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Resistance of European Spring 2-Row Barley Cultivars to *Pyrenophora graminea* and Detection of Associated Loci

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Abstract: *Pyrenophora graminea* is the seed-borne pathogen causal agent of barley leaf stripe disease. In this work, we screened a collection of 206 spring two-row barley cultivars from Europe for their resistance to the fungal pathogen. Artificial inoculation with the highly virulent isolate Dg2 revealed a continuous variation for the incidence of infection, with few highly resistant or highly susceptible genotypes. On average, old cultivars showed higher resistance than the more modern ones. Genome-Wide Association Scan was performed by exploiting available molecular data for >4000 SNP markers and revealed a single, highly significant association on the short arm of chromosome 6H, in a genomic position where quantitative trait loci (QTL) for barley resistance to *P. graminea* were not detected before. Based on the last version of the reference barley genome, genes encoding for proteins with a kinase domain were suggested as candidates for the locus.

Keywords: barley; leaf stripe; GWAS

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1. Introduction

Leaf Stripe is a seed-borne, single cycle disease of barley caused by the fungus *Pyrenophora graminea* (S. Ito and Kurib.) [anamorph: *Drechslera graminea* (Rabenh. exSchlecht.) Shoemaker (=*Helminthosporium gramineum* Rabh.)]. The pathogen can survive in the host pericarp as mycelium, and it penetrates during seed germination through the coleorhizae, developing systemically along with the plant [1]. The fungus infects barley seedlings, especially when soil temperatures during seed germination are below 12 °C, such as in the Mediterranean countries under winter sowing or in the Nordic countries under spring sowing. Disease symptoms start with the occurrence of yellow stripes on seedling leaves; they turn into chlorotic and necrotic stripes in time. Serious infections cause plants to desiccate and develop sterile spikes, up to premature death, thus affecting yield and quality of the crop [2,3]. Many countries reported severe yield losses because of the fungal disease [4–7].

Generally, leaf stripe is controlled by seed dressing with chemical compounds; however, the identification and exploitation of resistant genotypes is fundamental to decrease the use of pesticides in agriculture and for organic farming. Both race-specific [8,9] and partial resistance [10–12] to barley leaf stripe were reported. The first study was carried out by Skou and Haahr [13], who tested the response of 1029 different barley accessions to *P. graminea* infection, allowing the identification of several resistance sources; many of them postulated to be originally derived from *Hordeum laevigatum* through the cv. Vada. Giese et al. [14] reported a semi-dominant single gene on chromosome 2H as responsible of the "Vada Resistance" in European spring barleys. Two mapping populations developed from the crosses Alf × Vogelsanger Gold and L94 × Vada independently mapped

the "Vada resistance" gene (named *Rdgla*) on the long arm of chromosome 2H [8,11]. Biselli et al. [15] analyzed the L94 (susceptible) × Vada (resistant) and Arta (susceptible) × *Hordeum spontaneum* 41-1 (resistant) mapping populations and mapped *Rdg1a* to a smaller genomic interval of chromosome 2H. Their result also suggested that *Rdg1a* might be derived from *Hordeum spontaneum*, but the causal gene is yet undetermined.

Following Rgd1a, the qualitative resistance gene *Rdg2a* was mapped on the short arm of the chromosome 7H in highly resistant six-row winter barleys [9,16]. The locus showed resistance to a minimum of three highly virulent Italian isolates; however, it was not efficient against the highly virulent Dg5 isolate. After fine mapping [17] the *Rdg2a* gene was cloned by a map-based approach [18], and the organization and evolution of locus were characterized, together with a histological/molecular dissection of the *Rdg2a*-based barley leaf stripe resistance.

It was frequently reported that spring barleys have quantitative resistance to barley leaf stripe. Pecchioni et al. [10] studied the reaction of the population derived from two-row spring barley cultivars Proctor (hulled) and Nudinka (hulless) to *P. graminea* and mapped the so-called "Proctor-resistance" as a quantitative trait locus (QTL) on the centromeric region of chromosome 7H. The relation between hulless seed and susceptibility to *P. graminea* was emphasized in this study. A second QTL was detected on chromosome 2H. Similarly, Arru et al. [11] mapped two QLTs for leaf stripe resistance in the L94 × C123 mapping population: the QTL on chromosome 7H overlapped with Proctor-resistance, while the QTL on chromosome 2H did not. Finally, Arru et al. [12] tested the Steptoe × Morex population for susceptibility to the highly virulent *P. graminea* isolates Dg2 and Dg5, in order to investigate the isolate specificity of partial resistance to the fungus. They found that a QTL on the long arm of chromosome 2H and two QTLs located on chromosome 3H conferred resistance to both the isolates. Furthermore, it was determined that a QTL on the short arm of chromosome 2H was specific to the isolate Dg2, while a QTL located on chromosome 5H was specific to the isolate Dg5.

In this work, a highly virulent isolate of *Pyrenophora graminea* was used to artificially inoculate a collection of >200 spring barley cultivars from Europe to identify novel sources of resistance to the fungal pathogen and map the involved loci through a Genome-Wide Association Scan approach.

2. File, and Methods

2.1. Plant Material

A collection of 206 spring 2-row barley cultivars, fully representative of the diversity of spring barleys bred in Europe in the 20th century [19], was used in the present work (Supplementary File, Table S1). The panel was previously genotyped with the barley 9k Infinium iSelect array [20], and the description of population structure, genetic diversity and linkage disequilibrium was reported by Tondelli et al. (2013). In the present work, 4016 informative SNPs were maintained, after filtering for call rate (CR) >95% and minor allele frequency (MAF) >5% (Supplementary File, Table S2). Their physical position on the most recent version of the Morex reference genomic sequence [21] was retrieved from the Germinate Barley SNP Platforms hosted at the James Hutton Institute (https://ics.hutton.ac.uk/50k/index.pl, accessed on 19 February 2021).

2.2. Inoculation Test and Disease Evaluation

The resistance of spring 2-row barley cultivars was assessed by artificial inoculation with the highly virulent *Pyrenophora graminea* isolate Dg5 [5], using the "sandwich" method described in Pecchioni et al. [10]. For each accession, 60 seeds were surface-sterilized in 70% ethanol for 30 s and 5% NaOCl for 10 min, then rinsed in deionized water, left to dry and incubated in two Petri dishes (30 seeds each) between two potato dextrose agar (PDA) layers colonized by an actively growing mycelium of the fungus. Seeds were incubated for 20 days at 6 °C in the dark, and emerged seedlings were transplanted to six 12 cm pots (10 plants/pot) and grown in a greenhouse, following a randomized complete-block

design with two replications (three adjacent pots/replication). At the fourth leaf stage, the incidence of infection (i.e., the percentage of plants showing leaf stripes symptoms) was recorded for each genotype. The whole experiment was replicated in three different years, with transplanting occurring at the begin of March. Repeatability across experiments was estimated from the variance components calculated after fitting a fully random effects model as $H^2 = V_g / [V_g + (V_{gy}/y) + (V_e/ry)]$, where V_g is the genotypic variance, V_{gy} is the genotype x year interaction variance, V_e is the error variance, y is the number of years and r is the number of replicates.

2.3. Genome-Wide Association Scans

Markers with CR > 95% and MAF > 5% were used for detecting loci associated with spring barley resistance to *P. graminea*. Genome-Wide Association Scans (GWAS) were carried out using a mixed linear model (MLM) approach in Tassel v3.0 [22], which considers population substructure and genetic relatedness by including a kinship matrix as a random term. MLM was run without compression and P3D estimation. Thresholds for detecting significant SNP associations were calculated by False Discovery Rate (FDR) with the R package "qvalue". Genes with a putative role in the response of barley to pathogen infection were searched among the High Confidence genes annotated at associated genomic regions, based on the Morex reference genomic sequence [21].

3. Results and Discussion

3.1. Incidence of P. graminea Infection in Spring 2-Row Barley Cultivars

In this work, a three-year experiment of artificial inoculation under controlled conditions with a high virulent isolate of *P. graminea* was performed to assess the resistance of a collection of 206 spring two-row European barley cultivars. Together with the isolate Dg2, the isolate Dg5 used here was the most virulent when tested on a large collection of barley cultivars [5]. A continuous variation in the average incidence of infection was observed, with a distribution skewed towards resistant phenotypes (Figure 1). Similar distribution of phenotypic classes frequency suggesting quantitative resistance to leaf stripe disease has been already observed in barley [10–12,16].

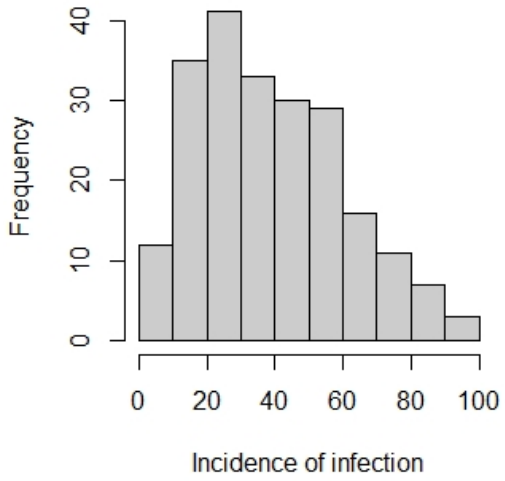

Figure 1. Frequency distribution of the incidence of *P. graminea* infection (i.e., the percentage of plants showing leaf stripes symptoms, averaged across the three experiments) in 206 spring 2-row barley cultivars.

Analysis of variance revealed significant effects for both the experiment factor, with a lower incidence of infection in the experiment no.3 (Supplementary File, Figure S1), and the genotype x experiment interaction (Supplementary File, Table S3). The repeatability value was 0.69, suggesting a highly relevant genetic control on the scored phenotype,

hence a high probability to detect significant marker-trait-associations through GWAS (see Section 3.2).

Tondelli et al. [19] observed a clear temporal trend in the diversity of the barley collection employed in this work, with modern and old varieties belonging to distinct subgroups (K1 and K2, respectively) and several admixed genotypes positioned between the two subgroups in a SNP-based Principal Coordinate plot. When K1 and K2 subgroups were considered in the analysis of *P. graminea* infection data, old barley cultivars showed a significantly lower incidence of the disease with respect to the modern ones ($p < 0.05$), with average values of 33.9% and 43.4%, respectively (Table 1). It could be hypothesized that recently deployed seed dressing chemical compounds allowing leaf stripe disease control decreased the need of selection pressure towards leaf stripe resistance in more recently released barley cultivars.

Table 1. Incidence of *P. graminea* infection in spring barley cultivars grouped according to STRUCTURE analysis and country of origin.

Country of Origin	No. Genotypes	Mean Infection Incidence
STRUCTURE group 1		
Austria	1	20.3
Czech Republic	12	37.0
Denmark	21	41.5
Estonia	1	7.0
France	1	20.9
Germany	21	42.9
Latvia	1	94.8
Netherlands	3	30.6
Slovak Republic	2	43.4
United Kingdom	15	56.5
Total	78	43.4
STRUCTURE group admixed		
Austria	2	43.3
Czech Republic	13	34.3
Denmark	8	40.1
Estonia	2	59.1
Finland	2	32.5
France	4	39.3
Germany	6	44.3
Italy	1	73.4
Latvia	7	35.0
Netherlands	8	40.9
Slovak Republic	1	37.4
Sweden	4	40.9
United Kingdom	8	28.3
Total	66	38.4
STRUCTURE group 2		
Denmark	11	23.1
Finland	6	56.5
France	1	83.3
Italy	2	33.7
Latvia	5	18.0
Netherlands	4	37.7
Norway	1	48.4
Sweden	27	34.0
United Kingdom	5	30.3
Total	62	33.9

When considering the most represented countries in the collection, higher resistance to leaf stripe was noticed in old genotypes from Latvia, Denmark and Sweden (STRUCTURE group 2 in Table 1) with respect to more recent cultivars from the United Kingdom, Germany and Denmark (STRUCTURE group 1 in Table 1; Supplementary File, Table S1). In more detail, 10 genotypes (Nordal, Vada, Ida, Golf, Alva, Imber, Lud, Tyra, Idumeja, Drost and Leeni) displayed symptoms in <10% of the plants (Supplementary File, Table S1). Except for the Estonian modern cultivar Leeni, the other nine cultivars belong to the structure group 2 (old varieties) or admixed and were released in Denmark (n = 3), the UK (n = 3), Sweden (n = 2), the Netherlands (n = 1) and Latvia (n = 1).

3.2. Genome Wide Association Scan

The phenotypic data collected on the spring barley population were associated with 4016 informative genome-wide SNP markers to identify genomic regions associated with *P. graminea* resistance. When considering the mean incidence of infection over the three replications of the experiment or data from the single experiments, mixed linear models in Tassel identified a single, highly significant SNP (−log10(P) = 11.2; FDR-corrected $p < 1 \times 10^{-4}$) on the short arm of chromosome 6H, at position 9,712,203 (Figure 2 and Supplementary File, Figures S2 and S3). This SNP (BOPA1_5993-2383) does not segregate uniformly in the barley population under analysis; in fact, the reference "A" allele was observed in 192 genotypes out of 206 (93.2%), while the alternative "T" allele was present in 11 genotypes (5.3%). For 3 cultivars, it was not possible to assign an allele at this locus.

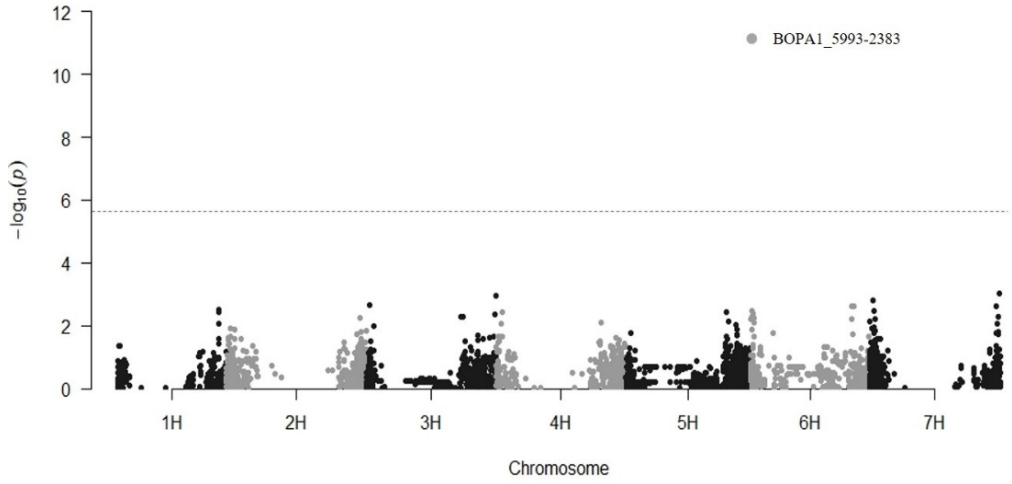

Figure 2. Manhattan Plot showing the results of the Genome-Wide Association Scan for spring barley resistance to leaf stripe. SNP markers mapping to the seven barley chromosomes are in different colors. The dashed line represents the 0.05 FDR threshold.

The average incidence of infection for the cultivars with the "T" allele—namely, Alexis, Alis, Birka, Doublet, Elo, Felicitas, Gate, Meltan, Roland, Saana, Triumph—was more than double the value observed for cultivars carrying the reference allele (76.5% vs. 36.9%). Among these highly susceptible genotypes is the German cultivar Triumph (also known as Trumpf), a widespread donor of yield and malting quality traits [23], and also a parent of Alexis, Alis, Doublet, Elo and Meltan (Supplementary File, Table S1). Alis showed a lower value of 43.5% infected plants across the three experiments, although a higher incidence of infection of 65.2% was registered in the third experiment, which on average was less severe.

The most resistant cultivars harbored the "A" allele, although within this class fall also 10 cultivars with >75% of infected plants (Supplementary File, Table S1). This resistance allele was the most frequent in the collection, while the rare alternative one was related to susceptibility; this may be due to a quantitative resistance gene being almost fixed in spring barleys. Since leaf stripe is not considered among the major diseases of barley, and breeding programs in Europe usually do not specifically focus on *P. graminea* resistance; genetic

linkage between BOPA1_5993-2383 and other important selected genes may support the hypothesis of a resistance locus unconsciously selected because of its linkage to valuable traits in spring barley.

Although a continuous phenotypic variation and high heritability of the measured trait were observed, no other significant loci were detected in this work when the mean values across the three experiments were used for GWAS. No significant association was observed, for example, at the *Rdg1a* locus, mapping at the telomere of chromosome 2HL [15]. *Rdg1a* is commonly referred to as the "Vada resistance" gene, because it was introduced into spring barleys through the cultivar Vada [16]. In the collection screened here, Vada is the parent of five cultivars only (i.e., Abacus, Alva, Egmont, Lud and Salka), and these are themselves in the pedigree of the cultivars Claret, Dandy, Roland and Golf (Supplementary File, Table S1). Although 7 out of 9 of these cultivars show leaf stripe symptoms in <15% of the plants, *Rdg1a* is most probably segregating at low frequency (i.e., <5%) in the population, and for this reason, markers associated with *Rdg1a* were filtered out in GWA analysis. Similarly, *Rdg2a* on chromosome 7H was detected in winter six-row barleys [9] and may not be present in the collection under study. Other low-frequency resistance genes may be responsible for the low susceptibility to leaf stripe observed in cultivars, such as Nordal (Heine 4808 × Dana, from Carlsberg, Denmark) or Ida (Arla M1 × Tellus, from Weibull, Sweden), but their detection needs different genetic approaches, such as the development of bi-parental mapping population and linkage analysis. On the other hand, the partial resistance to *P. graminea* observed in this work might be based on different mechanisms with respect to qualitative resistance.

Despite the moderate levels of linkage disequilibrium observed in the population [19], the low marker density especially around the centromeric regions (Figure 2) may preclude the identification of *P. graminea* resistance loci. This may be the case of the "Proctor-resistance" QTL mapped at the centromere of chromosome 7H [10].

3.3. Identification of Putative Candidate Genes

Thirty-six SNP markers are physically mapped in a 15 Mb genomic interval surrounding the leaf stripe resistance locus identified on chromosome 6H. None of these SNP shows high levels of linkage disequilibrium with BOPA1_5993-2383 (Figure 3), and small LD blocks are detected through Haploview analysis, which is consistent with other observations on telomeric regions in barley [24]. Based on this observation, we defined a confidence interval for the detected locus that spans from the SNP more distal to BOPA1_5993-2383 (at 9.18 Mb) to the more proximal one (at 10.49 Mb).

Based on the most recent version of the reference barley genome sequence (cultivar Morex, [21]), 28 high-confidence genes are annotated in this 1.3 Mb genomic interval of chromosome 6H (Table 2). The single, highly significant SNP detected in this work maps in the 3′ untranslated region of the gene *HORVU.MOREX.r2.6HG0451070* that is annotated as U5 small nuclear ribonucleoprotein helicase (U5 snRNP). snRNPs are components of the spliceosome for pre-mRNA splicing, but to our knowledge, their possible role in the plant response to pathogen has not been described; nine genes are annotated as U5 snRNP in the barley genome.

Among the 13 high-confidence genes neighboring *HORVU.MOREX.r2.6HG0451070*, 12 encode for protein kinase family proteins (n = 5), wall-associated kinase-like proteins (n = 4), kinase family proteins (n = 2) or receptor-like protein kinase (n = 1; Table 2). Plant wall-associated kinases (WAKs) are a class of receptor-like kinases that bind to the cell wall through an extracellular and a transmembrane domain; a serine threonine kinase intracellular domain functions as activator of a signaling cascade. Other than regulating cell expansion by binding to cell wall pectin polymers [25], it has been suggested that WAKs can sense cell wall integrity under stressing conditions [26] and can thus play important roles in host basal resistance [27]. In wheat, the wall-associated receptor kinase-like protein TaWAKL4 was identified by map-based cloning of the Stb6 locus, conferring race-specific resistance to *Zymoseptoria tritici* [28]. The rice *OsWAK (Xa4)* gene confers

resistance to bacterial blight by enhancing the biosynthesis of cellulose and strengthening the cell wall [29], and quantitative resistance to corn leaf blight is associated with the maize *ZmWAK-RLK1 (Htn1)* gene that reduces pathogen penetration into host tissues [30]. By comparing the transcriptomes of two barley genotypes during infection with *P. graminea*, Ghannam et al. [31] identified candidate genes possibly involved in callose deposition and different oxidation processes with a major role in the interaction between the host plant and the pathogen. Similarly, a histological and transcriptome analysis of barley to leaf stripe infection identified differential responses for cell wall modifications and induction of cell-wall reinforcement-related genes when compatible and incompatible interactions were compared [32].

Interestingly, the *Parastagonospora nodorum* resistance locus *Snn1* of wheat encodes for a member of the wall-associated kinase class of receptors that acts as a susceptibility factor, since it recognizes the SnTox1 toxin produced by the necrotrophic fungal pathogen to activate cell death, thus allowing *P. nodorum* to proliferate [33]. Based on these observations, the four wall-associated kinase-like proteins located at the leaf stripe resistance locus detected here could represent candidate genes for the resistance function encoded by the 6H leaf stripe resistance locus. Nevertheless, expression analyses, re-sequencing and allele mining experiments will be necessary to better resolve the complexity of the locus. It should finally be noted that an NBS-LRR resistance-like protein, which represents the proteins encoded by the major classes of plant disease resistance genes, maps at position 9.18 Mb, at the distal side of the genomic interval associated with *P. graminea* resistance. Conversely, *HORVU.MOREX.r2.6HG0450720*, the ortholog of the wheat kinase-pseudokinase *WTK1* gene underlying the yellow rust resistance locus *Yr15* [34], maps at position 8.45 Mb on chromosome 6H, outside of the LD-based confidence interval defined above.

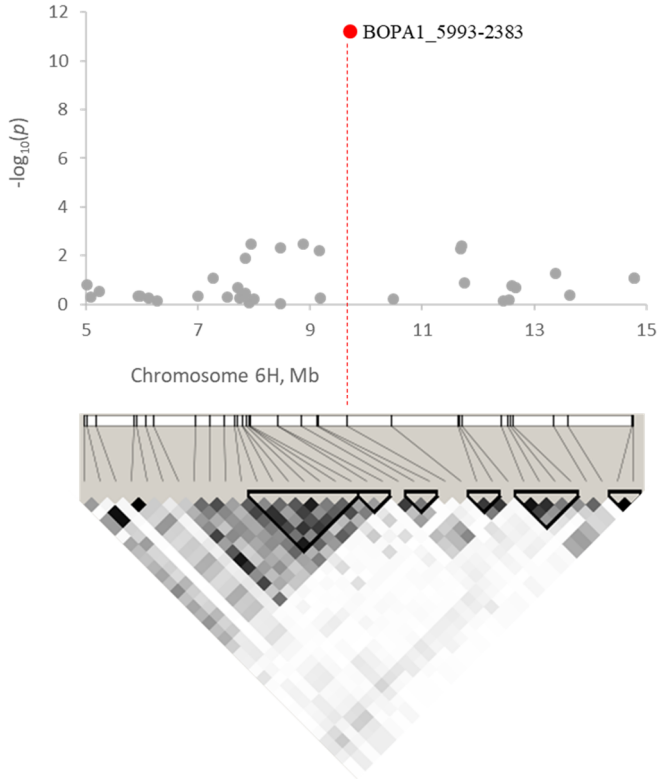

Figure 3. Levels of linkage disequilibrium at the genomic interval associated with leaf stripe reScheme 6. H, from 9.18 Mb to 10.49 Mb. A white-to-black color scale indicates the correlation between 36 SNP markers, based on Haploview analysis.

Table 2. High-confidence barley genes annotated in the 1.3 Mb genomic interval of chromosome 6H associated with the resistance to *P. graminea* in the spring barley cultivar collection.

Gene	Position_Start	Position_End	Annotation
HORVU.MOREX.r2.6HG0450940	9181207	9183058	NBS-LRR resistance-like protein
HORVU.MOREX.r2.6HG0450970	9287388	9289667	Zinc finger with UFM1-specific peptidase domain protein
HORVU.MOREX.r2.6HG0450990	9292853	9293407	Heat shock protein 70 (Hsp 70) family protein
HORVU.MOREX.r2.6HG0451000	9308835	9309251	Serine/threonine protein phosphatase 7 long form isogeny
HORVU.MOREX.r2.6HG0451010	9383225	9385148	F-box domain containing protein
HORVU.MOREX.r2.6HG0451040	9453725	9455308	Kinase family protein
HORVU.MOREX.r2.6HG0451050	9547723	9551610	Transmembrane protein
HORVU.MOREX.r2.6HG0451060	9552594	9554093	Kinase family protein
HORVU.MOREX.r2.6HG0451070	9704603	9712201	U5 small nuclear ribonucleoprotein helicase
HORVU.MOREX.r2.6HG0451080	9742211	9744382	Protein kinase family protein
HORVU.MOREX.r2.6HG0451090	9745927	9746731	Wall-associated kinase-like protein
HORVU.MOREX.r2.6HG0451100	9772601	9824626	Receptor-like protein kinase
HORVU.MOREX.r2.6HG0451110	9872022	9873296	Protein kinase family protein
HORVU.MOREX.r2.6HG0451160	9900818	9901225	Wall-associated receptor kinase 5
HORVU.MOREX.r2.6HG0451170	9943083	9946721	Protein kinase family protein
HORVU.MOREX.r2.6HG0451210	9988000	9988962	Wall-associated receptor kinase 5
HORVU.MOREX.r2.6HG0451220	10050772	10055369	Protein kinase family protein
HORVU.MOREX.r2.6HG0451270	10170204	10175665	Protein kinase family protein
HORVU.MOREX.r2.6HG0451280	10175679	10176481	wall-associated receptor kinase-like protein
HORVU.MOREX.r2.6HG0451290	10211221	10212693	3-ketoacyl-CoA synthase
HORVU.MOREX.r2.6HG0451310	10236832	10238547	Chloroplast stem-loop binding protein
HORVU.MOREX.r2.6HG0451320	10270618	10272058	F-box domain containing protein, expressed
HORVU.MOREX.r2.6HG0451340	10297765	10299204	Plant/F1M20-13 protein
HORVU.MOREX.r2.6HG0451360	10421589	10427171	Two-component response regulator
HORVU.MOREX.r2.6HG0451370	10443935	10449495	Two-component response regulator
HORVU.MOREX.r2.6HG0451400	10462773	10466795	Holliday junction ATP-dependent DNA helicase RuvB
HORVU.MOREX.r2.6HG0451420	10470836	10480123	Lysine-specific demethylase 3B
HORVU.MOREX.r2.6HG0451430	10490144	10493431	Protein kinesin light chain-related 3

In conclusion, in the present work, spring two-row barley cultivars from Europe with high resistance to *Pyrenophora graminea* were identified, and a novel highly significant locus associated with resistance/susceptibility to the fungus was mapped on the short arm of chromosome 6H. Taken together, these results may help resistance breeding towards this important seed-borne pathogen, with a particular interest for organic farming and for decreasing the use of chemical compounds in seed dressing.

Supplementary Materials: The following are available online at https://www.mdpi.com/2073-4395/11/2/374/s1, Figure S1: box-plot of incidence of infection in the three independent experiments; Figure S2: Manhattan Plots for resistance to *P. graminea* in the three independent experiments; Figure S3: Q-Q plot of *p*-values from the GWAS with the average phenotypic values across the three experiments; Table S1: list of spring barley cultivars tested in the present work, with incidence of *P. graminea* infection and allele at the SNP marker BOPA1_5993-2383; Table S2: SNP matrix used for GWAS. Table S3: ANOVA table for the incidence of *P. graminea* infection collected in the present work.

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Data Availability Statement: All the data are provided in Supplementary Files.

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Conflicts of Interest: The authors declare no conflict of interest.

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