

Near-isogenic lines developed for a major QTL on chromosome arm 4HL conferring *Fusarium* crown rot resistance in barley

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Abstract Ten pairs of near-isogenic lines (NILs) were developed for a major quantitative trait locus conferring *Fusarium* crown rot (FCR) resistance in barley. The locus locates on the long arm of chromosome 4H and the presence of the resistance allele reduced FCR severity by between 32.8 and 63.4 % with an average of 43.9 % across these NILs. Histological and quantitative PCR analyses all confirmed that the rates of *Fusarium* infection and disease development were much slower in the resistant isolines compared with those in the susceptible isolines. The contrasting phenotypes in FCR severity between the isolines for each of these NIL pairs would facilitate efforts in cloning and functional analyses of genes conferring resistance to this disease.

Keywords *Fusarium* crown rot · Disease resistance · Near isogenic lines · Barley

Introduction

Fusarium crown rot, caused by various *Fusarium* species, is a severe and chronic disease of cereals found in many parts of the semiarid regions of the world (Chakraborty et al. 2006). Several methods have been evaluated to reduce damage inflicted by this disease but none of these practices seem to be very effective as the frequency of FCR has increased recently in Australia as well as many other cereal growing regions worldwide. About 13 % of yield lost has been reported in the Pacific Northwest of USA (Smiley et al. 2005) and a recent survey in Australia found an estimated annual yield loss of \$97 million Australian dollars in wheat and barley combined (Murray and Brennan 2009, 2010).

It was recognised a long time ago that growing resistant cultivars has to be an integral component in effectively managing crown rot (Wildermuth and Purss 1971). Working toward the breeding of resistant varieties, several QTL have been detected in both wheat and barley (Liu and Ogonnaya 2015). However, QTL mapping offers only limited resolution (Tanksley 1988) due to the heterogeneity in genetic backgrounds in mapping populations in regard to a targeted locus thus markers obtained from QTL mapping studies can often not be reliably used to tag the targeted locus. As for FCR, it has been reported that both plant height and heading date have significant effects on FCR assessment in both wheat (Li et al. 2009; Liu et al. 2010) and barley (Li et al. 2009;

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Liu et al. 2010; Chen et al. 2013a; Bai and Liu 2015; Chen et al. 2015). Segregation of these non-targeted traits need to be fixed in developing reliable markers targeting a FCR locus. One of the approaches to obtain populations with uniformed genetic backgrounds is to develop and exploit a series of NILs (Tanksley 1988; Kaeppler et al. 1993). NIL-derived populations, segregating primarily for a targeted locus, allow the conversion of a quantitative trait into a Mendelian factor thus making accurate positioning of a QTL possible (Liu et al. 2006).

Several genotypes with high levels of resistance were identified from systematic screening in barley (Liu et al. 2012a). Genetics of FCR resistance in two of the most resistant genotypes, one belonging to *Hordeum spontaneum* (L.) and another to a landrace (*H. vulgare* L.) from Japan, has been investigated by QTL mapping. Three large-effect QTL, located on chromosomal arms 1HL, 3HL and 4HL, were detected from these two genotypes (Chen et al. 2012, 2013b). Among these QTL, the one on 4HL seems to have the largest effect and explained up to 45 % of the phenotypic variance with a LOD value of 16.4 (Chen et al. 2012). As part of our long term objectives to develop diagnostic markers for and investigate functions of FCR resistance genes in cereals, we have developed NILs for this large-effect QTL in different genetic backgrounds and describe them in this paper.

Materials and methods

Plant materials

The method of heterogeneous inbred family (HIF) (Tuinstra et al. 1997) combined with the fast-generation technique (Zheng et al. 2013) was used to develop NILs in glasshouses at Queensland Bioscience Precinct, Brisbane. Three segregating populations including ‘Baudin/AWCS276’, ‘Lockyer//AWCS276/AWCS079’ and ‘Commander//AWCS276/AWCS079’ were used in this study. The genotype AWCS276 is the resistance donor identified from a screening of 1047 genotypes representing different geographical origins and plant types (Liu et al. 2012b).

Molecular marker analysis

SSR markers linked most closely to the QTL were used to identify individual lines with or without resistance allele in each of the three populations (Chen et al. 2013a). The SSR marker *WMS6* (forward primer CGTATCACCTCCTAGCTAACTAG and reverse primer AGCCTTATCATGACCCTACCTT) was used for identifying heterozygous lines from the segregating populations of ‘Lockyer//AWCS276/AWCS079’ and ‘Commander//AWCS276/AWCS079’. As this marker was not polymorphic in the population of ‘Baudin/AWCS276’, another SSR marker linked closely to the peak of the QTL, ‘*HVM67*’ (forward primer GTCGGGCTCCATTGC TCT and reverse primer CCGGTACCCAGTGAC GAC) (Chen et al. 2013b), was used for this third population. The primer sequences for these markers were obtained from Varshney et al. (2007). The methods used for DNA isolation and marker analysis were as described by Chen et al. (2012). Polymerase chain reactions (PCR) were carried out in a total volume of 12 µL containing 25 ng genomic DNA, 0.2 µM of forward and reverse primers, 3 mM MgCl₂, 0.2 µM dNTPs and 0.5 U *Taq* DNA polymerase. PCR products were labelled with α -³³[P]dCTP (3000 ci/mmol). Reactions were run on a Gene Amp PCR system 2700 thermocycler (PE Applied Biosystems, Foster City, Calif) programmed with the cycling conditions of: one cycle for 3 min at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at the appropriate annealing temperature (ranging from 50 °C to 56 °C depending on specific markers) and 1 min at 72 °C, with a final extension of 5 min at 72 °C. The PCR products were mixed with an equal volume of loading dye, denatured at 95 °C for 5 min, and 3.8 µl from each sample was run on a denaturing 5 % polyacrylamide gel (20:1) at 90 W for 2 h. The gels were subsequently dried using a gel dryer for 30 min at 80 °C and exposed to Kodak X-Omat X-ray film for 5–7 days.

Identified heterozygous plants were then self-pollinated and eight to ten plants derived from each of the heterozygous plants were used for next round of selection. This process of selecting heterozygous plants and self-pollination was repeated until F₈ generation. Two isolines, one with and another

without the resistance allele from AWCS276, were then selected from each of the F_8 heterozygous plants and were treated as a pair of putative NILs. Seeds from these putative NIL pairs were then increased and their FCR severities assessed.

FCR inoculations and assessments

A highly aggressive *F. pseudograminearum* isolate (CS3096) collected in northern New South Wales and maintained in the CSIRO collection (Akinsanmi et al. 2004) was used in this study. Inoculum preparation, inoculations and FCR assessments were as described by Li et al. (2008). Briefly, inoculum was prepared using plates of $\frac{1}{2}$ strength potato dextrose agar. Inoculated plates were kept for 12 days at room temperature before the mycelium in the plates were scraped and discarded. The plates were then incubated for a further 7–12 days under a combination of cool white and black fluorescent lights with 12-h photoperiod. The spores were then harvested using double distilled water and the concentration of spore suspension was adjusted to 1×10^6 spore ml^{-1} . The spore suspensions were then used directly for inoculation or stored in minus 20 °C freezer until needed and Tween 20 was added (0.1 % v/v) to the spore suspension prior to use.

Seeds were germinated in Petri dishes on three layers of filter paper saturated with water. Seedlings of 3-day-old were immersed in the spore suspension for 1 min and two seedlings were planted into each 5 cm square punnet (Rite Grow Kwik Pots, Garden City Plastics, Australia) containing sterilized University of California mix C (50 % sand and 50 % peat v/v). The punnets were arranged in a randomized block design in either a glasshouse or a controlled environment facility (CEF). Settings for the glasshouse were: 25/18 (± 1) °C day/night temperature and 65/80 % (± 5) % day/night relative humidity, with natural sunlight levels and variable photoperiod depending on the time of year. The settings for the CEF were: 25/16 (± 1) °C day/night temperature and 65/85 % day/night relative humidity, and a 14-h photoperiod with $500 \text{ mol m}^{-2} \text{ s}^{-1}$ photon flux density at the level of the plant canopy. To promote FCR development, inoculated seedlings were watered only when wilt symptoms appeared.

Morphological and histological analyses

The first leaf sheaths were collected at 4, 9, 14, 21 dpi and used for histological analysis. Three plants were used for each of the time points for examining fungal mycelium density. Infected leaf sheaths were cleared and fixed according to Schäfer et al. (2004). Briefly, the samples were placed in a clearance solution (0.15 % trichloroacetic acid [w/v] in ethanol: chloroform [4:1, v/v]) for 48 h, with the solution being changed once during this time. The tissues were then washed 2×15 min with 50 % ethanol, 2×15 min with MilliQ H_2O , consequently followed by 30 min of incubation in 0.1 M Tris/HCl (pH 8.5). Samples were then either stained immediately with a cell wall-specific fluorescent brightener 28 (FB28) or stored in 50 % (v/v) glycerol. Cells were then analysed with an Axio Imager Upright Microscope (Germany) and the Axio Vision Release 4.8.2 (2010) Program.

Quantitative-polymerase chain reaction analysis

In parallel with the trials conducted for the histological and morphological analyses, two additional trials were conducted for quantifying pathogens in seedlings infected by FCR pathogens using quantitative polymerase chain reaction (qPCR). Each of the trials consisted of two replicates. Each replicate comprised a pool of 14 shoot bases. Samples used for qPCR analysis were collected at 4, 9, 14 and 21 dpi, respectively, by cutting the shoots between 0 and 2 cm above the soil surface with a pair of sharp scissors. Tissue samples were frozen immediately in liquid nitrogen and stored in -80 °C freezers until needed. Frozen samples were ground in a Retsch MM300 Ball mill (Retsch GmbH, Haan, Germany) for DNA extraction. DNA was extracted using the QIAGEN Plant DNeasy extraction kit (Doncaster, Victoria, Australia) following the manufacture's protocol. DNA from cultured mycelium of *F. pseudograminearum* and disease free barley tissue were also obtained using the same protocol and used as controls in the qPCR analysis (Bai and Liu 2015).

Fungal biomass was estimated using two sets of primers: (i) the *Tri5* gene from the trichothecene cluster responsible for trichothecene production by *Fusarium* species (forward 5'-GCGCATCGA GAATTTGCA-3'; reverse 5'-TGGCGAGGCTGAGC

AAAG-3') and (ii) the fungal ribosomal 18 s gene (forward 5'-GTCCGGCCGGGCTTTCC-3' and reverse 5'-AAGTCCTGTTTCCCCGCCACGC-3'). Both of these genes have been previously used to detect *Fusarium* species associated with barley and wheat CR. Barley actin-binding protein (AY145451, forward 5'-GAACAGGAGCTGGAGACTGC-3' and reverse 5'-ATCATGGATGGCTGGAAGAG-3') was used as the reference for barley (Liu et al. 2012a).

The volume used for qPCR amplification was 10 μ L containing 5 μ L SYBR Green PCR master mix (Applied Biosystems, Scoresby, Victoria, Australia), 1 μ L of a 3 μ M mix of forward and reverse primers, and 4 μ L of sample DNA diluted 1:10 in sterile water. Following an initial denaturation at 95 °C for 10 min, 45 cycles each of 15 s denaturing at 95 °C and 1 min annealing/elongation step at 60 °C were used for qPCR. A final denaturation step at 95 °C for 2 min, annealing at 60 °C for 15 s, and denaturing at 95 °C for 15 s was added to determine the melting temperature of the amplified product in the form of a dissociation curve. QPCR was performed on 384 well plates; each DNA extract (biological replication) was analyzed in three replicated wells. The average value from the three replicated wells was used as data for each biological replication. *Fusarium* DNA relative to barley DNA was calculated as an estimate of relative biomass using the following equation (Yuan et al. 2006):

$$\text{Relative biomass} = \frac{E_{\text{Fungal}}^{-C_t}}{E_{\text{Plant}}^{-C_t}}$$

where E_f is PCR amplification efficiency determined using LINREGPCR 7.5 (Ruijter 2003) and C_t is the crossing threshold.

FCR severity assessment

FCR severity for each of the lines was estimated from the two trials conducted for the histological and fungal biomass assessments. Just before the samples were taken at 21 dpi, FCR severity was assessed with a 0–5 scale according to Li et al. (2008), where “0” representing no symptom and “5” whole seedling completely necrotic. A disease index (DI) was then calculated for each line following the formula of $DI = (\sum_n X / 5 N) \times 100$, 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

Statistical analyses were performed using the SPSS statistics 19.0 for Windows statistical software package (SPSS Inc., Chicago, IL). Homogeneity of variance was tested using one-way analysis of variance (ANOVA) to determine whether the data could be combined across trials for further analyses. An analysis of variance was used to detect the significance of genetic effects for FCR resistance. For each trial, the following mixed-effect model was used: $Y_{ij} = \mu + r_i + g_j + w_{ij}$. Where Y_{ij} is the trait value on the j th genotype in the i th replication, μ is the general mean, r_i is the effect due to i th replication, g_j is the effect due to the j th genotype, w_{ij} is the error or genotype by replication interaction, where genotype was treated as a fixed effect and that of replicate as random. The means of FCR severity were calculated within each trial and the general means were used to compare the difference between isolines with or without the resistant alleles for each of the NIL pairs analysed with the student t test. Pearson correlation coefficients were estimated between DI values and fungal biomass.

Results

Development and evaluation of NILs for FCR resistance

A total of 13 heterozygous plants were initially identified from the three segregating populations using SSR markers WMS6 and HVM67, and a single pair of putative NILs was developed from each of these heterozygous plants. One isolate for each of the NIL pairs possesses the allele from the resistant parent ‘AWCS276’ and other isolines possess an alternative allele from one of the susceptible cultivars used in each of the populations. No significant differences in plant morphology were found between isolines for each of the NIL pairs but disease assessments revealed that significant differences in FCR severity between the isolines were detected for 10 of the 13 putative NIL pairs (Table 1). Seven of these ten NIL pairs were derived from the population ‘Lockyer//AWCS276/AWCS079’, two from the population ‘Commander//AWCS276/AWCS079’ and the other one from the population ‘Baudin/AWCS276’. As expected, the isolines possessing the allele from the resistant parent

Table 1 A comparison of the disease index for the ten pair of barley near-isogenic lines developed for a major 4HL locus conferring Fusarium crown rot resistance

NILs	Genetic background	Trial 1	Trial 2	Mean	DI %	P value
NIL_CR4HL_1R	Baudin/AWCS276 F9	28.6	35	31.8	48.0	<0.05
NIL_CR4HL_1S		64.5	57.7	61.1		
NIL_CR4HL_2R	Lockyer//AWCS276/AWCS079 F8	39.2	39.2	39.2	35.2	<0.05
NIL_CR4HL_2S		61.4	59.5	60.5		
NIL_CR4HL_3R	Lockyer//AWCS276/AWCS079 F8	31.4	32.3	31.9	54.7	<0.05
NIL_CR4HL_3S		73.0	67.8	70.4		
NIL_CR4HL_4R	Lockyer//AWCS276/AWCS079 F8	44.2	42.5	43.4	32.8	<0.01
NIL_CR4HL_4S		65.5	63.4	64.5		
NIL_CR4HL_5R	Lockyer//AWCS276/AWCS079 F8	38.4	33.2	35.8	41.6	<0.05
NIL_CR4HL_5S		55.9	66.6	61.3		
NIL_CR4HL_6R	Lockyer//AWCS276/AWCS079 F8	26.6	22.2	24.4	63.4	<0.01
NIL_CR4HL_6S		67.8	65.5	66.7		
NIL_CR4HL_7R	Lockyer//AWCS276/AWCS079 F9	33.2	29.5	31.4	40.2	<0.01
NIL_CR4HL_7S		50.8	54.2	52.5		
NIL_CR4HL_8R	Lockyer//AWCS276/AWCS079 F9	36.7	35.8	36.3	34.7	<0.05
NIL_CR4HL_8S		58.6	52.5	55.5		
NIL_CR4HL_9R	Commander//AWCS276/AWCS079 F8	25.5	32.3	28.9	39.1	0.05
NIL_CR4HL_9S		42.5	52.5	47.5		
NIL_CR4HL_10R	Commander//AWCS276/AWCS079 F8	22.2	24.5	23.3	49.5	<0.01
NIL_CR4HL_10S		46.6	45.8	46.2		

'R' lines represent those with allele from the resistant parent AWCS276 and 'S' lines are those with an alternative allele from the susceptible parents; 'DI' represents disease index

AWCS079 all gave better FCR resistance than their respective counterparts for each of these ten pairs of NILs. The average DI value for the isolines possessing resistant alleles was 32.6, whereas it was 58.6 for susceptible isolines (Table 1). Significant differences in FCR resistance were detected between the two trials and the interaction between the genotypes and trials was also significant (Table 2). Error variances were homogenous over trials so data from individual trials were combined and used to compare the differences between the isolines for each of the 10 pairs of NILs by the student *t* test (Fig. 1).

Histological and qPCR analyses of NILs during FCR infection

Three of the 10 NIL pairs were randomly selected for the histological analysis. Samples were collected at four time points (4, 9, 14 and 21 dpi, respectively). As

Table 2 Analysis of variance for FCR severity across the two independent trials

Source of variation	d.f.	s.s.	m.s.	v.r	<i>F</i> pr.
Genotype	19	12,680.4	667.3	3.2	<0.001
Trial	1	23	23	0.1	0.043
Genotype × trial	19	1057.9	55.6	0.2	0.085
Residual	76	15,881	208.9		

d.f. Degree of freedom, *s.s.* sum of squares of deviations, *m.s.* mean square, *v.r.* variance ratio obtained by dividing the treatment mean square by the residual mean square, *F* pr. Significance probability estimated by *F* test

most of the infected plants were found dead after 21 dpi, no other time points were considered after this one. As expected, fungal mycelium was detected earlier and its density was higher in the susceptible isolines at each of the time points analysed as compared with that in the resistant isolines. The most



Fig. 1 Plants of a pair of the near isogenic lines (NIL_CR4HL_6S and NIL_CR4HL_6R), showing the similar morphology of the non-inoculated plants (*left*) and the

difference in resistance to *Fusarium pseudograminearum* at 21 days post inoculation (*right*). *S* represents lines without the resistant allele and *R* represents lines with the resistant allele

contrasting difference between each of the NIL pairs was detected at the last time point assessed (Fig. 2).

The differences in the fungal biomass between the isolines for the three pairs of NILs were further assessed using qPCR. This assessment detected more fungal biomass in the susceptible isolines compared with that in resistant isolines at each of the four time points (4, 9, 14 and 21 dpi) sampled during FCR development. This was the case with the use of either *18s* gene or *Tri5* (Fig. 3) as the reference gene.

Discussion

NILs are an important genetic resource in studying gene loci conferring a wide array of characteristics (Pumphrey et al. 2007; Chen et al. 2014). We report in this paper the development of 10 pairs of NILs for a major locus located at distal end of chromosome 4HL that confers FCR resistance in barley. Phenotypes contrasting in FCR severity were detected between the resistant and susceptible isolines for each of the 10

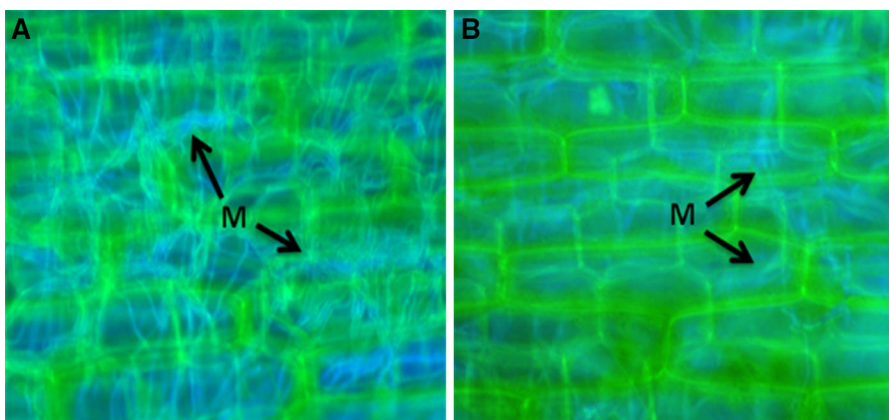
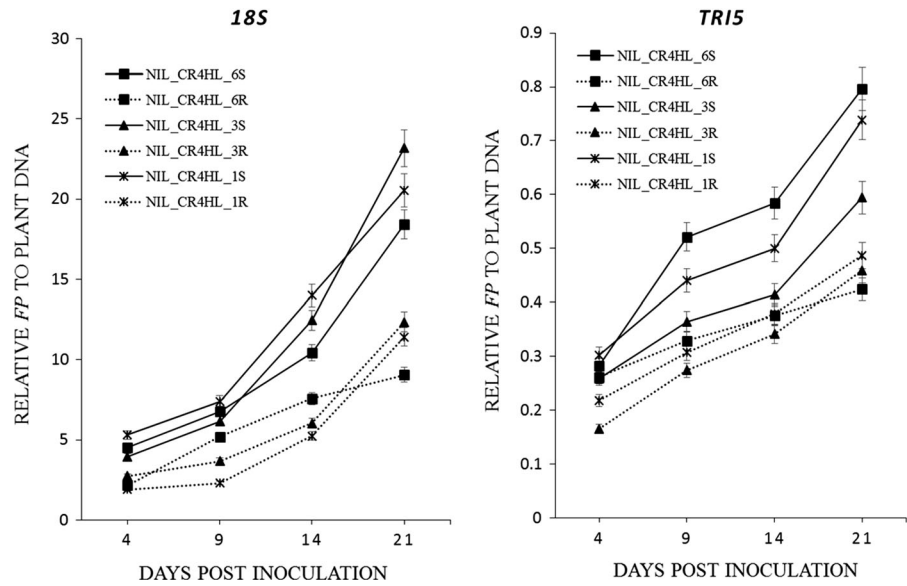


Fig. 2 Differences in the level of fungal (*Fusarium pseudograminearum*) mycelium colonization between the two isolines NIL_CR4HL_6S and NIL_CR4HL_6R at 21 days post inoculation, showing higher density of mycelium (*M* fixed and cleared

tissues after staining with fluorescent brightener 28 and pointed with *arrows*) in the susceptible isolate (**a**) compared with that in the resistant isolate (**b**)

Fig. 3 Relative *Fusarium pseudograminearum* biomass estimated with the fungal *18s* rDNA (left) or *Tri5* (right) as reference genes at four different time points during *Fusarium* crown rot infection and development. Error bars represent percentage (5.0) of error



NIL pairs. Among these NIL pairs, the presence of the FCR allele reduced FCR severity by 32.8–63.4 % with an average of 43.9 %. Histological analysis against three of NIL pairs revealed that more mycelium was detected in the susceptible isolines compared with that in each of the resistant isolines. These observations were further confirmed by the qPCR analysis which detected high concentrations of *Fusarium* in the susceptible isolines at each of the time points assessed.

Previous studies showed that both plant height (Li et al. 2010; Liu et al. 2010; Chen et al. 2013b; Bai and Liu 2015) and growth rate (Liu et al. 2010, 2012a, b; Chen et al. 2013a) affect FCR severity. Thus NILs developed in genetic backgrounds differing in these characteristics would be more valuable in further work aimed at clarifying functions of gene(s) underlying the targeted locus. In an effort to increase the variability of genetic background for the NILs generated, three different segregating populations were used in developing the NILs reported here. Similar to that reported for those NILs in wheat (Ma et al. 2012), the different genetic backgrounds must have contributed to the significant differences in the effect of the 4HL FCR locus detected among the NIL pairs.

In contrast to the traditional approach of using markers flanking a QTL in generating NILs (Pumphrey et al. 2007), we used single markers linked to the targeted locus in generating the NILs. As discussed in an earlier study (Ma et al. 2012), the advantage of

using one linked marker only is that NILs generated this way likely contain smaller sizes of the ‘non-desirable’ chromosomal segments differentiating the isolines. However, due to limited resolution of QTL mapping, markers selected from QTL mapping studies may not be highly reliable in tagging a given locus (Tanksley 1988). This could result in a recombination between the linkage marker and its target. Thus, using a single linked marker could lead to false NILs which do not segregate at the targeted locus. This is the most likely reason why difference in FCR severity was not detected between isolines for three of the initial 13 pairs of putative NILs generated for the locus on 4HL.

Fusarium crown rot is one of the many important plant diseases caused by necrotrophic pathogens. Despite its enormous importance in plant protection, a mechanistic understanding of how resistance to necrotrophic pathogens functions remains mostly unknown. To our knowledge, no genes conferring resistance to any necrotrophic pathogen has been cloned in any major crop species. One of the main difficulties is that such resistance is often quantitatively inherited and controlled by multiple genes each with a relatively small effect. The contrasting phenotypes in FCR severity between the isolines for each of the NIL pairs obtained in this study further confirm data from QTL mapping studies showing that gene loci with major effects do exist in conferring resistance to FCR in both wheat (Ma et al. 2010; Li et al. 2010;

Zheng et al. 2014) and barley (Chen et al. 2013a, 2013b). There is little doubt that the huge differences in FCR severity between the isolines for the NIL pairs developed in this study would facilitate future efforts in cloning and functional studies of genes underlying FCR resistance.

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