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Delineating a locus conferring *Fusarium* crown rot resistance on chromosome arm 1HL in barley by developing and analysing a large population derived from near isogenic lines☆

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ABSTRACT

Fusarium crown rot (FCR), a chronic and severe disease caused by various *Fusarium* species, is prevalent in semi-arid cropping regions worldwide. One of the major QTL conferring FCR resistance was detected on chromosome arm 1HL (*Qcrs.cpi-1H*) in barley. To develop markers that can be reliably used to incorporate the resistance locus into breeding programs, we developed and assessed a near-isogenic line-derived population consisting of 1180 recombinant inbred lines targeting the locus. Using this population, we delineated *Qcrs.cpi-1H* into an interval of 0.4 cM covering a physical length of about 487 kb. Six markers co-segregating with this locus were generated. Co-linearity for genes located in this interval between the genome of barley and those of either rice or *Brachypodium distachyon* is poor. Three genes with non-synonymous variations between the resistant and susceptible lines were identified within the interval. The results reported in this study not only provide markers for integrating *Qcrs.cpi-1H* into breeding programs, but also form a solid foundation for cloning the causal gene(s) underlying this locus.

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1. Introduction

Fusarium crown rot (FCR), caused by various species of *Fusarium*, is a chronic disease for cereal production in arid and semi-arid cropping regions worldwide [1,2]. Initial infection of FCR is characterized by brown lesions in the crown and lower stem regions and inside leaf sheaths. Under moisture stress, especially during the period between anthesis and milky ripening, 'whiteheads' containing shrivelled or no grains could occur in FCR infected fields. As a result, grain yield and hence crop value can be significantly affected [1,3]. Significant yield losses due to this disease on wheat and barley have been reported in many countries [4–7]. Incidence and severity of FCR have been exacerbated in Australia in recent years, likely due to the increase in the intensity of cereal production for economic reasons and the wide adoption of reduced tillage for moisture conservation [1]. Reduced frequency of precipitation and increased temperature during crop growth have also been identified as possible factors contributing to the exacerbation of the disease [8].

Reducing inoculum load, including crop rotation and stubble burning, has been the focus of management practices for reducing FCR damage [9,10]. This is based on the belief that physical contact of the stem base with infested stubble of the preceding years facilitates the pathogen infection [11,12]. These practices, however, have serious limitations. Stubble burning is not only a serious environmental concern but also leads to loss of soil moisture and reducing beneficial soil microbes. Crop rotation is not always practical as the FCR pathogens can survive several years in stubble [11,12] and growing less valuable crops may also lead to the loss of income. With the wide adoption of precision farming, inter-row sowing has also been recommended for minimizing yield loss from the disease [13].

It was realized for a long time that growing resistant varieties is a critical component in effectively managing FCR [14]. Sources of resistance were identified, and numerous QTL conferring FCR resistance has been detected in both barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.) [15]. However, QTL mapping can only provide limited resolution [16] and, therefore, markers obtained from such studies cannot be reliably used for marker-assisted selection. This is mainly because that some undesirable traits also segregate with the targeted trait in a given mapping population. Plant height and growth rate have been found affecting FCR assessment in both barley [17–22] and wheat [23,24]. Clearly, segregations of these characteristics in a mapping population would make accurate assessment of FCR severity difficult.

The phenotypic difference between the two lines of a near-isogenic line (NIL) pair mainly depends on the difference between their genomes at target locus. This unique feature of NILs makes them highly effective in validating QTL conferring various characteristics [25]. Combined with techniques that can speed up life cycles [26–29], NILs can now be conveniently and quickly obtained for different crop species. These techniques have been used to develop NILs targeting loci conferring FCR resistance in both wheat [30] and barley [31]. Importantly, a NIL-derived population can be conveniently used to develop markers tightly linked with a given locus as, different from those routinely used for QTL mapping, such populations segregate mainly for the targeted locus under investigation. These approaches have been used to investigate an FCR resistance locus on chromosome arm 1HL. This locus, termed as *Qcrs.cpi-1H*, was initially identified from a landrace originated from Japan. Comparing to popular cultivars which are all susceptible to FCR, the Japanese landrace is highly resistant to FCR pathogen inoculation [20]. *Qcrs.cpi-1H* explained up to 33.4% of FCR severity variance [20]. NILs targeting this QTL were generated and used to validate its effects in different genetic backgrounds, and transcriptomic differences between the resistant and susceptible lines for three pairs of the NILs were investigated in a previous study [32]. In the study reported here, we delineated the locus in a refined interval and obtained markers co-segregating with the locus by generating and characterising a large NIL-derived population. By analysing differentially expressed genes located in the refined interval, a small number of candidate genes underlying *Qcrs.cpi-1H* were also identified.

2. Materials and methods

2.1. Plant materials

A NIL-derived population consisting of 1180 lines was generated and used in this study. This population was derived from five different heterozygous F_7 plants obtained in generating the NILs (1H_NIL1) targeting this locus based on the marker *WMC1E8* [32]. A single-seed-descent approach was used to process the F_7 heterozygous plants by five further rounds of self-pollination using the fast-generation method [27]. Seeds from each of the lines were then increased in large pots and used for this study. The population was processed in glasshouses at Queensland Bioscience Precinct in St Lucia, Australia.

2.2. Preparation of inoculum and evaluation of FCR resistance

A highly aggressive *F. pseudograminearum* isolate CS3096, collected in northern New South Wales, Australia [33], was used to assess plant resistance to FCR. Preparation of inoculum, inoculation of pathogen and assessment of disease severity were performed according to the method described by Li et al. [34]. In short, half strength potato dextrose agar plates were used to prepare inoculum. Inoculated plates were kept at room temperature for 12 days before scraping and discarding the mycelium. The plates were then incubated for another 7–12 days under a 12-h photoperiod light combination that consists of cool white and black fluorescent. Spores were then collected and adjusted to spore suspension with concentration at 1×10^6 spores mL^{-1}. To maintain the activity of spores, the suspension was kept in a –20 °C freezer and used within two weeks. Before inoculation, Tween-20 was added (0.1%, v/v) to the thawed spore suspension.

Seeds were germinated on water-saturated filter paper in Petri dishes. After immersed in the spore suspension for 1 min, two three-day-old seedlings were planted into a 3 cm square punnet containing sterilized "University of California

mix C" (50% sand and 50% peat, v/v). The punnets were randomly placed in a controlled environment facility (CEF). The environmental settings for the CEF included 25/18 (±1) °C day/night temperature, 65%/80% (±5)% day/night relative humidity, and a 14-h photoperiod with 500 μmol m^{-2} s^{-1} photon flux density at the plant canopy level. We only watered the inoculated seedlings when they began to wilt.

To confirm the location of the targeted locus and identify markers flanking it, a subpopulation containing 88 of the NIL-derived lines was assessed in three independent inoculation trials. Each trial contained two replicates and 14 seedlings per line were used in each of the replicates. Markers flanking the targeted locus developed based on the subpopulation were then used to identify recombinant lines from the whole NIL-derived population. Five independent trials were conducted on the recombinant lines identified. The resistant and susceptible isolines of the NIL pair 1H_NIL1 were used as positive and negative controls, respectively, in each inoculation trial. FCR severity was assessed four weeks post inoculation with a 0–5 scorings, where 0, no obvious symptom; 1, obvious necrotic lesion on coleoptile or 1st leaf sheath; 2, the 1st leaf sheath and below subcrown internode partially necrotic; 3, the 2nd leaf sheath and the below sub-crown internode completely necrotic with clear reduction (but no more than 50%) of plant/seedling height; 4, the 3rd leaf or leaf sheath and the below sub-crown internode partially or completely necrotic with severe (>50%) reduction in plant/seedling height; 5, whole plant severely to completely necrotic [34].

2.3. Phenotypic data analysis

Statistical analyses of all phenotypic data were performed using the R programming language [35]. For each trial, the following mixed-effect model was used: $Y_{ij} = \mu + r_i + g_j + w_{ij}$, where Y_{ij} is trait value on the jth genotype in the ith replication, μ is general mean, r_i is effect due to ith replication, g_j is effect due to the jth genotype, w_{ij} is error or genotype by replication interaction; genotype and replication was treated as a fixed and random effect, respectively. The disease scores from all seedlings for each of the NIL-derived lines were averaged to determine whether a given line is resistant (<2.5) or susceptible (>2.5) to FCR infection.

2.4. Identification of the targeted interval and marker development

The *Qcrs.cpi-1H* had been mapped into a physical interval of ~11 Mb in a previous study based on RNA-seq analysis against several sets of the NILs targeting the locus [32]. Insertion/deletion (InDel) and Kompetitive allele specific PCR (KASP) markers targeting this interval were developed and used in this study. The InDel variants were identified based on the variants between the pseudomolecule of 'Morex' [36] and an assembly of a wild barley (*H. spontaneum* L.) genotype AWCS276 [37] using SSR-finder (https://github.com/GouXiangJian/SSR_finder). For KASP markers, SNPs within the interval were detected using RNA-seq sequence from three of these NIL pairs [32] on CLC genomic workbench platform V11.0 (CLC Bio, Aarhus, Denmark). All primers were designed using the Primer-BLAST [38] and the primer sequences are listed in Tables S1 and S2. MSTmap Online [39] was used to build linkage maps with the following parameters: grouping logarithm of the odds ratio (LOD) criteria, single LG; population type, RIL10+; no mapping missing threshold, 0%; no mapping distance threshold, 1 centiMorgan (cM); no mapping size threshold, 2; try to detect genotyping errors, yes; and genetic mapping function, Kosambi. The genetic linkage map was plotted using MapDrawJZ (https://github.com/pinbo/MapDrawJZ), a modified version of MapDraw V2.1 [40].

2.5. DNA extraction and genotyping

Leaf tissue from each line of the NIL-derived population was collected and vacuum dried for DNA extraction using the CTAB protocol [41]. KASP assay were conducted using 384-well set on the Vii 7 Real-Time PCR system (Applied Biosystems, Foster City, California, USA) following the "KASP genotyping trial kit user guide" (https://biosearch-cdn.azureedge.net/assetsv6/KASP-genotyping-trial-manual.pdf) and "Guide to running KASP genotyping reactions on the ABI Viia7 instrument" (https://biosearch-cdn.azureedge.net/assetsv6/running-KASP-on-ABI-Viia7.pdf). InDel makers were assessed according to the method described by Zheng et al. [42].

2.6. Identification of candidate genes, nonsynonymous SNPs and collinearity analysis

Annotations of both high (HC) and low confidence (LC) genes in the genomic interval defined by the two flanking markers for the *Qcrs.cpi-1H* locus were extracted from the barley archive in Ensembl Plants (http://plants.ensembl.org/Hordeum_vulgare/Info/Index). SNPs contained in these genes were identified using snpEff 4.3q [43]. The variant database was built based on the international barley reference genome of 'Morex' and its annotation file [36]. Orthologs for candidate genes surrounding the 1HL locus in *Brachypodium distachyon* and rice (*Oryza sativa* L.) were extracted using Ensembl Plant BioMart [44].

3. Results

3.1. Validation of the chromosomal interval containing Qcrs.cpi-1H based on analysing the subpopulation with 88 NIL-derived lines

Based on results from the RNA-seq analysis [32], four InDel markers targeting the interval were developed using the sequence differences between the resistant and susceptible NILs. Together with *WMC1E8* (the marker initially used for generating the NILs), the five markers all segregated in the subpopulation (Fig. 1a). Linkage analysis showed that they spanned a genetic distance of ~7.3 cM and covered a length of ~5.2 Mb in the barley reference genome of 'Morex'. FCR severity assessment of this subpopulation showed that all lines fell into a binary pattern, i.e., their FCR severity scores belonged to either the resistant or susceptible classes (Fig. S1).

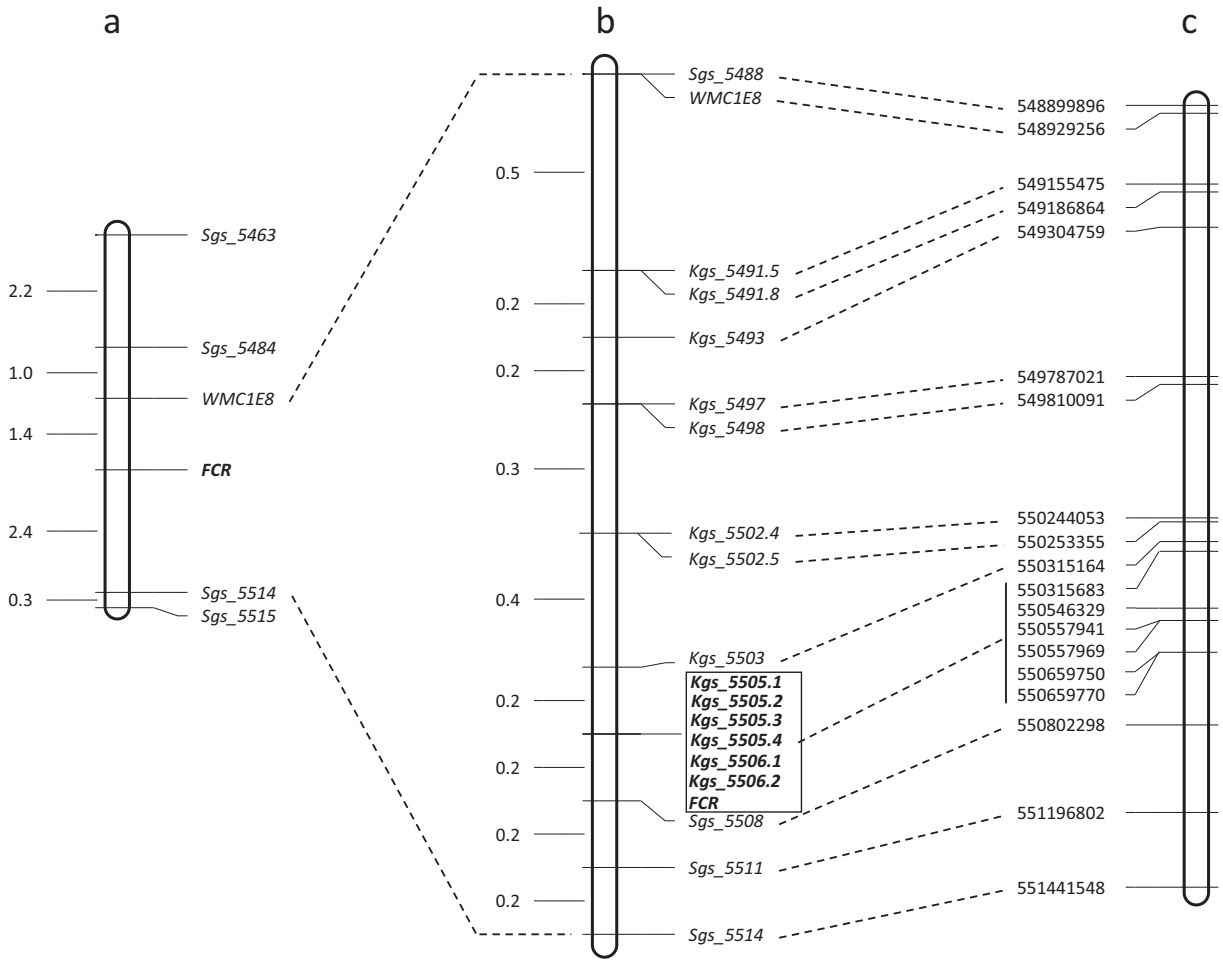

Fig. 1 – Genetic and physical maps surrounding the *Fusarium* crown rot resistance locus *Qcrs.cpi-1H* in barley. (a) The targeted interval based on the assessment of a subpopulation consisting of 88 NIL-derived lines. (b) The high-density linkage map surrounding *Qcrs.cpi-1H* based on the analysis of the whole population consisting of 1182 NIL-derived lines. Markers co-segregating with the locus are in bold and placed in a box. (c) Physical positions of markers surrounding *Qcrs.cpi-1H* on the 1H pseudomolecule of the 'Morex' genome.

After analysing the marker profiles and the phenotypic data, *Qcrs.cpi-1H* was mapped into a ~2.6 Mb interval flanked by *WMC1E8* and *Sgs_5514* (Fig. 1a).

3.2. Fine mapping of the *Qcrs.cpi-1H* locus using the NIL-derived population

WMC1E8 and *Sgs_5514* were used to screen the whole NIL-derived population containing 1180 lines. Twenty-five recombinant lines were identified between these two markers. FCR assessments against these recombinants found that six of them were FCR resistant and the other 19 susceptible (Fig. 2). The difference in FCR severity between the two groups of recombinants was highly significant ($P < 0.01$; Student's *t*-test).

To construct a high-density map spanning the targeted interval, 14 KASP markers and three InDel markers between the two flanking markers were generated and assessed against the 25 recombinant lines. Linkage analysis showed that the FCR locus co-segregated with six of these markers and it was placed at 0.2 cM proximal to *Kgs_5503* and 0.2 cM distal to *Sgs_5508* (Figs. 1b, 2). The linkage order of the markers surrounding *Qcrs.cpi-1H* was identical with their relative physical positions in the 'Morex' genome (Fig. 1b, c). Based on the physical positions of its flanking markers, *Kgs_5503* and *Sgs_5508*, *Qcrs.cpi-1H* was delimited to a ~487 kb genome interval from 550.3 Mb to 550.8 Mb on the 1H pseudomolecule of 'Morex'.

3.3. Identification of candidate genes in the targeted region

Based on the 'Morex' genome, the targeted interval contained 13 high confidence (HC) and 15 low confidence (LC) genes (Table S3). The LC genes were not taken into further consideration due to lack of clear functional annotation. Expression profiles and single nucleotide variants of the 13 HC genes were examined using RNA-seq data generated from the three pairs of the NILs targeting *Qcrs.cpi-1H* [32]. Six of these HC genes differentially expressed in at least one of the NIL pairs were used (Fig. 3). SNPs between resistant and

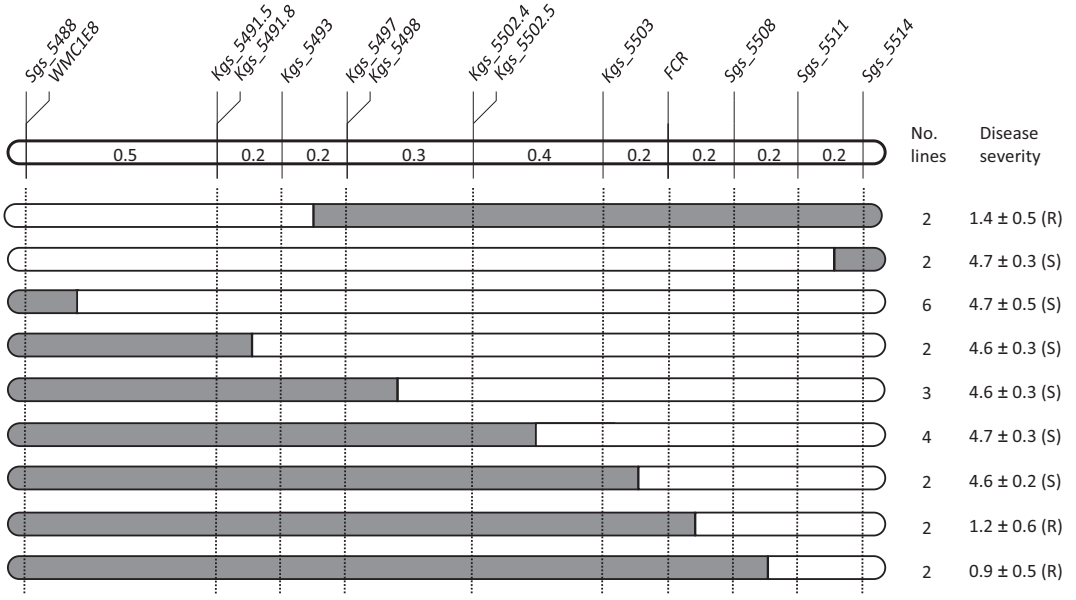

Fig. 2 – Genotypes and phenotypes of the key recombinant lines identified with markers surrounding the *Qcrs.cpi-1H* locus. The solid regions represent alleles from the resistant parent and the empty ones for alleles from the susceptible parent. Numbers of recombinant lines and corresponding FCR severity (mean ± SE) were provided on the right-hand side of the diagram. The numbrs between makers stand for genetic distance (cM).

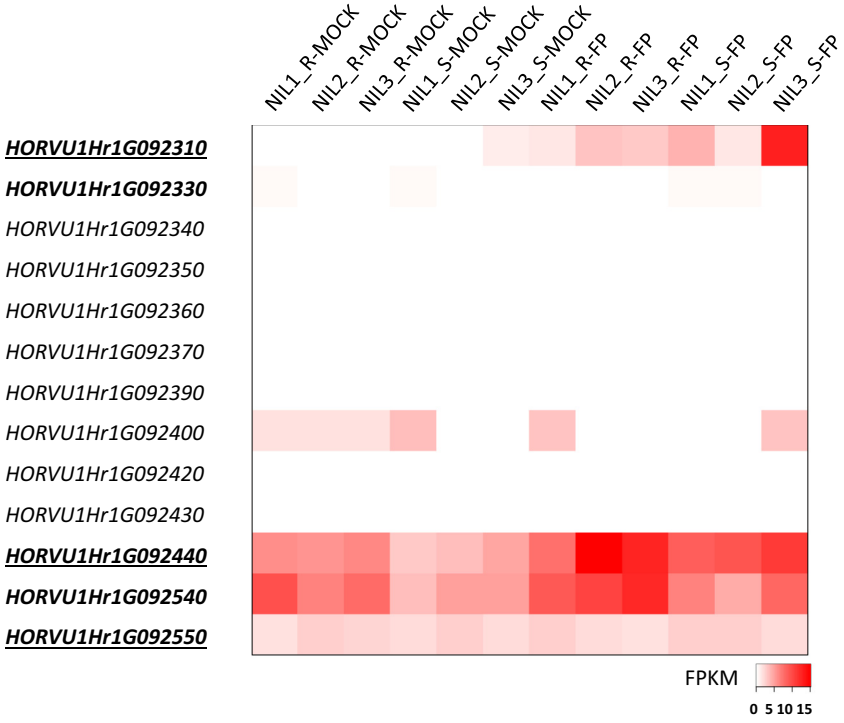

Fig. 3 – Expression profiles of the high confidence (HC) genes among the three pairs of near-isogenic lines targeting the FCR resistance locus *Qcrs.cpi-1H*. The heatmap shows the levels of absolute expression of these genes in resistant (R) and susceptible (S) NILs 4 days post water (MOCK) or *Fusarium pseudograminearum* (FP) treatment. Genes carrying SNP(s) were in bold and the genes containing non-synonymous SNP(s) were underlined.

susceptible isolines were identified in five of the HC genes. SNPs in three of the genes led to non-synonymous variations (Fig. 3; Table S4). Two of these genes *HORVU1Hr1G092310*, encoding a glucan endo-1,3-beta-glucosidase, and *HORVU1Hr1G092440*, encoding a P-loop containing nucleoside triphosphate hydrolases superfamily protein, carrying non-synonymous SNPs were up-regulated in each of the NILs following FCR inoculation. The third gene containing a non-synonymous SNP, *HORVU1Hr1G092550*, encodes a receptor-like kinase. This gene expressed consistently in all the NILs, with or without FCR inoculation (Fig. 3).

3.4. Collinearity between the genes in the targeted interval and those in *Brachypodium* and rice

Collinearity for genes located in the identified interval containing the targeted locus *Qcrs.cpi-1H* was assessed with their corresponding regions in the genomes of *Brachypodium* and rice (Table 1; Fig. S2). This analysis found that gene collinearity in this interval among these three species was poor. Orthologs for only three of the 13 barley genes were found in the corresponding interval in the *Brachypodium* genome, and the orders of the genes were different between the two genomes. Of the 10 genes in the *Brachypodium* genome, orthologs for seven of them were not found in the barley genome. Similarly, orthologs for only four of the 13 barley genes in the targeted interval were detected in the corresponding region of the rice genome and the orders were different between the two genomes. Orthologs for six of the 10 genes in the rice genome were not found in the barley genome (Table 1, Fig. S2). Of the *Brachypodium* and rice genes absent in the barley genome, six were annotated with a wide range of functions. Two of them were found in both genomes of *Brachypodium* and rice, one encoding a late embryogenesis abundant protein and the other with unknown functions (Table 1).

4. Discussion

In this study, we developed and assessed a large NIL-derived population that was constructed for a major FCR QTL *Qcrs.cpi-1H*. Six markers co-segregated with this locus were developed and the candidate genes were refined. These results should be helpful not only in incorporating the resistance locus into breeding programs but also in identifying the causal gene(s) underlying the locus.

Reproducible and reliable phenotypic data are critical for high quality mapping of any locus [45]. Previous studies have repeatedly shown that several characteristics affect the accurate assessment of FCR severity. This includes both plant height [18,23,46] and flowering time [19,42]. For minimizing the interference from the segregations of these non-targeted characteristics in FCR assessment, a NIL-derived population targeting the *Qcrs.cpi-1H* locus was developed and used in the study reported here. As expected, FCR severities among the lines of this large NIL-derived population were easily categorized into ether a resistant or susceptible class,

Table 1 – Candidate genes surrounding the *Qcrs.cpi-1H* locus and their orthologs in *Brachypodium distachyon* and *Oryza sativa*.

Brachypodium distachyon	*Hordeum vulgare* [a]	*Oryza sativa* [a]	Putative function [b]
			F-box family protein
		Os05g0583551	Hypothetical conserved gene
BRADI_2g15400v3	*HORVU1Hr1G092310*		Glucan endo-1,3-beta-glucosidase 13
	HORVU1Hr1G092330		Sugar transporter protein 7
	HORVU1Hr1G092340	*Os05g0584900*	S-type anion channel SLAH2
	HORVU1Hr1G092350		Sulfate transporter 4;2
BRADI_2g15405v3	*HORVU1Hr1G092360*	*Os05g0583600*	WRKY DNA-binding protein 27
		Os05g0583950	Hypothetical conserved gene
		Os05g0584200	Late embryogenesis abundant protein
	HORVU1Hr1G092370		Ycf68
	HORVU1Hr1G092390		Cell wall-associated hydrolase
BRADI_2g15410v3	*HORVU1Hr1G092400*		Cell wall-associated hydrolase
	HORVU1Hr1G092420		30S ribosomal protein S15, chloroplastic
	HORVU1Hr1G092430		Unknown function
		Os05g0584750	Hypothetical protein
BRADI_2g15490v3	*HORVU1Hr1G092440*	*Os05g0584600*	P-loop containing nucleoside triphosphate hydrolases superfamily protein
BRADI_2g15480v3			Similar to Dihydrodipicolinate synthase 1, chloroplast precursor
		Os05g0584450	Hypothetical gene
BRADI_2g15471v3		*Os05g0584400*	Conserved hypothetical protein
BRADI_2g15460v3			Glycosyl transferase
BRADI_2g15450v3			Glycosyl transferase
BRADI_2g15440v3			Glycosyl transferase
BRADI_2g15420v3	*HORVU1Hr1G092540*	*Os05g0584300*	Late embryogenesis abundant hydroxyproline-rich glycoprotein family
	HORVU1Hr1G092550		LRR receptor-like serine/threonine-protein kinase GSO2

[a] The barley genes were listed as their physical order on the 1H pseudomolecule of 'Morex'; positions of the *Brachypodium* and rice orthologs were adjusted according to the positions of their orthologs in the barley genome.
[b] Putative functions of the genes were retrieved from Ensembl Plants (http://plants.ensembl.org/index.html).

making it possible to accurately place the targeted locus in a well-defined genomic interval.

Of the 13 HC genes located within the targeted interval containing the FCR resistance locus *Qcrs.cpi-1H*, six were detected in the transcriptomic data obtained in studying genes responsive to FCR infection using three pairs of the NILs targeting this locus [32]. It was identified that SNPs caused nonsynonymous mutations between resistant and susceptible alleles in the three HC genes, and that these genes played a key role in plant defence against pathogens. One of the genes, *HORVU1Hr1G092550*, encodes a receptor-like kinase (RLK) which has been identified in various immune systems of plants [47]. RLK, usually located on either plasma or cytoplasmic membrane, is able to recognize elicitors generated by pathogens and triggers downstream defence responses in the plant to avirulent pathogens [48]. The second gene, *HORVU1Hr1G092310*, encodes a glucan endo-1,3-beta-glucosidase which is known to be involved in systemic acquired resistance [49]. This enzyme plays an important role in seed plant defence against pathogen attack through the degradation of fungal cell wall polysaccharides [50]. The third gene *HORVU1Hr1G092440* encodes a P-loop containing nucleoside triphosphate hydrolase (P-loop NTPase) which is known to negatively regulate the abiotic stress and plant defence response in both rice and *Arabidopsis* [51,52].

Recently, an updated Morex genome assembly was released [53]. We analysed the collinearity of the candidate genes between this new and the earlier versions. Most of the LC genes disappeared in the new version. However, the two versions showed a highly conserved collinearity for the HC genes.

Clearly, the three genes with non-synonymous variations between the resistant and the susceptible NILs must be carefully examined in identifying gene(s) underlying FCR resistance at the targeted locus. However, recent studies show that non-classical NBS-LRR genes can also be responsible for resistance to a wide range of pathogens in plants [54,55]. They include the *Fhb1* gene conferring *Fusarium* head blight (FHB) in wheat [56,57]. Results from previous studies showed that *Fusarium* pathogens causing FHB can also lead to FCR [1]. Similar to the situation for FHB [58], host resistance to FCR is also not pathogen species-specific [23,46]. The common aetiology between FHB and FCR raises the possibility that resistance to the latter may also be conferred by non-classical NBS-LRR genes.

It has become clear in recent studies that large numbers of genes in a given species are 'dispensable', thus gene(s) underlying the FCR resistance locus *Qcrs.cpi-1H* may not necessarily be present in the genome of 'Morex' which is highly susceptible to FCR. For example, the components of dispensable genes are about 50% in maize (*Zea mays* L.) [59], 43% in rice [60]; 36% in bread wheat [61,62] and 38% in barley [63]. To identify additional genes likely located in the targeted interval, we analysed the corresponding genome regions in both *Brachypodium* and rice. This analysis found that synteny for the targeted genomic regions among the three species is poor. Several of the genes in the 'Morex' genome were not found in the orthologous regions of either *Brachypodium* or rice, and the orders for the few shared orthologs are often different.

Declaration of competing interest

The authors declare no conflicts of interest.

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Author contributions

Chunji Liu and Meixue Zhou conceived the experiments. Shang Gao and Zhi Zheng designed the experiments. Shang Gao, Yunfeng Jiang and Miao Liu developed the NIL-derived population. Shang Gao, Zhi Zheng and Haiyan Hu conducted the inoculation experiments. Shang Gao and Zhi Zheng designed SSR and KASP markers and genotyped the population. Shang Gao and Jiri Stiller analysed the data. Shang Gao wrote the first version of the manuscript. Chunji Liu revised the manuscript and wrote its final version. All authors reviewed and approved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cj.2020.03.008.

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