis using 223 wheat accessions. Out of the 54 SNPs identified, 40 SNPs (74.07%) were repeatedly detected in all the three trials conducted (Fig. 2; Table 4). The DI values among the three trials were highly correlated (Table 2). Four Chinese wheat germplasms including Heng 4332, Luwanmai, Pingan 998 and Yannong 24 showed similar resistance levels to FCR as the resistant control varieties 04zhong36 and CSCR6 (Table S3). These consistent results at phenotypic and genotypic level demonstrated the potential value of our injection assay in further genetic analysis of FCR resistance.

Of the 10 QTLs represented by the 54 SNPs loci identified in the study, only three were located near known FCR loci. *Qfcr.cau.5D,* which were targeted by four SNPs located between 548.86 and 548.94 Mb on chromosome 5D, was close to the SNP locus at 546.92 Mb reported by Pariyar et al. (2020). Two SNPs associated with *Qfcr.cau.3B* at 821.08 and 822.11 Mb were also located within the genomic region of *Qcrs.cpi-3B* from Spelt wheat CSCR6 reported by Ma et al. (2010). In addition to these known loci, the other seven loci on chromosomes 2B, 3A, 3D, 4A, 7A and 7B may be novel considering their long distances to the known FCR loci on same chromosomes (Table 4) (Bovill et al. 2006;

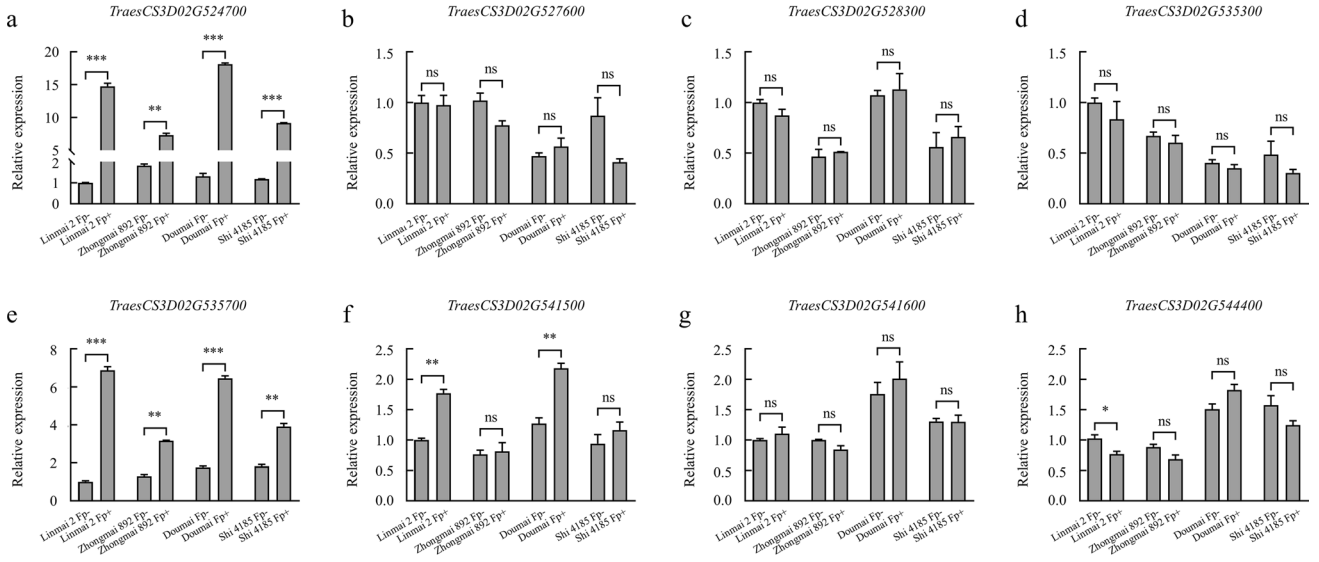


**Fig. 4** Relative expression level of eight selected genes located within the region of *Qfcr.cau.3D-3*. **a** *TraesCS3D02G524700*; **b** *TraesCS3D02G527600*; **c** *TraesCS3D02G528300*; **d** *TraesCS3D02G535300*; **e** *TraesCS3D02G535700*; **f** *TraesCS3D02G541500*; **g** *TraesCS3D02G541600*; **h** *TraesCS3D02G544400*. *, statistically significant at $P<0.05$; **, statistically significant at $P<0.01$; ***, statistically significant at $P<0.001$; ns, not significant; Fp−, mock-inoculated control plants; Fp+, inoculated with *Fusarium pseudograminearum*

Poole et al. 2012; Martin et al. 2015; Jin et al. 2020; Pariyar et al. 2020; Rahman et al. 2020, 2021; Su et al. 2021; Hou et al. 2023). The high proportion of novel loci in the current analysis (70.00%) maybe due to the inoculation method. It was known that wheat resistance to Fusarium head blight, which was another important *Fusarium* disease in wheat, had two major resistance types including resistance to initial pathogen penetration (Type I) and resistance to spread of FHB symptoms (Type II) (Bai et al. 2018). In injection assay, the wound on the leaf sheath created during injection may help FCR pathogens to avoid the resistance mechanisms at the epidermal level (Lai et al. 2020; Li et al. 2022). The loci identified via this method may mainly confer resistance to spread of the pathogens on wheat stems, whereas those identified previously using seedling dip, stem droplet or colonized grains may confer resistance to initial pathogen penetration and spread of pathogens to various extent (Wildermuth and McNamara 1994; Mitter et al. 2006; Li et al. 2008; Yang et al. 2010, 2019). In line with this hypothesis, three partial resistant varieties including Sunco, CSCR6 and 04zhong36, which all showed stable resistance to FCR using seedling dip or colonized grain methods, showed large variations in FCR reactions in injection assay. While CSCR6 and 04zhong36 remained resistant with the DI values of 30.83 and 33.33, the DI value of Sunco reached 56.67 (Ma et al. 2010; Poole et al. 2012; Jin et al. 2020) (Table S1). Similar to this study, significant variations in FCR severity of some other resistant varieties inoculated with different methods were also reported (Erginbas-Orakci et al. 2018). Therefore, it is possible that genes identified by different inoculation methods may confer different types of resistance. Further gene mapping studies using same set of germplasms will help to confirm this hypothesis.

More than half of the SNPs (70.37%) identified in this study were clustered in a region of approximately 5.57 Mb on chromosome arm 3DL. The locus *Qfcr.cau.3D-3* associated with these 38 SNPs was consistently detected in three trials. A previous GWAS analysis also identified a SNP locus *wsnp_Ex_c14027_21925404* in a similar region using 161 diverse international wheat genotypes (Pariyar et al. 2020). The significant amounts of SNPs associated with *Qfcr.cau.3D-3* in this study and the repeatedly identification of this locus via spore suspension injection (this study) and colonized grains (Pariyar et al. 2020) indicated its potential value in FCR breeding program. In addition, it was known that several FCR QTLs have significant interactions with their nearby PH or HD QTLs that also segregated in the same mapping populations (Wallwork et al. 2004; Li et al. 2009; Chen et al. 2013a, 2013b; Zheng et al. 2014). In current study, a total of five QTLs on chromosome 1B, 2D, 3A, 4D and 5B were associated with PH (Table S5). Among these QTLs, *Qph.cau.2D* were located near *Rht8* which was a major gene controlling plant height (Chai et al. 2022). Similarly, of the eight QTLs associated with HD, *Qhd.cau.5A-2* was located near *Vrn1* gene (Table S5) (Yan et al. 2003). Fortunately, none of these QTLs for PH and HD was on chromosome 3D, suggesting *Qfcr.cau.3D-3* is a 'pure' resistance locus independent from the effects of PH and HD (Table 4 and Table S5). On the other hand, as the effects and stability of loci identified from GWAS analysis

usually need to be confirmed in bi-parental populations, we further validated *Qfcr.cau.3D-3* in two RILs populations between four Chinese wheat varieties. The results showed that the presence of this locus could reduce FCR severity up to 21.55% (Fig. 3 and Table S7). Interestingly, collinearity analysis indicated that the collinear positions of sequences containing SNPs associated with two other FCR loci (*Qfcr.cau.3A and Qfcr.cau.3B*) on chromosome 3D were within the regions of *Qfcr.cau.3D-3* (Fig. S2). Similar results were obtained in previous QTL mapping studies, in which FCR loci located on chromosome 3BL in wheat and 3HL in barley were identified (Li et al. 2009; Ma et al. 2010; Liu et al. 2011). It was likely that genes underlying the three loci on group 3 chromosomes in this study are homologous but further experiments are required.

To further investigate the putative candidate genes for *Qfcr.cau.3D-3*, we investigated the expression levels of eight disease-related genes located within the genomic regions of this locus. The expressions of *TraesCS3D02G524700* encoding pathogenesis-related protein PR4 in Linmai 2 and Doumai were much higher than that of Zhongmai 892 and Shi 4185 at 72 hpi. A nearly 18-fold and 15-fold changes of this gene was induced by FCR in Doumai and Linmai 2 at 72 hpi (Fig. 4). PR4 proteins were shown to inhibit the growth of *Fusarium culmorum* and *Fusarium graminearum* (Caruso et al. 1993, 1996, 2001; Bertini et al. 2009). These two pathogens are also casual agents of FCR in wheat (Kazan and Gardiner 2018). These results suggested that *TraesCS3D02G524700* is a strong candidate gene for the trait. However, the roles of other FCR induced genes including *TraesCS3D02G535700 and TraesCS3D02G541500* were also worth further investigations. The products of these two genes were also known to be related to disease resistance (Hall et al. 2009; Ao et al. 2014).

## Conclusion

In this study, we characterized FCR resistance of 223 wheat germplasms using the spore suspension injection method. Four genotypes including Heng 4332, Luwanmai, Pingan 998 and Yannong 24 with stable FCR seedling resistance were identified. GWAS analysis identified 54 SNPs associated with FCR resistance. Based on LD decay distance, these 54 SNPS represented 10 putative QTLs which potentially confer resistance to the spread of FCR pathogen in wheat stems. Of these loci, *Qfcr.cau.3D-3*, which was targeted by 38 SNPs clustered within a region of approximately 5.57 Mb (609.12–614.69 Mb), could reduce up to 21.55% of disease severity in different genetic backgrounds. qRT-PCR analysis confirmed the involvement of several genes inside the region of *Qfcr.cau.3D-3* for FCR resistance. Interestingly, the collinear regions of other two FCR loci (*Qfcr.cau.3A and Qfcr.cau.3B*) on chromosome 3D were within the regions of *Qfcr.cau.3D-3*, suggesting that genes underlying these three loci may be homologous. Our results provide useful genetic resources for further improvement of FCR resistance in the breeding programs and insights on cloning the casual genes underly the *Qfcr.cau.3D-3* locus.

## Author Contribution statement

JM and JL designed the research and drafted the manuscript; JL performed experiments and conducted data analysis; JL, SZ, XX, YS, JY, YG and JY performed phenotypic evaluation; ZZ, BL, QS, CX, JL and JM revised the manuscript. All authors have read and approved the final manuscript.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00122-024-04553-9.

**Acknowledgements** We thank Dr. Xianchun Xia from Chinese Academy of Agricultural Sciences for providing the seeds of two RILs populations of Doumai/Shi 4185 and Linmai 2/Zhongmai 892.

**Funding** This work was supported by National Key Research and Development Program of China (2023YFD2301500).

**Data availability** The datasets used in the current study are available from the corresponding author upon reasonable request.

## Declarations

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

**Ethical standards** The experiments were performed in compliance with the current laws of China.

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