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ORIGINAL ARTICLE

Host and pathogen genetics reveal an inverse gene-for-gene association in the *P. teres* f. *maculata*–barley pathosystem

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Abstract

Key message **Pathogen and host genetics were used to uncover an inverse gene-for-gene interaction where virulence genes from the pathogen Pyrenophora teres f. maculata target barley susceptibility genes, resulting in disease.**

Abstract Although models have been proposed to broadly explain how plants and pathogens interact and coevolve, each interaction evolves independently, resulting in various scenarios of host manipulation and plant defense. Spot form net blotch is a foliar disease of barley caused by *Pyrenophora teres* f. *maculata*. We developed a barley population (Hockett × PI 67381) segregating for resistance to a diverse set of *P. teres* f. *maculata* isolates. Quantitative trait locus analysis identified major loci on barley chromosomes (Chr) 2H and 7H associated with resistance/susceptibility. Subsequently, we used avirulent and virulent *P. teres* f. *maculata* isolates to develop a pathogen population, identifying two major virulence loci located on Chr1 and Chr2. To further characterize this host–pathogen interaction, progeny from the pathogen population harboring virulence alleles at either the Chr1 or Chr2 locus was phenotyped on the Hockett × PI 67381 population. Progeny harboring only the Chr1 virulence allele lost the barley Chr7H association but maintained the 2H association. Conversely, isolates harboring only the Chr2 virulence allele lost the barley Chr2H association but maintained the 7H association. Hockett × PI 67381 F_2 individuals showed susceptible/resistant ratios not significantly different than 15:1 and results from F_2 inoculations using the single virulence genotypes were not significantly different from a 3:1 (S:R) ratio, indicating two dominant susceptibility genes. Collectively, this work shows that *P. teres* f. *maculata* virulence alleles at the Chr1 and Chr2 loci are targeting the barley 2H and 7H susceptibility alleles in an inverse gene-for-gene manner to facilitate colonization.

Introduction

The relationship between a host and pathogen is often described as an evolutionary arms race, in which the host is under constant pressure to resist novel pathogen colonization strategies while the pathogen must perpetually overcome these resistance measures through genetic adaptation. Oftentimes the evolution or attainment of a single gene is enough to tip the balance in favor of one side over the other, but this shift in balance is often only temporary. The prevailing model used to describe the genetics of host–pathogen interactions was originally put forth as the gene-for-gene model (reviewed in Flor 1971), which supposes that for every gene conferring resistance in the host, there is a corresponding gene conferring avirulence in the pathogen. Over the last several decades, the gene-for-gene concept has been broadened to show that the avirulence "effectors" that were eliciting a defense response resulting in effector triggered immunity (ETI) originally had a role in virulence. Evolution

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of the host and pathogen was summarized in the zig-zag model (Jones and Dangle 2006; Chisolm et al. 2006), which also accounted for the host recognition of pathogen associated molecular patterns (PAMPs) resulting in PAMP-triggered immunity (PTI), an early defense response thought to involve different defense response pathways. More recent work has shown crosstalk between the ETI and PTI pathways (Ngou et al. 2021; Yuan et al. 2021), demonstrating the complexity of the host–pathogen interaction.

Localized programmed cell death (PCD) is typically thought of as a plant defense response resulting from ETI that impedes biotrophic pathogen colonization. However, pathogens displaying characteristics of necrotrophic or hemibiotrophic lifestyles often induce and use PCD to acquire nutrients from dying cell tissue, hijacking this host defense response in a model described as an inverse gene-for-gene interaction (Friesen and Faris 2021). In this model, pathogens use necrotrophic effectors to target plant susceptibility genes—which in some cases have similarities to classical resistance genes—to trigger PCD, facilitating necrotrophic pathogen nutrient acquisition, colonization, and sporulation.

Pyrenophora teres is the causal agent of net blotch of barley (*Hordeum vulgare*). This filamentous fungal pathogen is endemic worldwide and can be found in two different forms, *Pyrenophora teres* f. *teres* and *Pyrenophora teres* f. *maculata*. Although both forms of *P. teres* are present in all major barley growing regions, it is common for one form to be locally dominant, with the predominant form often changing over time (Liu and Friesen 2010; Louw et al. 1996; McLean et al. 2009). Though morphologically similar, the two forms are distinguished based on the physiology of disease symptoms observed on the surface of barley leaves. *Pyrenophora teres* f. *maculata* is responsible for spot form net blotch (SFNB), characterized by initial small necrotic lesions which, over time, progress to larger round or elliptical necrotic lesions with accompanying chlorosis. A resistant or incompatible interaction is characterized by dark pinpoint lesions generally unaccompanied by tan necrosis or chlorosis, indicative of a failure of the pathogen to penetrate and/or colonize beyond the initial penetration. In a highly susceptible or compatible interaction, necrotic lesions spread to the point of coalescence and may result in the death of the leaf (Liu et al. 2011). The severity of SFNB is dependent on the genetics of the pathogen and host as well as environmental variables such as temperature and humidity (Liu et al. 2011).

P. teres f. *maculata* has recently increased in prominence in the USA and Australia, causing yield losses of up to 44% (Liu and Friesen 2010; Marshall et al. 2015; McClean et al. 2010; Jayasena et al. 2007). In Australia, potential losses caused by this pathogen have been valued at AU$192 million per year (Murray and Brennan 2010). Additionally, reports of the pathogen causing disease on wheat (*Triticum aestivum* L.) have emerged from Hungary (Tóth et al. 2008), Russia (Mikhailova et al. 2010), and Brazil (Perelló et al. 2019).

Efforts have been undertaken from both host and pathogen sides to identify and validate genomic regions involved in the *P. teres* f. *maculata*–barley interaction, though most research has focused on host loci associated with resistance/susceptibility (Reviewed in Clare et al. 2020). To date, a combination of quantitative trait loci (QTL) analyses and genome-wide association studies (GWAS) have identified resistance/susceptibility loci on all barley chromosomes (Reviewed in Clare et al. 2020). Comparatively less research has been directed toward understanding the genetics of pathogen virulence/avirulence. To date, there have only been three studies mapping virulence in *P. teres* f. *maculata*. Carlsen et al. (2017) crossed *P. teres* f. *maculata* isolates FGOB10Ptm-1 and SG1 and inoculated the progeny of the cross on four commonly used SFNB differential barley lines, resulting in the identification of eight QTL associated with virulence. One virulence locus on chromosome 2 was contributed by SG1, and seven on chromosomes 1, 3, 4, and 5 were contributed by FGOB10Ptm-1. Three associations on chromosome 1 were present in a closely linked region, and while these QTL may be representative of several individual genes, it is also possible that a single gene was contributing this virulence (Carlsen et al. 2017). Clare et al. (2022) used association mapping with a natural population of 103 North American *P. teres* f. *maculata* isolates inoculated on 30 SFNB differential barley lines to identify 26 novel QTL on chromosomes 1, 2, 3, 4, 5, 8, 10, and 11. Most recently, Dahanayaka et al. (2022) created a hybrid *P. teres* f. *teres*/*P. teres* f. *maculata* population which was phenotyped on eight barley cultivars and used to identify two unique QTL associated with virulence on chromosomes 9 and 12, which were determined to be contributed by the *P. teres* f. *maculata* parent.

In the current study, we created a barley recombinant inbred population by crossing the SFNB-susceptible barley line Hockett by the resistant line PI 67381 to identify two major QTL associated with resistance/susceptibility. Additionally, we generated a biparental *P. teres* f. *maculata* mapping population by crossing the virulent isolate P-A14 by the avirulent isolate CAWB05-Pt-4 to evaluate the genetics of virulence, resulting in the identification of two major virulence loci. Single-virulence progeny genotypes from the P-A14×CAWB05-Pt-4 pathogen population were then evaluated on the Hockett×PI 67381 barley population to reveal an inverse gene-for-gene interaction in the *P. teres* f. *maculata*–barley pathosystem.

Materials and methods

Barley population development

One hundred and eighteen $F_{2:6}$ recombinant inbred lines were developed by single-seed descent from a cross between

the barley two-row breeding line PI 67381 (Muñoz-Amatriaín et al. 2014) and the two-row cultivar Hockett, developed by Montana State University in 2008, resulting in a Hockett × PI 67381 recombinant inbred line population. F_2 individuals were similarly derived from crosses of Hockett and PI 67381.

Barley genotypic analysis

DNA extraction and genotyping for barley progeny and parental lines was performed by the North Central Small Grains Genotyping Lab (Fargo, ND). Genotyping was performed using the barley 50K Illumina iSelect single-nucleotide polymorphism (SNP) array, and genotype calling was done using GenomeStudio software v2.0 (https://support.illumina.com/array/array_software/genomestudio/downloads.html) developed by Illumina (San Diego, CA). Markers with greater than 30% missing data across all lines were removed, and remaining markers were filtered for allele frequencies between 25 and 75% of each parental type.

Barley genetic map construction

Mapping of the Hockett × PI 67381 population was performed using the Microsoft Excel-based software program MapDisto v2.1.7 (http://mapdisto.free.fr/Download_Soft/) (Heffelfinger et al. 2017) with the previously described filtered SNP markers. The "Find linkage groups" command was used to determine linkage groups with a LOD_{min} of 5.0 and an r_{max} of 0.3. The "Order a linkage group" command was used to establish the ordering of markers for each group, and the "Check inversions," "Ripple order," and "Drop locus" commands were used to further refine these orders. Linkage groups were compared with "MorexV3" and "MorexGenome" in Barleymap (https://floresta.eead.csic.es/barleymap/find/) to identify the barley chromosomes corresponding to each linkage group as well as to resolve large gaps. Co-segregating markers were thinned to a single best marker based on missing data content.

P. teres f. *maculata* population development

P. teres f. *maculata* isolate P-A14 was collected from Pinnacle barley in Montana, USA, in 2012 (Wyatt and Friesen 2021), and *P. teres* isolate CAWB05-Pt-4 was collected from an unknown wild barley species in Pacific Grove, CA, USA, in 2005 (Lu et al. 2012). CAWB05-Pt-4 is morphologically similar to other *P. teres* f. *maculata* and *P. teres* f. *teres* isolates but is avirulent on all known barley lines. CAWB05-Pt-4 intercrosses under controlled laboratory conditions with both *P. teres* f. *maculata* and *P. teres* f. *teres*; therefore, we refer to it as only *P. teres*. P-A14 and CAWB05-Pt-4 isolates were crossed to produce a P-A14 × CAWB05-Pt-4 biparental population consisting of 135 progeny as described in Shjerve et al. (2014). Briefly, sterile wheat and barley stems were placed on Sach's media (1 g $CaNO_3$, 0.25 g $MgSO_4$ $7H_2O$, trace $FeCl_3$, 0.25 g K_2HPO, 4 g $CaCO_3$, 20 g agar, ddH_2O to 1 L) and 100 µL of inoculum at a concentration of 4000 spores/mL from each parental isolate was placed on opposite ends of each stem. The inoculum was allowed to converge in the center of the stems, and the plates were incubated in the dark for 12 days at 15 ℃ and then at 13 ℃ with a 12-h photoperiod until pseudothecia began to develop. After the development of pseudothecia, individual stems were transferred to the lids of water agar plates and further incubated at 13 ℃ with a 12-h photoperiod while inverted to allow pseudothecia to discharge ascospores vertically onto the water agar. Once ascospores were detected (approximately 2 weeks), a single ascospore was then picked from each group of ascospores to avoid clones due to the mitotic division during the final stage of ascospore production. Ascospores were placed on V8-PDA medium (150 mL V8 juice, 10 g Difco PDA, 3 g $CaCO_3$, 10 g agar, ddH_2O up to 1 L) and allowed to grow and sporulate. To ensure genetic purity, two rounds of conidia isolation were performed.

P. teres f. *maculata* genotypic analysis

DNA was extracted from fungal progeny and parental isolates as described in Shjerve et al. (2014). Briefly, approximately 50 mg of lyophilized fungal tissue for each isolate was ground with liquid nitrogen using a drill and pestle in a 2-mL tube. Seven hundred µL of Qiagen RLT buffer and 2 µL of RNAse (20 mg/mL) was added to the ground tissue, and the resulting mixture was then homogenized with repeated pipetting. The homogenized mixture was incubated in a water bath at 65 °C for 1 h followed by centrifugation for 8 min at 16,300 × G in a benchtop centrifuge to pellet cell debris. Supernatant was transferred to a new 1.7-mL test tube, and 600 µL of phenol:chlorophorm:isoamyl alcohol (25:24:1) solution was added. The mixture was inverted several times until evenly mixed followed by centrifugation at 16,300 × G for 8 min. The resulting aqueous layer was transferred to a new 1.7-mL test tube where 0.1 × volume of 3 M NaOAc (pH 5.2), and 2.5 × volume of 95% EtOH was added and mixed by inversion to precipitate DNA. The solution was centrifuged for 10 min at 16,300 × G, pelleting the DNA. Ethanol and sodium acetate were then decanted, and the DNA pellet was washed twice with 500 µL of 70% EtOH followed by centrifugation at 6000 × G. Residual ethanol was removed, and the pellet was dried in a laminar flow hood for up to 5 min. Dried DNA pellets were dissolved in 60 µL of molecular biology grade water. Genomic DNA libraries were constructed using a RAD-GBS method as described in Koladia et al. (2017). Sequencing of genomic libraries was carried out using an Ion Torrent PGM system, and sequence

files were obtained from the Ion Torrent Server (Leboldus et al. 2015). Sequence files in FASTQ format were passed into a SNP calling pipeline. Within the SNP calling pipeline, progeny sequences were aligned to parental sequences using the Burrows-Wheeler Aligner (https://sourceforge.net/projects/bio-bwa/files/) and were exported as sequence alignment/map (SAM) files (Li and Durbin 2009). SAM files were then converted to binary alignment/map (BAM) files, and a list of BAM files was generated for downstream use. Next, Samtools v0.1.19 (http://www.htslib.org/download/) was used to create a sequence dictionary of the P-A14 parental reference FASTA for SNP calling (Wyatt and Friesen 2021). The Genome Analysis Toolkit Kit v3.7 HaplotypeCaller (https://github.com/broadinstitute/gatk/releases) was used to call SNPs which were exported in Variant Call Format (VCF) (McKenna et al. 2010). The subsequent VCF file was then filtered based on GATK best practices (FS > 60, MQ < 40, MQRankSum < − 2.5, QD < 2, QUAL < 10, ReadPosRankSum < − 12.5, SOR > 3).

The resulting VCF file was passed to Microsoft Excel for manual marker filtering. Markers with greater than 30% missing data across all isolates were removed, and the remaining markers were filtered for allele frequencies between 25 and 75% of each parental type.

P. teres f. maculata genetic map construction

The P-A14 × CAWB05-Pt-4 genetic map was constructed using MapDisto. Markers were placed in accordance with their physical location in the P-A14 reference genome, eliminating the need for further re-ordering. Co-segregating markers were thinned to a single best marker based on missing data content.

Phenotypic analysis

Inoculations were performed as described by Carlsen et al. (2017). Briefly, *P. teres* f. *maculata* isolates were grown on V8-PDA medium in the dark for 5 to 7 days at room temperature before being placed under light at room temperature for 24 h and then placed in the dark at 15 ℃ for 24 h. Plates were then flooded with 100 mL sterilized distilled water and brushed with an inoculation loop to loosen spores. The resulting solutions were then diluted to 2000 spores/mL, and Tween 20 (J.T. Baker Chemical Co.) was added to them at a rate of 1 drop/50 mL to prevent spore clumping. Barley lines were planted in racks containing 96 conetainers (Stuewe & Sons, Inc.), with Tradition barley planted in the outside border to reduce edge effect. Inoculations of the P-A14 × CAWB05-Pt-4 population were performed in randomized groups on barley lines Hockett, Innovation (Busch Agricultural Resources LLC), Lacey (University of Minnesota), Stellar-ND (North Dakota State University), and Pinnacle (North Dakota State University), with barley lines Tradition (Busch Agricultural Resources Inc.), and PI 67381 used as susceptible and resistant checks, respectively. These lines were used because they are popular cultivars in the local region where P-A14 was collected. Inoculations of the Hockett × PI 67381 RIL population with isolates P-A14 and C-A17, collected in Montana, USA (Wyatt and Friesen 2021), FGOB10Ptm-1 collected in North Dakota, USA (Carlsen et al. 2017; Syme et al. 2018), Den2.6 collected in Denmark (Wyatt and Friesen 2021), NZKF2 collected in New Zealand (Wyatt and Friesen 2021), and P-A14 × CAWB05-Pt-4 progeny isolates #16, #56, #101, and #105 were performed using parental lines PI 67381 and Hockett as checks. Inoculations of Hockett × PI 67381 F_2 individuals were performed only once, with isolates P-A14 or the P-A14 × CAWB05-Pt-4 progeny isolates #101 and #105. Fifty-eight F_2 seeds were planted for each inoculation, along with PI 67381 and Hockett as checks. All seedlings were inoculated using a paint sprayer (DeVilbiss, model# SRIPRO-635G-10) when secondary leaves had become fully expanded (14 to 16 days). Leaves were covered homogenously with a heavy mist of inoculum but prior to runoff. After inoculation, plants were placed in mist chambers at 100% relative humidity and 21 ℃ for a 24-h light cycle. Plants were then transferred to a growth chamber under a 12-h photoperiod at 21 ℃. Disease reactions were evaluated after 7 days on a 1 to 5 scale as in Neupane et al. (2015), where a 1-type reaction represented a high level of resistance/low level of virulence, and a 5-type reaction represented a low level of resistance/high level of virulence. At least three replications were performed for each mapping population combination.

QTL analysis

For both barley and *P. teres* f. *maculata* populations, QTL analyses were performed with the mapping data generated from MapDisto and the corresponding average reaction type data using Qgene v4.4.0 (https://www.qgene.org/qgene/download.php) (Joehanes and Nelson 2008). Permutation tests with 1000 iterations were performed to establish initial critical LOD thresholds at a significance level of $\alpha = 0.01$ for each trait. QTL analyses were performed using composite interval mapping with forward cofactor selection.

Statistical analysis

Genotypic classes for both Hockett × PI 67381 and P-A14 × CAWB05-Pt-4 progeny were analyzed for significant variation in average reaction type using Fisher's least significant difference test. For both populations, a one-way ANOVA was performed in Microsoft Excel and the resulting

data were used to determine least significant differences at both an $\alpha = 0.05$ and $\alpha = 0.01$ level of significance.

Results

Barley population development and genetic mapping

One hundred and eighteen Hockett × PI 67381 $F_{2:6}$ progeny were obtained via single-seed descent. Genotyping of the population and parents using the barley 50K Illumina iSelect SNP Array resulted in 44,040 single-nucleotide polymorphism (SNP) markers. Markers for which a genotype could not be called or for which a heterozygous genotype was called were removed, resulting in 14,394 markers. Markers were filtered for segregation distortion based on the relative abundance of either parent's genotype at any one locus within the population, with a maximum allelic ratio of 3:1 (75%) and minimum allelic ratio of 1:3 (25%) used as cutoffs. Additionally, markers with greater than 30% missing data were removed, leaving 9120 markers. Markers lacking a chromosomal designation were discarded, leaving 7523 markers comprising seven linkage groups corresponding to the seven barley chromosomes. Finally, co-segregating markers and markers causing high levels of linkage distortion were removed, resulting in 1047 high-quality markers to be used in QTL analysis (Table 1). With a total map size of 963.85 cM, the resulting average marker density was one marker/1.09 cM.

P. teres population development and genetic mapping

One hundred and thirty-five progeny were isolated from the cross of *P. teres* isolates P-A14 and CAWB05-Pt-4. A total of 26,317,764 reads were generated via Ion Torrent RAD-GBS sequencing for parental and progeny isolates. Reads were de novo assembled into 11,724 sequence tags and 8657 sequence tags with average sizes of 232–313 bp, respectively. Average read count per progeny isolate was 192,100, and these reads were used for the SNP calling pipeline. Markers with greater than 30% missing data and markers with segregation distortion greater than 75% or less than 25% were removed, resulting in 9791 filtered markers. Markers were placed and ordered based on their physical position in the P-A14 genome assembly, resulting in 12 linkage groups representing the 12 *P. teres* chromosomes. Co-segregating markers were discarded, resulting in 1,210 high-quality markers to be used in QTL analysis (Table 2). With a total map size of 2198.86 cM, the resulting average marker density was one marker every 1.82 cM (one marker/34.7 kb).

Table 1 Hockett × PI 67381 mapping statistics

Chromosome	Markers	Size (cM)
1H	137	127.46
2H	161	150.39
3H	212	157.29
4H	113	104.49
5H	176	185.99
6H	94	98.71
7H	154	139.52
Total	1047	963.85

Barley chromosomes are shown with corresponding marker counts and map distances

Phenotypic analysis

Hockett was more susceptible to *P. teres* f. *maculata* isolate P-A14 with an average reaction type of 3.83 compared to PI 67381 with an average reaction type of 1.67, whereas both Hockett and PI 67381 were resistant to CAWB05-Pt-4 with average disease reaction types of 1.00 and 1.33, respectively (Fig. 1). Hockett × PI 67381 RIL progeny exhibited a range of average reaction types to P-A14 with a maximum reaction type of 4.50 and a minimum of 1.33. *P. teres* isolate CAWB05-Pt-4 was markedly less virulent on the Hockett × PI 67381 population, with an average reaction type of 1.05. The P-A14 × CAWB05-Pt-4 population exhibited a range of average reaction types on Hockett, with a maximum average reaction type of 3.83 and a minimum of 1.00.

Table 2 P-A14 × CAWB05-Pt-4 mapping statistics.

Chromosome	Markers	Size (cM)
1	174	305.40
2	142	231.86
3	154	280.59
4	101	190.52
5	144	238.76
6	87	151.53
7	106	180.15
8	81	146.25
9	76	140.01
10	31	95.67
11	67	123.39
12	47	114.73
Total	1210	2198.86

P. teres f. *maculata* chromosomes are shown with corresponding marker counts and map distances

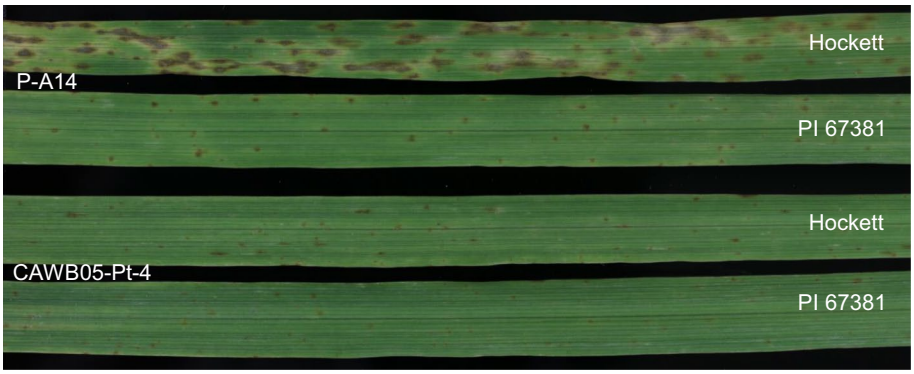

Fig. 1 Phenotypic response of Hockett and PI 67381 barley to *P. teres* f. *maculata* isolates P-A14 and CAWB05-Pt-4. The Hockett-P-A14 combination showed a compatible interaction resulting in host susceptibility, whereas all other combinations were incompatible and resulted in resistance

QTL analysis

Mapping data were used in conjunction with reaction type data to perform QTL analysis for both the pathogen and host population. Using composite interval mapping with forward cofactor selection and a critical logarithm of odds (LOD) threshold of 3.9 ($\alpha = 0.01$) for the Hockett × PI 67381 RIL barley population, major QTL involved in resistance/susceptibility to P-A14 were identified on Chr2H and 7H, with the QTL on Chr2H accounting for 34% of the population's phenotypic variation (LOD = 15.6) and the QTL on Chr7H accounting for 19% of the barley population's phenotypic variation (LOD = 8.5)(Fig. 2).

Similarly, mapping and reaction type data obtained for the P-A14 × CAWB05-Pt-4 population were used to perform pathogen-side QTL analysis. A critical LOD threshold of 4.1 was established ($\alpha = 0.01$), and major QTL involved in virulence/avirulence on Hockett were identified on Chr1 and 2, with the QTL on Chr1 accounting for 25% of the population's phenotypic variation (LOD = 16.1) and the QTL on Chr2 accounting for 51% of the population's phenotypic variation (LOD = 29.4) (Fig. 3).

The Hockett × PI 67381 population was additionally inoculated with *P. teres* f. *maculata* isolates C-A17, FGOB-10Ptm-1, Den2.6, and NZKF2. QTL analyses for C-A17 and FGOB10Ptm-1 showed major QTL at the same Chr2H and 7H loci as were previously identified for P-A14. The analyses for isolates Den2.6 and NZKF2 showed a major QTL at the Chr2H locus but not at the 7H locus. Additionally, a QTL on Chr6H was identified using C-A17, FGOB10Ptm-1, and NZKF2 reaction type data (Online Resource 1).

In addition to barley line Hockett, the P-A14 × CAWB05-Pt-4 population was also inoculated on lines Innovation, Lacey, Pinnacle, and Stellar-ND. QTL analyses for all four lines showed major QTL at the same Chr1 and 2 loci as were previously identified for Hockett. Additional minor QTL corresponding to these four lines were identified on Chr1, 3, 5, and 10 (Online Resource 2).

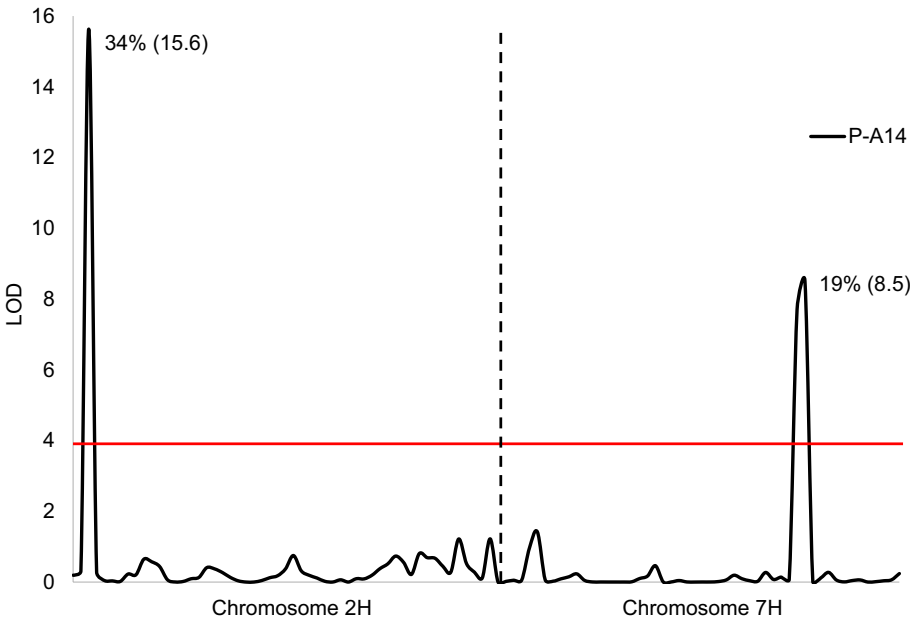

Fig. 2 Hockett × PI 67381 population data analysis. Hockett × PI 67381 QTL involved in resistance/susceptibility to *P. teres* f. *maculata* isolate P-A14. QTL analysis performed using composite interval mapping with forward cofactor selection. The red horizontal line represents the calculated critical LOD threshold ($\alpha = 0.01$) of 3.9. R^2 and LOD values corresponding to each isolate are listed next to significant QTL. Only chromosomes with significant QTL are shown

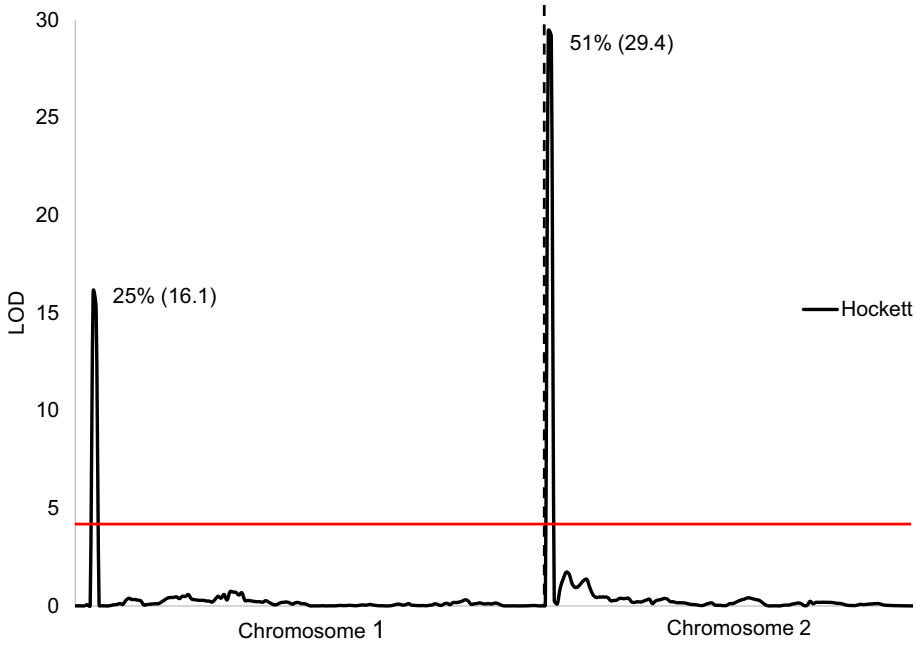

Fig. 3 P-A14 × CAWB05-Pt-4 population data analysis. P-A14 × CAWB05-Pt-4 QTL involved in virulence/avirulence on barley line Hockett. QTL analysis performed using composite interval mapping with forward cofactor selection. The red horizontal line represents the calculated critical LOD threshold ($\alpha = 0.01$) of 4.1. Only chromosomes with significant QTL are shown. R^2 and LOD values are listed next to significant QTL. Only chromosomes with significant QTL are shown

Genotypic class analysis

After performing QTL analysis of the P-A14 × CAWB05-Pt-4 population on Hockett, progeny isolates were grouped into genotypic classes based on their parental allele for the Chr1 and Chr2 QTL. Alleles were determined based on the genotypes present for markers associated with the QTL region, resulting in four distinct classes: $Chr1^{P\text{-}A14}Chr2^{P\text{-}A14}$ possessing the P-A14 (virulent) allele at both loci, $Chr1^{P\text{-}A14}Chr2^{CAWB}$ possessing the P-A14 allele at the Chr1 locus and the CAWB05-Pt-4 (avirulent) allele at the Chr2 locus, $Chr1^{CAWB}Chr2^{P\text{-}A14}$ possessing the CAWB05-Pt-4 allele at the Chr1 locus and the P-A14 allele at the Chr2 locus, and $Chr1^{CAWB}Chr2^{CAWB}$ possessing the CAWB05-Pt-4 allele at both loci. Fisher's least significant difference test was used to show that all four classes significantly differed from each other based on their average reaction type on Hockett (Table 3).

A similar analysis conducted on the host population showed that the host progeny possessing the Hockett (susceptibility) allele at only one of the Chr2H or 7H loci differed significantly in average reaction type compared to progeny harboring the Hockett allele at either both or neither loci. Unlike the pathogen population genotypic classes, the single-susceptibility host progeny classes did not differ significantly from each other, indicating that the Hockett susceptibility alleles at each locus contributed similarly to the disease reaction when the PI 67381 allele was present at the other locus (Table 3).

P-A14 × CAWB05-Pt-4 progeny isolates #56 and #101 (genotypic class $Chr1^{CAWB}Chr2^{P\text{-}A14}$) and isolates #16 and #105 (genotypic class $Chr1^{P\text{-}A14}Chr2^{CAWB}$) were inoculated on the RIL population, and QTL analyses performed using the resulting reaction type data (Fig. 4). A QTL on Chr7H corresponding to the same 7H locus identified for parental isolate P-A14 was identified for isolates #56 and #101, which harbor only the Chr2 virulence allele (Fig. 4b). This QTL accounted for 36% of phenotypic variation for isolate #101 (LOD = 12.1) and 33% of the phenotypic variation for isolate #56 (LOD = 10.3). A QTL on Chr2H corresponding to the same 2H locus identified for parental isolate P-A14 was identified for isolates #16 and #105, which harbor only the Chr1 virulence allele (Fig. 4c). This QTL accounted for 23% of the phenotypic variation for isolate #105 (LOD = 6.9) and 16% of the phenotypic variation for isolate #16 (LOD = 5.0). Additionally, a single QTL was identified on Chr6H for isolate #105, accounting for 17% of the phenotypic variation (LOD = 4.7) (data not shown). No significant association was identified on either Chr2H or 7H using isolate CAWB05-Pt-4 (Fig. 4d).

Hockett × PI 67381 F_2 analysis

As the two major QTL present in the Hockett × PI 67381 population accounted for a combined 53% of the phenotypic variation when inoculated with P-A14, it was hypothesized that at least two genes were strongly involved in resistance/susceptibility. To test this hypothesis and determine gene action, Hockett × PI 67381 F_2 progeny was inoculated with isolate P-A14 and P-A14 × CAWB05-Pt-4 progeny isolates #101 (genotypic class $Chr1^{CAWB}Chr2^{P\text{-}A14}$) and #105 (genotypic class $Chr1^{P\text{-}A14}Chr2^{CAWB}$). It was expected that a 15:1 ratio of susceptible/resistant or resistant/susceptible F_2 progeny would be observed in response to P-A14 if two major genes were

Table 3 Phenotypic score comparisons of genotypic classes derived from the host and pathogen populations

(a) Comparison of host genotypic classes		
Chr2H Locus	Chr7H Locus	Average reaction type
Hockett	Hockett	3.53a
Hockett	PI 67381	3.07b
PI 67381	Hockett	2.78b
PI 67381	PI 67381	2.23c
(b) Comparison of pathogen genotypic classes		
Chr1 Locus	Chr2 Locus	Average reaction type
P-A14	P-A14	2.95a
CAWB05-Pt-4	P-A14	2.20b
P-A14	CAWB05-Pt-4	1.87c
CAWB05-Pt-4	CAWB05-Pt-4	1.20d

a Host genotypic groups are shown with average reaction types when inoculated with *P. teres* f. *maculata* isolate P-A14. **b** Pathogen genotypic groups are shown with their average reaction type when inoculated on Hockett barley. Groups with different letters following their average reaction type were significantly different at the 0.01 level of probability

conferring this phenotype. Likewise, a 3:1 ratio of susceptible/resistant or resistant/susceptible F_2 progeny was expected to be observed in response to pathogen progeny harboring only one virulence allele contributed by P-A14. F_2 progeny was determined to be resistant if they displayed a reaction type ≤ 1.5 or susceptible if they displayed a reaction type ≥ 2. Of 51 F_2 seedlings inoculated with P-A14, three were determined to be resistant and 48 determined to be susceptible. Of 49 F_2 seedlings inoculated with progeny #101 ($Chr1^{C\text{-}AWB}Chr2^{P\text{-}A14}$), 15 were determined to be resistant and 34 determined to be susceptible. Of 49 F_2 seedlings inoculated with progeny #105 ($Chr1^{P\text{-}A14}Chr2^{CAWB}$), 16 were determined to be resistant and 33 determined to be susceptible. Chi-square analysis indicated that none of the observed ratios of resistant/susceptible reaction types differed significantly from the expected ratios (Table 4), indicating that effectors associated with the *P. teres* f. *maculata* Chr1 and 2 QTL are likely targeting susceptibility genes underlying the barley Chr2H and 7H QTL, respectively. Additional data used for all statistical analyses are provided in Online Resource 3.

Together, these results provide support for the role of the inverse gene-for-gene model in the *P. teres* f. *maculata*-barley pathosystem, in which dominant virulence genes in the pathogen target dominant susceptibility genes in the host, resulting in the completion of the pathogenic life cycle.

Discussion

Necrotrophic pathogens of cereals have emerged as a major problem for breeders and growers globally due to these pathogens having the potential to successfully thrive in new environments, adapt to current and novel hosts, and adapt to global climate change (McLean et al. 2009; Chakraborty 2013; Valásquez et al. 2018). The threat of these diseases has led to the application of large amounts of fungicides worldwide resulting in the development of fungicide-resistant pathogen populations (Mair et al. 2020), contributing to the need for alternate control practices. Spot form net blotch (SFNB), first reported in Denmark in the 1970s (Smedegård-Petersen 1971), has recently emerged as a major disease of barley throughout many important barley growing regions of the world, including the USA, Canada, Australia, Denmark, Norway, and South Africa (Reviewed in McLean et al. 2009 and Liu et al. 2011). More recently, *P. teres* f. *maculata*, the causal agent of SFNB, has emerged as a pathogen of wheat (Perelló et al. 2019; Uranga et al. 2020), showing that this pathogen can adapt to its host and environment and is therefore a considerable threat to global cereal production.

Due to their ease of development and screening, several barley populations have been used to evaluate resistance/

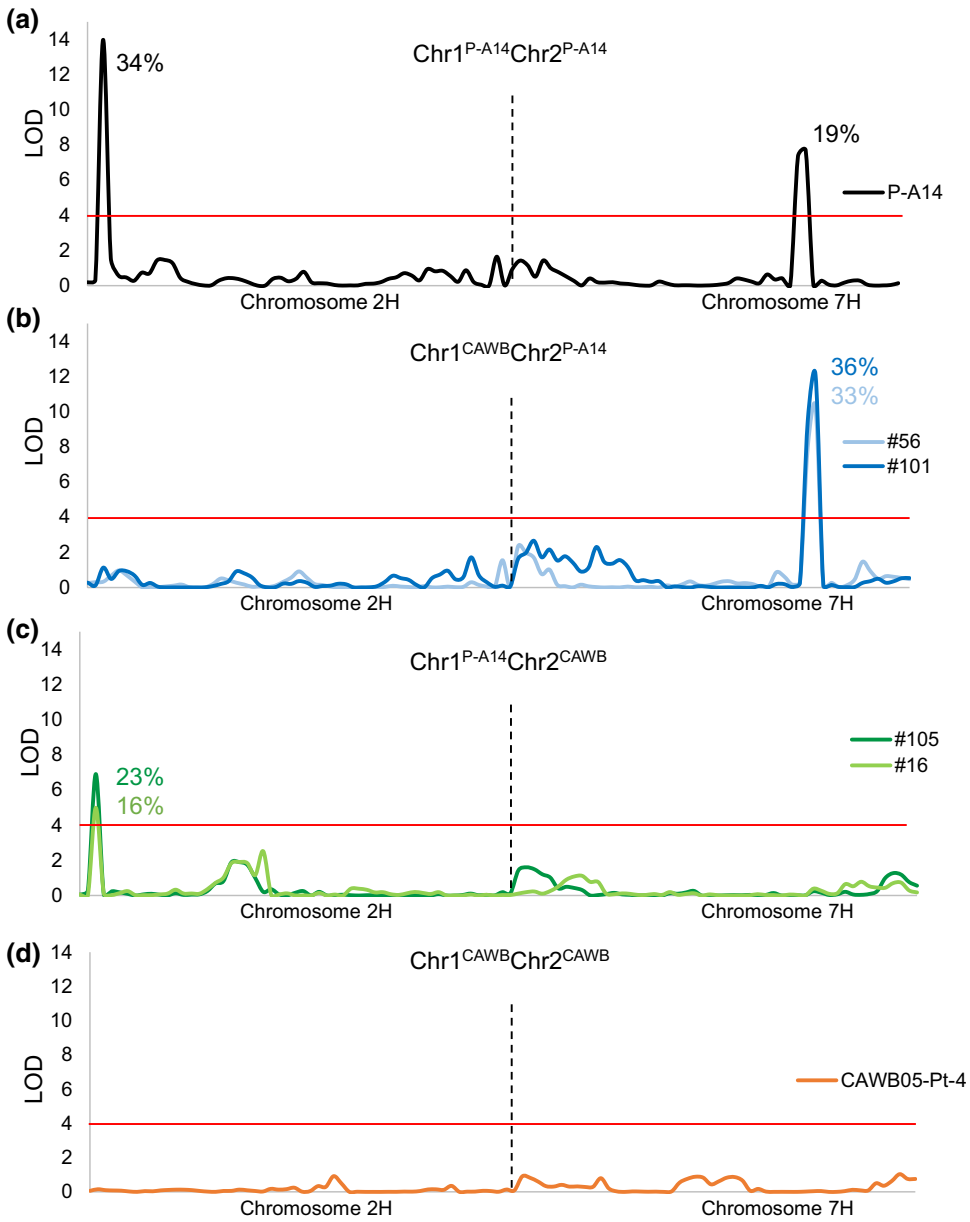

Fig. 4 Hockett × PI 67381 QTL analysis with *P. teres* f. *maculata* P-A14 × CAWB05-Pt-4 parental and progeny isolates. **a** Isolate P-A14 targets the resistance/susceptibility loci at chromosome 2H and chromosome 7H. **b** Isolates #56 and #101 with genotype $Chr1^{CAWB}Chr2^{P\text{-}A14}$ target the resistance/susceptibility locus at chromosome 7H. **c** Isolates #105 and #16 with genotype $Chr1^{P\text{-}A14}Chr2^{CAWB}$ target the resistance/susceptibility locus at chromosome 2H. **d** Isolate CAWB05-Pt-4 doesn't show a significant association with resistance/susceptibility on either chromosome. The red horizontal lines indicate the critical LOD threshold ($\alpha = 0.01$) of 4.0. Only chromosomes 2H and 7H are shown. Color-coded R^2 values corresponding to each isolate are listed next to the QTL

susceptibility to *P. teres* f. *maculata* to identify QTL associated with disease severity on six of the seven barley chromosomes (reviewed in Clare et al. 2020). Relatively fewer studies have evaluated the genetics of *P. teres* f. *maculata* virulence due to the labor-intensive work needed to develop, phenotype, and map fungal populations. Using both host and pathogen mapping populations in conjunction with QTL analysis provides the opportunity to identify both barley susceptibility loci and the corresponding pathogen virulence loci targeting the putative susceptibility genes, the first step toward identifying and characterizing the causal genes. The work presented here shows not only the importance of understanding the pathogen side of a disease interaction, but also the value of jointly evaluating the host and pathogen to define an interaction more accurately.

To evaluate this interaction, we chose host and pathogen parental genotypes that displayed a wide range of reaction types, allowing for easier identification of genetic regions associated with susceptibility and virulence. Using the host and pathogen disease phenotyping data, it was clear that both virulence and susceptibility were additive; that is, when the pathogen harbored both Chr1 and Chr2 virulence alleles, the pathogen was significantly more virulent than when it only harbored one or the other. Similarly, when the host progeny harbored both the 2H and 7H susceptibility alleles, these lines were more susceptible. Additionally, we showed that the Chr1 virulence allele in the pathogen was targeting

Table 4 Hockett × PI 67381 F_2 reaction type data.

Pathogen	P-A14	#101	#105
# F_2 progeny tested	51	49	49
# Resistant (RT ≤ 1.5)	3	15	16
# Susceptible (RT ≥ 2.0)	48	34	33
Expected 1:15	3.19	–	–
Expected 15:1	47.81	–	–
Expected 1:3	–	12.25	12.25
Expected 3:1	–	36.75	36.75
X^2 value	0.011765	0.823129	1.530612
p-value	0.914	0.364	0.216

Data shows observed numbers of resistant and susceptible Hockett × PI 67381 F_2 progeny when inoculated with P-A14 and P-A14 × CAWB05-Pt-4 progeny isolates #101 and #105. F_2 progeny were evaluated based on a 1 to 5 reaction type (RT) scale (Neupane et al. 2015). RT of ≤ 1.5 were defined as resistant and RT of ≥ 2 were defined as susceptible. Expected ratios of resistant:susceptible and susceptible:resistant F_2 progeny given two genes conferring virulence (P-A14) and one gene conferring virulence (#101, #105) are shown. X^2 and corresponding *p*-values indicate no significant variation of observed ratios from expected ratios

the Chr2H susceptibility allele in the host and the Chr2 virulence allele was targeting the barley Chr7H susceptibility allele, indicating a gene-for-gene interaction.

Recombinant inbred host populations are powerful tools in evaluating resistance to pathogens because the progeny are fixed lines where inoculations can be replicated, and multiple pathogen genotypes can be evaluated side-by-side. However, the shortcoming of RIL populations is that they are not useful for evaluating gene action. F_2 population inoculations cannot be replicated; however, due to heterozygosity, they can be used to evaluate dominance and therefore predict gene function. Here F_2 populations were used to show that susceptibility was dominant over resistance, another indication of effectors targeting a susceptibility gene product and therefore indicating an inverse gene-for-gene interaction. This inverse gene-for-gene interaction is reminiscent of the *Parastagonospora nodorum*-wheat interaction where multiple necrotrophic effectors directly or indirectly target individual susceptibility gene products resulting in pathogen colonization of the host (reviewed in Friesen and Faris 2021; Faris and Friesen 2020). The necrotrophic effectors in the *P. nodorum*-wheat interaction also act additively in several instances (Friesen et al. 2007, 2008) but in some other cases the effectors act epistatically (Friesen et al. 2008; Peters et al. 2019; Peters-Haugrud et al. 2022). Additional research into this *P. teres* f. *maculata*–barley interaction is being performed, including gene cloning and functional characterization of the effectors and the corresponding susceptibility genes in barley to fully understand the effector mode of action and the function of the host response. In the current study, major resistance/susceptibility loci were identified on the short arm of barley Chr2H and the long arm of Chr7H in the Hockett × PI 67381 barley population. The major QTL identified on Chr2H was previously identified in three different barley biparental populations (Tamang et al. 2019), as well as by association mapping (Burlakoti et al. 2017). A major QTL identified in a similar region on Chr7H has also been previously described by Tamang et al. (2019) in the same three populations, as well as by Williams et al. (1999, 2003), Grewal et al. (2008), Wang et al. (2015), and Tamang et al. (2015), indicating the importance of these genes in multiple breeding lines and cultivars. However, little was known about gene action or the association between these host resistance/susceptibility genes and the possibility of corresponding pathogen avirulence/virulence genes recognized by or targeting these host genes.

Effector genes have been localized to sub-telomeric regions in *P. teres* f. *teres* (Wyatt et al. 2020), and based on the telomeric localization of both virulence loci presented here, it appears that this localization may also be common for *P. teres* f. *maculata*. Wyatt et al. (2020) speculated that this localization of effector genes to sub-telomeric regions was due to these regions having the ability to rapidly evolve, providing a mechanism for rapid adaptation of the pathogen to its changing host and environment. Similar QTL regions on both Chr1 and 2 identified here were recently identified in a broad association mapping study in which 30 loci associated with *P. teres* f. *maculata* virulence were identified (Clare et al. 2022), indicating the prevalence of these two loci as well as the complexity of this host–pathogen interaction.

In addition to Hockett barley (released by Montana State University), barley lines from other US breeding programs were evaluated using the P-A14 × CAWB05-Pt-4 pathogen population, including Pinnacle (North Dakota State University, 2-row), Stellar-ND (North Dakota State University, 6-row), Innovation (Busch Agricultural Resources), and Lacey (University of Minnesota). All lines harbored susceptibility specific to the Chr1 and 2 virulences, suggesting that each harbored the Chr2H and 7H susceptibilities that were present in Hockett barley. This shows that these susceptibilities are prevalent in major US barley breeding programs, especially those releasing lines planted in the Northern Plains of the USA.

P. teres f. *maculata* isolates collected in Montana, North Dakota, Denmark, and New Zealand were also evaluated on the Hockett × PI 67381 population. Inoculations of C-A17 and FGOB10Ptm-1 showed that, like P-A14, these North American isolates harbored both virulences that targeted the Chr2H and 7H barley loci. The isolates from Denmark and New Zealand were less virulent on the susceptible parental line Hockett but did show a significant association with the Chr2H locus, suggesting that these isolates harbored the *P. teres* f. *maculata* Chr1 virulence.

However, the barley Chr7H locus was not significantly associated with disease, suggesting that the Danish and New Zealand isolates lacked the Chr2 virulence.

One additional barley locus associated with resistance/susceptibility specific to FGOB10Ptm-1, C-A17, and NZKF2, but not P-A14 or Den2.6, was identified on Chr6H. On the pathogen side, in addition to the major Chr1 and Chr2 loci identified, relatively minor loci associated with virulence were present on Chr1 (Lacey, Pinnacle), Chr3 (Innovation, Lacey), Chr3 (Pinnacle), Chr3 (Lacey, Pinnacle, Stellar-ND), Chr5 (Innovation, Pinnacle), and Chr10 (Innovation). Four of these minor QTL may have been previously identified by Clare et al. (2022). The minor QTL on Chr1 was identified at a distance of roughly 400 kb from the *Ptm_QTL2* locus. The QTL on Chr3 associated with virulence on lines Innovation and Lacey was present about 90 kb from the *Ptm_QTL9* locus, the Chr3 QTL corresponding only to Pinnacle was located 20 kb from the *Ptm_QTL10* locus, and the final Chr3 QTL was identified at approximately 240 kb from the *Ptm_QTL14* and *Ptm_QTL15* loci. The remaining minor QTL on Chr5 and Chr10 appear to be novel, as they were located over one Mb from any previously described locus (Clare et al. 2022). In addition to the interacting loci identified in the Hockett × PI 67381 and the P-A14 × CAWB05-Pt-4 populations, the presence of these additional minor associations underscores the unique complexities inherent in any individual SFNB interaction and provides targets for future investigation.

Given that all the susceptible barley lines used in this study were from different North American breeding programs, it is likely that the Chr2H and Chr7H susceptibilities are prevalent in many popular cultivars planted in North America, providing selection pressure for the pathogen populations in these regions to maintain the Chr1 and Chr2 virulence alleles. Further characterization of the genes conferring virulence will be critical to understanding how to breed for resistance to spot form net blotch, an economically important emerging disease of barley. Additionally, evaluations of global barley collections are needed to identify the prevalence of these susceptibilities outside of the USA.

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Author contribution statement RMS, NAW, and TLF designed the experiments. JDF and RSB generated sequencing data for the barley and *P. teres* markers, respectively. RMS, NAW, and GKK performed mapping, phenotyping and QTL analysis. RMS and TLF analyzed the data and wrote the manuscript. All authors edited and approved the manuscript.

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Data availability All data associated with this study are available in the supplementary data (Online Resource 3) or upon request from the corresponding author.

Declarations

Conflict of interest The authors declare that there are no conflicts of interest.

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