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ORIGINAL ARTICLE

Discovery and fine mapping of *Rph28*: a new gene conferring resistance to *Puccinia hordei* from wild barley

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Abstract

Key message **A new gene *Rph28* conferring resistance to barley leaf rust was discovered and fine-mapped on chromosome 5H from wild barley.**

Abstract Leaf rust is a highly destructive disease of barley caused by the fungal pathogen *Puccinia hordei.* Genetic resistance is considered to be the most effective, economical and eco-friendly approach to minimize losses caused by this disease. A study was undertaken to characterize and fine map a seedling resistance gene identified in a *Hordeum vulgare* ssp. *spontaneum*-derived barley line, HEB-04-101, that is broadly effective against a diverse set of Australian *P. hordei* pathotypes. Genetic analysis of an F_3 population derived from a cross between HEB-04-101 and the *H. vulgare* cultivar Flagship (seedling susceptible) confirmed the presence of a single dominant gene for resistance in HEB-04-101. Selective genotyping was performed on representative plants from non-segregating homozygous resistant and homozygous susceptible F_3 families using the targeted genotyping-by-sequencing (tGBS) assay. Putatively linked SNP markers with complete fixation were identified on the long arm of chromosome 5H spanning a physical interval between 622 and 669 Mb based on the 2017 Morex barley reference genome assembly. Several CAPS (cleaved amplified polymorphic sequences) markers were designed from the pseudomolecule sequence of the Morex assembly (v1.0 and v2.0), and 16 polymorphic markers were able to delineate the *RphHEB* locus to a 0.05 cM genetic interval spanning 98.6 kb. Based on its effectiveness and wild origin, *RphHEB* is distinct from all other designated *Rph* genes located on chromosome 5H and therefore the new locus symbol *Rph28* is recommended for *RphHEB* in accordance with the rules and cataloguing system of barley gene nomenclature.

Introduction

Barley leaf rust (BLR) is one of the most widespread and destructive foliar diseases of *Hordeum* spp. (Park et al. 2015). Caused by the biotrophic fungal pathogen *Puccinia hordei* Otth., leaf rust poses a significant threat to barley production causing yield reductions of up to 60% in susceptible cultivars, especially during severe epidemics (Cotterill et al. 1992). Genetic resistance is considered to be the most effective, economical and eco-friendly approach to minimize losses caused by BLR. Over the last 30 years, enormous efforts have been made to characterize and map genes conferring resistance to *P. hordei* in cultivated barley, resulting in the designation of 24 distinct all-stage resistance (ASR) and three adult plant resistance (APR) loci (Park et al. 2015; Kavanagh et al. 2017; Rothwell et al. 2020). Most of the 24 loci conferring ASR have been rendered ineffective due to rapid evolution of matching virulence in *P. hordei* populations, emphasizing the importance of ongoing efforts to both

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diversify and characterize new sources of resistance (Brooks et al. 2000; Park et al. 2015). The availability and utilization of diverse sources of effective resistance is crucial for successful barley breeding programmes (Singh et al. 2017). Therefore, it is essential to identify and map new resistance (R) genes and to develop breeder friendly molecular markers linked to these genes which would be helpful to identify and combine these genes through marker assisted selection.

Introgressing genes from various gene pools of *Hordeum* is an important approach to widen the genetic base of *Hordeum vulgare* (Vatter et al. 2018). Four genes (*Rph10, Rph11*, *Rph13* and *Rph15/Rph16*) have been sourced from wild barley, *H. vulgare* ssp. *spontaneum* (*Hvs*), and a further four *Rph* genes (*Rph17, Rph18, Rph22* and *Rph26*) have been sourced from bulbous barley, *H. bulbosum*; however, to date, none of these genes have been deployed in agriculture (Park et al. 2015). The wild progenitor, *Hvs*, has a higher level of genetic diversity for disease resistance genes compared to cultivated barley (Von Bothmer et al. 2003). A comparison of a sequenced wild barley genome (WB1) with the barley cultivar Morex revealed that WB1 carries more genes involved in resistance to various biotic and abiotic stresses (Liu et al. 2020), further highlighting the importance of the wild barley gene pool as a source of resistance.

Many different molecular marker systems have been employed for genetic mapping of *Rph* genes in barley, e.g. RAPD (random amplified polymorphic DNA), AFLP (amplified fragment length polymorphism), RFLP (restriction fragment length polymorphism) and SSR (simple sequence repeats) were used to map *Rph2* (Borovkova et al. 1997; Franckowiak et al. 1997), *Rph15* (Weerasena et al. 2004), *Rph19* (Park and Karakousis 2002) and *Rph21* (Sandhu et al. 2012), respectively. In addition to conventional molecular markers, NGS (next-generation sequencing) technologies such as DArT (Diversity arrays technology) and GBS (genotyping by sequencing) now provide high throughput and cost-effective genotyping platforms that facilitate fast detection and application of SNPs (single nucleotide polymorphisms) in barley (Darrier et al. 2019). DArT has been employed successfully in mapping several *Rph* genes including *Rph14, Rph20* and *Rph23* (Golegaonkar et al. 2009; Hickey et al. 2011; Singh et al. 2015). Furthermore, a complexity reduction GBS method- DArT-Seq platform was also successfully used to map *Rph9.am*, *Rph24, Rph25* and *Rph27* (Dracatos et al. 2014; Ziems et al. 2017; Kavanagh et al. 2017; Rothwell et al. 2020).

The NGS technologies usually identify the target interval at Megabase (Mb) levels (Kayam et al. 2017) and then chromosomal regions can be further saturated with markers of choice to fine map the target locus. Among the different molecular marker types used for fine mapping traits of interest in plants, co-dominant markers e.g. insertion-deletion and SNP [KASP (kompetitive allele-specific PCR) or CAPS (cleaved amplified polymorphic sequences)] are preferred due to their abundance and ability to distinguish homozygous from heterozygous marker loci (Liu et al. 2016). CAPS markers have been effectively employed in barley where high levels of polymorphism are available (Shavrukov 2014, 2016). Recently, Fazlikhani et al. (2019) performed high resolution mapping of the leaf rust resistance locus *RphMBR1012* using a combination of SSRs, insertion/deletion polymorphisms (InDels) and SNPs (KASP and CAPS marker systems). The recent availability of a reference genome assembly for barley cultivar Morex (Mascher et al. 2017) through increased access to the gene space has consequently improved the efficiency of molecular marker development for fine mapping studies. For instance, the *Rph13* locus (originally derived from *Hvs*) in barley accession PI 531849 was recently fine-mapped to the long arm of chromosome 3H using the Morex genome as a road map, despite its previously reported possible linkage with *Rph9* on 5H (Jost et al. 2020).

In order to diversify the genetic base of barley leaf rust resistance and utilize potential of available barley reference genome assembly and NGS, the present study was conducted to characterize BLR resistance in the *Hvs* derived barley line HEB-04-101 (referred to as *RphHEB*) identified from the wild barley nested association mapping population HEB-25 (Maurer et al. 2015). The barley line HEB-04-101 was used in this study because it is resistant to predominant Australian *P. hordei* pathotypes. The second objective of this study was to fine map *RphHEB* locus and develop CAPS markers and identify possible candidate genes.

Materials and methods

Plant materials and pathogen isolates

Maurer et al. (2015) developed a Nested Association Mapping (NAM) population for barley known as HEB-25 (HEB = Halle Exotic Barley) by crossing the European cultivar Barke (*H. vulgare*) with 24 highly divergent *Hvs* and one *agriocrithon* accession, with the aim of increasing genetic diversity. The resulting F_{1s} from each cross combination were then backcrossed with Barke as a female parent. BC_1 plants for each cross were selfed three times to produce 1420 BC_1S_3 (equivalent to $BC_1F_{3:4}$) lines. These HEB lines were introduced to Australia for detailed phenotypic evaluation of rust resistance and seed is currently maintained at the University of Adelaide, Australia. Initial rust testing of the HEB population at Plant Breeding Institute Cobbitty (PBIC) with *P. hordei* pathotype 5457 P+ identified over 100 lines with variable resistant infection type (IT) responses (D. Singh, unpublished). One of these lines (HEB-04-101) was used as a resistant parent and crossed to the seedling susceptible

Australian barley cultivar Flagship for inheritance and mapping studies.

Development of mapping population

An F_3 mapping population ($n = 125$) was developed by crossing HEB-04-101 and Australian barley cultivar Flagship (seedling susceptible to pathotype 5457 P+) for genetic analysis, characterization and mapping of the resistance in HEB-04-101 (tentatively designated as *RphHEB*). F_1 seed was harvested, threshed and sown to raise F_2 seed. Approximately 150 seeds from F_2 were space planted at the Horse Unit field site of PBIC, and the individual F_2 plants were harvested to generate F_3 families. The seeds were threshed and stored in dehumidified rooms under controlled temperature until further testing and sowing.

Sowings and inoculations

Twenty to 25 seeds from each F_3 family, including parents, were sown in 90-mm-diameter pots containing Grange Horticultural® soil premix (comprised of 80% 0–8 mm composted pine bark, 10% 0–3 mm composted pine bark, 10% propagating sand, 1 kg/m^3 gypsum, 1 kg/m^3 superphosphate, 0.25 kg/m^3 potassium nitrate, 0.25 kg/m^3 nitroform and 1.5 kg/m^3 magrilime). Seed of 23 *P. hordei* differentials (Park et al. 2015) was also sown as clumps as a control. All pots were fertilized with Aquasol (@) 25 g/10 L of water) at the time of sowing as well as one day before inoculation. The seedlings were raised in disease-free rooms maintained at 18–20 °C. Ten days old seedlings with fully expanded first leaves were inoculated with *P. hordei* pathotype 5457 P+. A suspension was prepared by adding 10 mg urediniospores to 10 ml of light mineral oil (Isopar L® Univar, Ingleburn, NSW, Australia) for 200 pots. The mixture was then homogenously atomized over the seedlings with a mist atomizer. Following inoculation, seedlings were incubated at temperature 20 ± 5 °C in a dark chamber for 24 h in which an ultrasonic humidifier created 100% humidity. After 24 h of incubation, seedlings were moved to microclimate rooms maintained at 22–24 °C with natural light and automatic drip irrigation (5 min cycle, 4 times a day).

Phenotyping and genetic analysis of mapping population

Disease assessments were made 10–12 days post-inoculation using a '0–4' infection type (IT) scale as outlined by Park and Karakousis (2002). ITs of "0", "; (fleck)", "1" and "2" were used to indicate the resistant response, while ITs of 3+ and above were used to indicate susceptible host response. Variation in the IT patterns was indicated by using the symbols + (more than average for the class), − (less than average for the class), C (chlorosis) and N (necrosis). F_3 lines were scored as homozygous susceptible (HS) or homozygous resistant (HR) when all the seedlings (approximately 25) of individual family produced susceptible or resistant ITs, respectively. Lines were scored as segregating (Seg) when both resistant and susceptible plants were detected within an individual F_3 family. For pooled analysis, resistant and susceptible plants from segregating F_3 families were counted and recorded. Chi-squared (χ^2) analysis was performed to determine the goodness of fit of observed ratios to expected ratios. *P* values were calculated from χ^2 values using the online calculator "Quickcalcs" (GraphPad Software Inc, USA).

Targeted genotyping-by-sequencing (tGBS) genotyping

Genomic DNA was extracted from a single leaf of individual plants selected from F_3 lines classified as HR and HS, including the parents, using the CTAB (cetyl trimethylammonium bromide) protocol as described by Fulton et al. (1995). All samples were quantified using a spectrophotometer (Nanodrop™, Biolab, Melbourne, VIC, Australia). DNA quality was assessed on a 0.8% agarose gel, and all samples were diluted to 100 ng/µl. Twenty-five HR and 25 HS lines and the parents were genotyped using the tGBS service provided by Agriculture Victoria Research, Bundoora, Australia. For tGBS analysis**,** samples were analysed using a custom bioinformatics pipeline. In brief, this pipeline processes sample reads from the tGBS assay to generate genotype calls for polymorphic loci. First, the sample read data are used to build an allele-specific reference (ASR) and then allelism among the ASR sequences is determined from their alignment to the Barley Morex IBSC reference genome assembly. Markers were classified as putatively linked when genotype calls were at least 70% fixed across samples within one or more of the phenotypic classes (HR and HS).

Development of molecular markers and conversion of identified SNPs to CAPS

Fifty-seven CAPS markers (Supplementary Table 1) were designed using Primer 3 Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) within the physical region (47 Mb, identified through tGBS) harbouring *RphHEB*, based on the Morex reference v1.0 and v2.0. DNA from the parents HEB-04-101 and Flagship was PCR amplified using CAPS oligonucleotides in a 50 µl reaction volume containing 20 µl genomic DNA (10 ng/µl), 10 µl MyFi Buffer (Bioline), 0.5 µl MyFi polymerase (Bioline), 10 µl (1.5 µM) each of forward and reverse primers and 9.5 µl double-distilled water. All reactions were conducted in a 96 well-plate in an automated thermocycler (Bio-Rad

T100) with an initial denaturation step of 95 °C at 10 min, followed by 30 cycles at 94 °C for 20 s, 60 °C for 30 s and 72 °C for 30 s with final extension at 72 °C for 10 min. Parental PCR products were then purified using Agencourt AMPure protocol-"000601v024" (Agencourt Bioscience Corporation) and sent to AGRF (Australian Genome Research Facility) for Sanger sequencing. To determine polymorphisms between the parents, the resulting parental sequences were analysed for the presence of SNPs using Sequencher 5.1 software (Gene Codes, Ann Arbor, MI, United States). Identified SNPs were converted into CAPS markers and further subjected to dCAPS (Derived Cleaved Amplified Polymorphic Sequences) Finder 2.0 (http://helix.wustl.edu/dcaps/) to identify restriction endonuclease sites (Neff et al. 2002). Restriction mapper version 3.0 (http://www.restrictionmapper.org/) was used to analyse the results from dCAPS finder. Of 57 designed CAPS markers, 16 were polymorphic (Table 1) and were used for fine mapping of *RphHEB*.

Marker genotyping, linkage analysis and map construction

Twenty to twenty-five plants from each of 125 F_3 families from HEB-04-101/Flagship mapping population were genotyped with 16 polymorphic markers (Supplementary Table 2) that were evenly distributed within the 47 Mb target region [based on Morex reference v1.0 and v2.0 (Mascher et al. 2017; Mascher 2019)] identified on the long-arm chromosome 5HL using tGBS data. PCR reactions were performed using 10 µl reaction volumes, and all other conditions were the same as described above. PCR products were digested for three hours using temperatures specific for the respective restriction enzymes used for each of the

Table 1 Details of 16 polymorphic CAPS markers, their regions based on the chromosome 5H Morex genome assemblies v1.0 2017 and v2.0 2019 and restriction enzymes used

Marker name	Locus Name	Position Morex (v1.0 2017)	Position Morex (v2.0 2019)	Forward primer sequence	Reverse primer sequence	Restriction enzyme
M1	HORVU5Hr1G111400	636,119,905	569,345,120	CCGATGCCGTACTTCTCCTG	GCAAAAGAAGCCTCGAGTTCG	BstEII
M2	HORVU5Hr1G112350	638,954,426	571,895,908	TGCAGTAGCATTTGGTTTGG	GGAACCAGAGGGAAGAAACA	BpiI
M3	HORVU5Hr1G112440	639,354,363	572,223,918	TGAGCTCATCGTCCCAGTG	GGCGGTAGAGAATCCTGATG	AciI
M4	HORVU5Hr1G112440	639,354,449	572,224,004	CATGGCTTCTACCCCAACGT	CGGCGGTAGAGAATCCTGAT	AciI
M5	HORVU5Hr1G112790	640,333,659	573,069,863	ATGATGTCACGGACGAGTCG	GCATCACCCTCCGTGTTGAT	MboII
M6	HORVU5Hr1G112840	640,366,473	573,102,877	CCCCCTTTCACTCATGTTTC	TGAAGTTGCAGCTTTCAGGT	HaeIII
M7	HORVU5Hr1G112950	640,562,732	573,253,857	ACACATCCACAGCCAATTCC	CGCACAAGTGAACCAGTGAA	MboI
M8	HORVU5Hr1G112970	640,595,738	573,300,562	GAGGAGGAGCCATTTGTGTC	TAGACGAACAGCTCCAGCAA	EciI
M9	HORVU5Hr1G113020	640,765,749	573,398,808	GAGCGAGGAGTGCGTGAG	AGCTGGCGTAGACCATCTTG	SacII
M10	HORVU5Hr1G113010	640,716,881	573,453,793	GCTCAAGTCCAAGAGGATGC	GATCACGTCCAGGCTGATTT	BstUI
M11	HORVU5Hr1G113000	640,714,952	573,456,469	GGACTGCCCATCTGCTTCTA	AGGGGCACTCGTACGACATA	AluI
M12	HORVU5Hr1G112980	640,699,963	573,471,458	AGGAATACCAGAGCGAAGCA	GCCGCAGGTCTTTGATTATG	MboI
M13	HORVU5Hr1G113240	641,284,985	573,689,190	GGTTCGCAGAATACGAAACG	ACGTAGTCGATCCGGAACAT	Tsp45I
M14	HORVU5Hr1G113260	641,551,861	573,919,245	AAAGCGCCACTTCTTGCATA	CTCGATGAATATCGGGATCG	AciI
M15	HORVU5Hr1G113740	642,608,654	574,814,525	GTCTGTGCCGATGTGATGG	CATGCCCAGCTCACAAGATA	AciI
M16	HORVU5Hr1G115340	646,305,420	577,812,402	AAGGTGTCCACGCCCAAT	AAGCCAGAGTGCACGACTTT	MboI

interrogated SNP variants according to manufacturer's instructions (New England Biolabs, Australia). Digested PCR products were loaded to a 2% agarose gel with 1 × TAE buffer and then subjected to electrophoresis for 60 min @ 120 V. After electrophoresis, gels loaded with products were visualized under UV light using Gel Doc IT imaging System (Model M-26, Bioimaging Systems, CA, USA). Genetic map was constructed using software Voorrips (2002) and physical map using the software Pretzel (https://plantinformatics.io/; Keeble-Gagnere et al. 2019).

Results

Phenotyping and genetic analysis of *RphHEB*/Flagship population

Parent HEB-04-101 produced a low to intermediate IT ('0;' to ';1 + CN') during rust tests at the seedling stage in response to five *P. hordei* pathotypes (200 P-, 220 P+ +13, 253 P−, 5653 P+ and 5457 P+). In contrast, the Flagship parent was susceptible (IT '3+') in response to all five pathotypes (Table 2; Fig. 1). Seedlings of the HEB-04-101/Flagship F_3 population ($n = 125$) segregated for a single gene when inoculated with *P. hordei* pathotype 5457 P+. Three distinct phenotypic classes (HR, Seg and HS) were observed among F_3 families, and Chi-squared analysis was best fit to a 1:2:1 (HR/Seg/HS) segregation ratio expected for monogenic inheritance ($\chi^2 = 0.98$, $P > 0.6$; Table 3). Further pooled analysis based on recording resistant (R) and susceptible (S) individuals within 68 segregating lines revealed segregation of 736 resistant (R) plants and 238 susceptible (S) plants conforming to the goodness of fit for a single gene (3R:1S) ratio (736R:238S, $\chi^2 = 0.16$, $P = 0.68$) and demonstrating the dominant nature of *RphHEB*.

Table 2 Infection types on HEB-04-101 and Flagship when tested with five pathotypes of *P. hordei*

Genotypes	*P. hordei* pathotypes				
	200 P−	220 P+ +13	253 P−	5653 P+	5457 P+
HEB-04–101	;	0;	;1+C	;1C	;1+CN
Flagship	3+	3+	3+	3+C	3+C

Infection types are based on "0" to "4" scale (Park and Karakousis, 2002). "0" = no visible symptoms, "; (fleck)" = small chlorotic or necrotic spots with under-developed uredinia, "1" = minute uredinia enclosed by necrotic tissue, "2" = small to medium sized uredinia enclosed by chlorotic and/or necrotic tissue, "3" = medium to large uredinia with or without chlorosis. The letters "C" and "N" indicate chlorosis or necrosis, respectively; "+" indicates higher infection types than normal. Infection types of "3+" or higher were considered to indicate host susceptibility

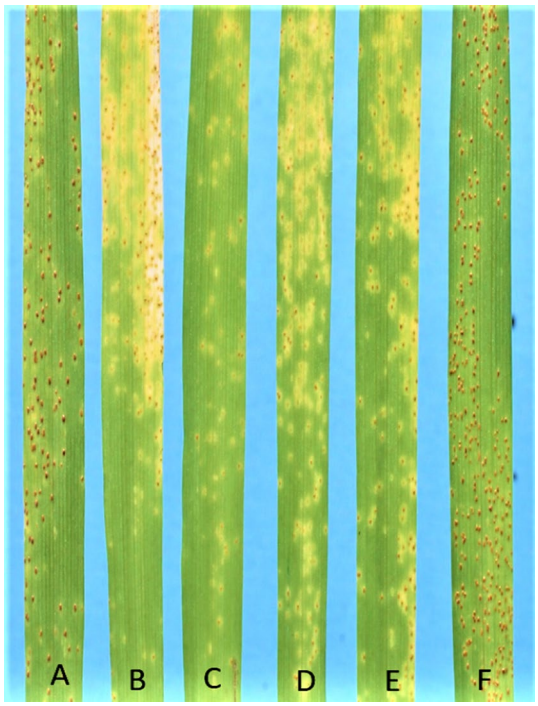

Fig. 1 Phenotypic response of F_3 families derived from HEB-04-101 × Flagship when infected with *P. hordei* pathotype 5457 P+ (PBI culture # 612). The susceptible parent Flagship (**a**) shows large uredinia compared to resistant parent HEB-04-101 (**b**) which shows chlorosis and necrosis with restricted uredinia. **c**, **d** and **e** represent lines from segregating population with various resistant infection types, while **f** represents a susceptible line from segregating population with large sporulating uredinia

Targeted genotyping by sequencing (tGBS)

Targeted genotyping-by-sequencing analysis was performed on 25 HR and 25 HS lines. A total of 119 putatively linked alleles were found on the long arm of chromosome 5H (Table 4). Of these, 23 had a predominance of the resistant parent genotype in the resistant progeny lines and a predominance of the susceptible parent genotype in the susceptible progeny lines, while additional 43 and 53 markers were prevalent for the resistant genotype only, or susceptible genotype only, respectively. Based on the Morex (v1.0) barley reference assembly, the distribution of putatively linked markers positioned the *RphHEB* locus within a 47 Mb physical interval (622–669 Mb) on the long arm of chromosome 5H (Fig. 2).

Fine mapping of *RphHEB*

In total, 57 CAPS derived from four rounds of marker design were developed within a physical interval of 47 Mb (based on tGBS data and Morex v1) on the long arm of chromosome 5H. Sixteen polymorphic markers were used for

Table 3 Distribution and Chi-squared analysis of F_3 families derived from HEB-04-101/Flagship when tested with *P. hordei* pathotype 5457 P+

Population	Number of F_3 families[#]			Tested ratio	χ^2	P-value
	HR	Seg	HS			
HEB-04-101/Flagship	29	68	28	1:2:1	0.98	0.61

[#]20–25 plants assessed per F_3 family; HR = homozygous resistant, Seg = segregating, HS = homozygous susceptible

χ^2 table value at $P = 0.05$ is 5.99 and $P = 0.01$ is 9.21 at 2 d.f

Table 4 Sum of linked alleles per chromosome identified through tGBS assay based on Morex reference v1.0

	1H	2H	3H	4H	5H	6H	7H	Unknown
HR* and HS*	0	0	0	0	23	0	1	0
HR	1	2	1	2	43	0	1	0
HS	0	0	1	1	53	4	1	0

**HR* homozygous resistant, **HS* homozygous susceptible (of 119 putatively linked alleles detected, 23 had a predominance of the resistant parent genotype in the resistant progeny lines and a predominance of the susceptible parent genotype in the susceptible progeny lines, while an additional 43 and 53 markers were prevalent for the resistant genotype only, or susceptible genotype only, respectively)

recombination-based fine mapping of the *RphHEB* locus. We genotyped single representative plants from each F_3 family from 125 individuals with these polymorphic markers (Fig. 3). Once the genotype of 125 families was determined, the progeny of the same samples were rust tested at the F_4 generation, and data were used to fine map *RphHEB*.

In the first round, 16 markers were developed, and four polymorphic markers were used to genotype the entire F_3 mapping population. Two flanking markers, M1 (636.11 Mb) and M16 (646.30 Mb), were identified that placed *RphHEB* at a genetic distance of 6.8 and 4.8 cM, respectively, corresponding to a physical interval of 10.20 Mb. In a second round, 21 further markers were developed between M1 and M16 to further narrow the *RphHEB* interval; eight polymorphic markers were used to genotype 125 F_3 families, which identified seven informative recombinants (Table 5) and allowed the identification of new flanking markers: M5 (640.33 Mb) and M10 (640.71 Mb) delimiting *RphHEB* to 380 kb. Development of further 14 markers in a third round and subsequent genotyping of the mapping population with polymorphic markers enabled *RphHEB* to be mapped between M7 (640.56 Mb) and M12 (640.69 Mb), restricting the physical window to 137 kb.

Based on a newly available iteration of the v1.0 2017 Morex genome assembly (referred to as v2.0), we were able to accurately predict the physical interval of the *RphHEB* locus. A 137 kb (640,562,732–640,699,963) interval between the flanking markers (M7 and M12) as determined based on Morex v1.0 corresponded to 217 kb (573,253,857–573,471,458) in v2.0. The quality of genome assemblies within this region on chromosome 5HL was determined for both Morex v1.0 and v2.0 using the genomic similarity search tool YASS (https://bioinfo.lifl.fr/yass/yass.php) and visualized using a dot plot analysis. The analysis revealed a putative 310 kb inversion from 640.59 to 640.90 Mb (Fig. 4). When the region between M7 and M12 was investigated for high confidence (HC) genes, only two HC genes were detected in Morex v1.0, while 10 HC genes were found in v2.0. Therefore, Morex v2.0 was used as a road map to design more markers between M7 and M12. Finally, six markers (between M7 and M12) within the 217 kb region were developed and used to genotype three critical recombinants (1749, 1819 and 1859) (Table 5). This genotypic analysis led to the finalization of two closely linked markers M8 (573.30 Mb) and M9 (573.39 Mb) that positioned *RphHEB* to a genetic distance of 0.4 and 0.8 cM in the F_3 and narrowed the physical interval to 98.6 kb (Fig. 5).

In order to further increase the map resolution at the *RphHEB* locus, M8 and M9 were used as flanking markers to genotype a further 970 F_2 plants derived from a cross between Flagship and HEB-04-101 and three recombinants were identified. Progeny testing and phenotyping of these three recombinants with pt. 5457 P+ allowed mapping of *RphHEB* at a genetic distance of 0.05 cM between M8 and M9 in the F_2. The physical window remained the same (98.6 kb) because 14 more markers (10 designed within gene r2.5HG0437510 and four within r2.5HG0437530) between M8 and M9 amplified DNA of Morex but did not amplify DNA from HEB-04-101.

Gene annotation

We compared and analysed CDS HC genes in the 98.6 kb region between M8 and M9 in Morex v2.0 2019 (https://webblast.ipk-gatersleben.de/barley_ibsc/). Gene annotation revealed the presence of five HC genes based on the v2.0 2019 Morex reference genome (Table 6). Among these five predicted genes,

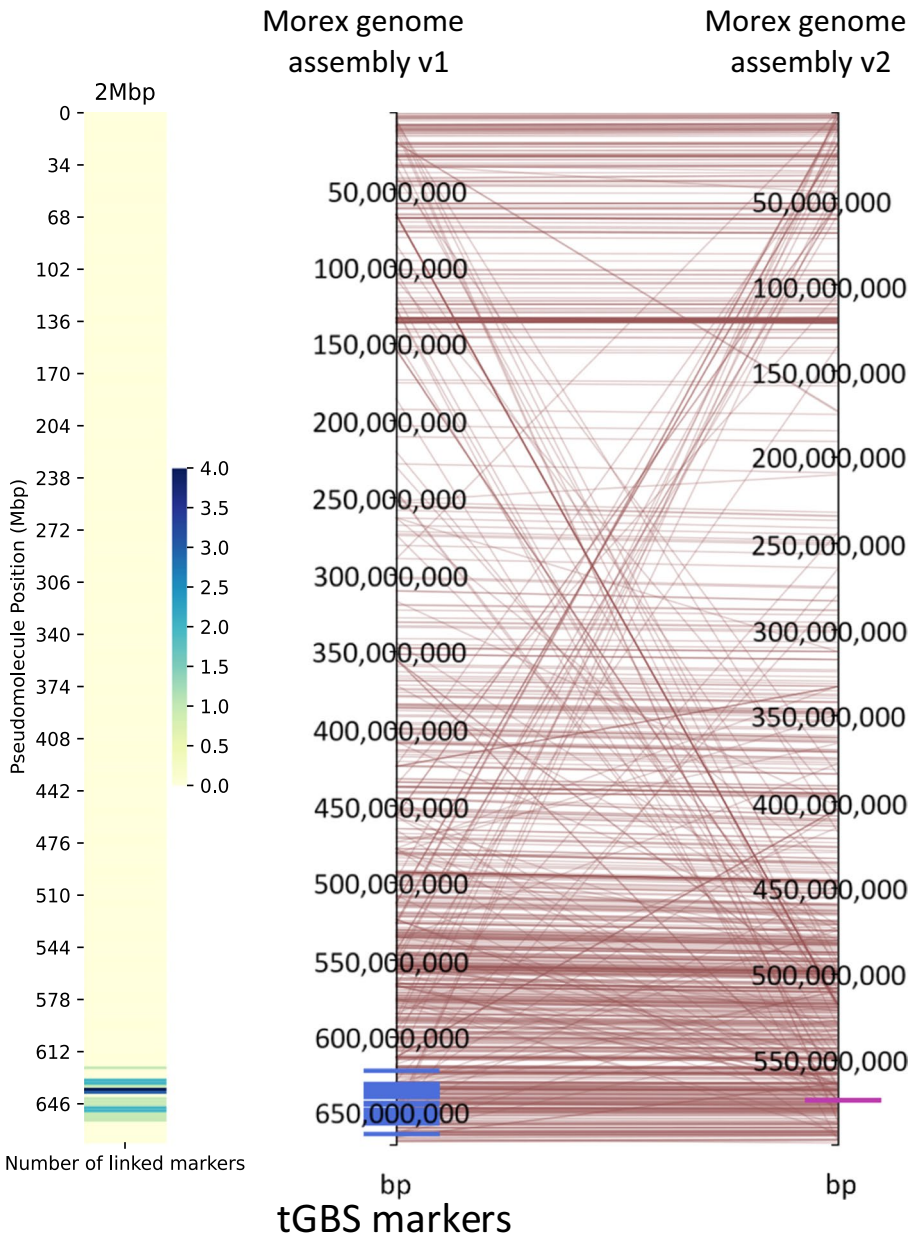

Fig. 2 Putatively linked markers on chromosome 5H identified through targeted genotyping by sequencing in v1 and v2 Morex genome assembly. Blue highlighted region indicated linked region from 622 to 669 Mbp in v1. Diagram generated using the software Pretzel (https://plantinformatics.io/; Keeble-Gagnere et al. 2019)

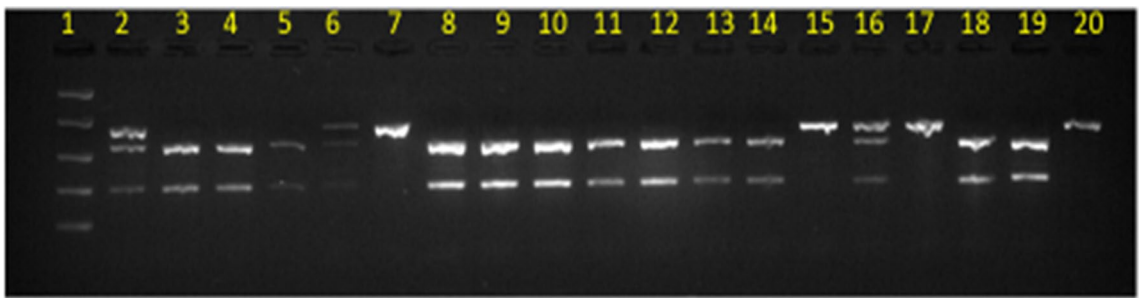

Fig. 3 Gel image showing digested PCR products in F_3 mapping population using enzyme AciI. From L–R (1 = easy ladder Bioline, 2–18 = lines from F_3 population, 19 = Flagship and 20 = HEB-04-101. After restriction digestion, susceptible parent Flagship (# 19) produced 2 bands of ~ 250 bp and 550 bp, resistant parent HEB-04-101 (# 20) produced a product size of ~ 900 bp. Well # 2, 6 and 16 show heterozygous lines carrying alleles from both parents

Table 5 Genotyping of recombinants with markers segregating at the *RphHEB* locus in an F_3 mapping population derived from a cross between HEB-04-101 and Flagship

ID	M5	M6	M7	M8	M9	M10	M11	M12	*RphHEB*
1749	H	H	H	H→	S	S	S	S	S
1751	H	H	R	R	R	R	R	R	R
1754	H	H	S	S	S	S	S	S	S
1819	S	S	S	S	R←	R	R	R	S
1852	S	S	R	R	R	R	R	R	R
1854	S	R	R	R	R	R	R	R	R
1859	R	R	R	R	H←	H	H	H	R

The allelic abbreviations R, S and H represent resistant, susceptible and heterozygous genotypes/phenotypes, respectively. Arrows indicate recombination break points

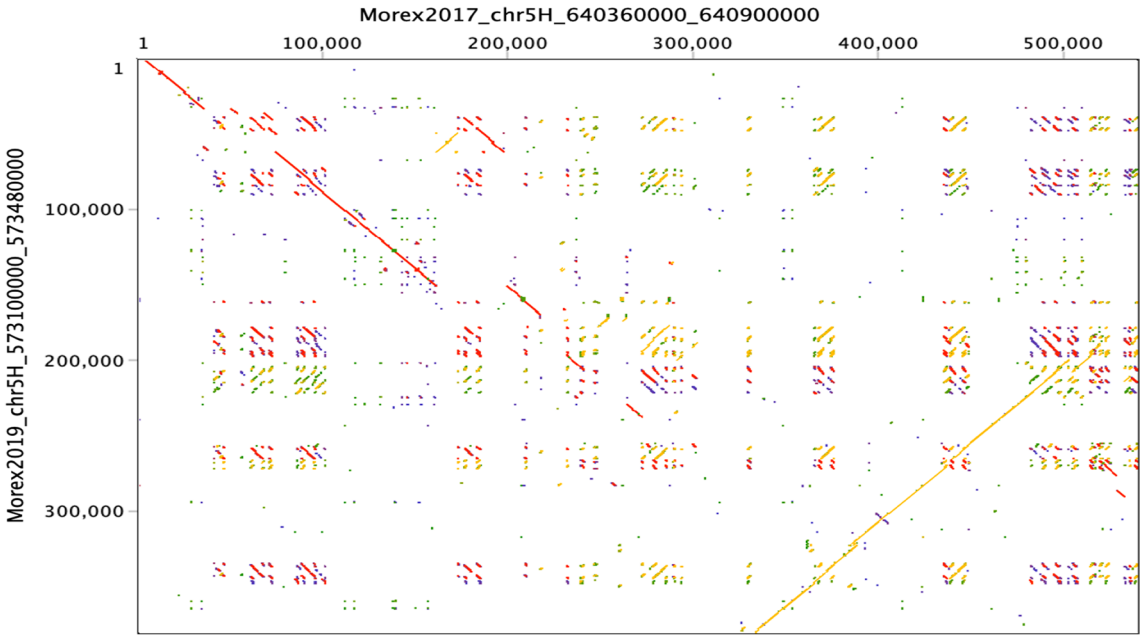

Fig. 4 Dot plot analysis representing a possible inversion through sequence alignment and comparison of Morex 2017 (640,360,000–640,900,000) versus Morex 2019 (573,100,000–573,480,000) using genomic similarity search tool YASS

two (r2.5HG0437510 and r2.5HG0437530) are predicted to encode NLR (nucleotide binding-site and leucine-rich repeat) immune receptors and were annotated as resistance-like proteins (https://doi.ipk-gatersleben.de/DOI/83e8e186-dc4b-47f7-a820-28ad37cb176b/d1067eba-1d08-42e2-85ec-66bfd5112cd8/2). Fourteen more CAPS markers were designed within the sequence of these two NLR gene candidates. PCR amplification was attempted using these 14 markers on HEB-04-101 DNA, using Morex as a control. All primer pairs successfully amplified products of expected size using Morex genomic DNA; however, none of the primers amplified products using the resistant parent (HEB-04-101). Similarly, an attempt was made to amplify PCR products from several other wild barley leaf rust resistant lines, HEB-05-053, HEB-03-055 and HEB-04-106 (Davinder Singh, unpublished). PCR amplification was unsuccessful on all HEB lines, suggesting possible sequence variation or the absence of these resistance genes in wild barley relative to the Morex reference. To explain the apparent sequence variation between the HEB lines and Morex at the *RphHEB* locus, the entire gene sequence (retrieved from IPK) from the two NLR genes in the target region was used as a query and compared to the homologous region of the recently sequenced wild barley reference genome-WB1 (Liu et al. 2020) using BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?BLAST_SPEC=blast2seqandLINK_LOC=align2seqandPAGE_TYPE=BlastSearch). Deletion of 912 bp was detected in one of the resistance genes (r2.5HG0437510) in Morex relative to WB1, suggesting the possibility of an insertion/deletion in *RphHEB* that may have led to amplification failure of markers. It is also possible that the variation detected between WB1 and Morex to the NLR genes is completely different to variation at the *RphHEB* locus.

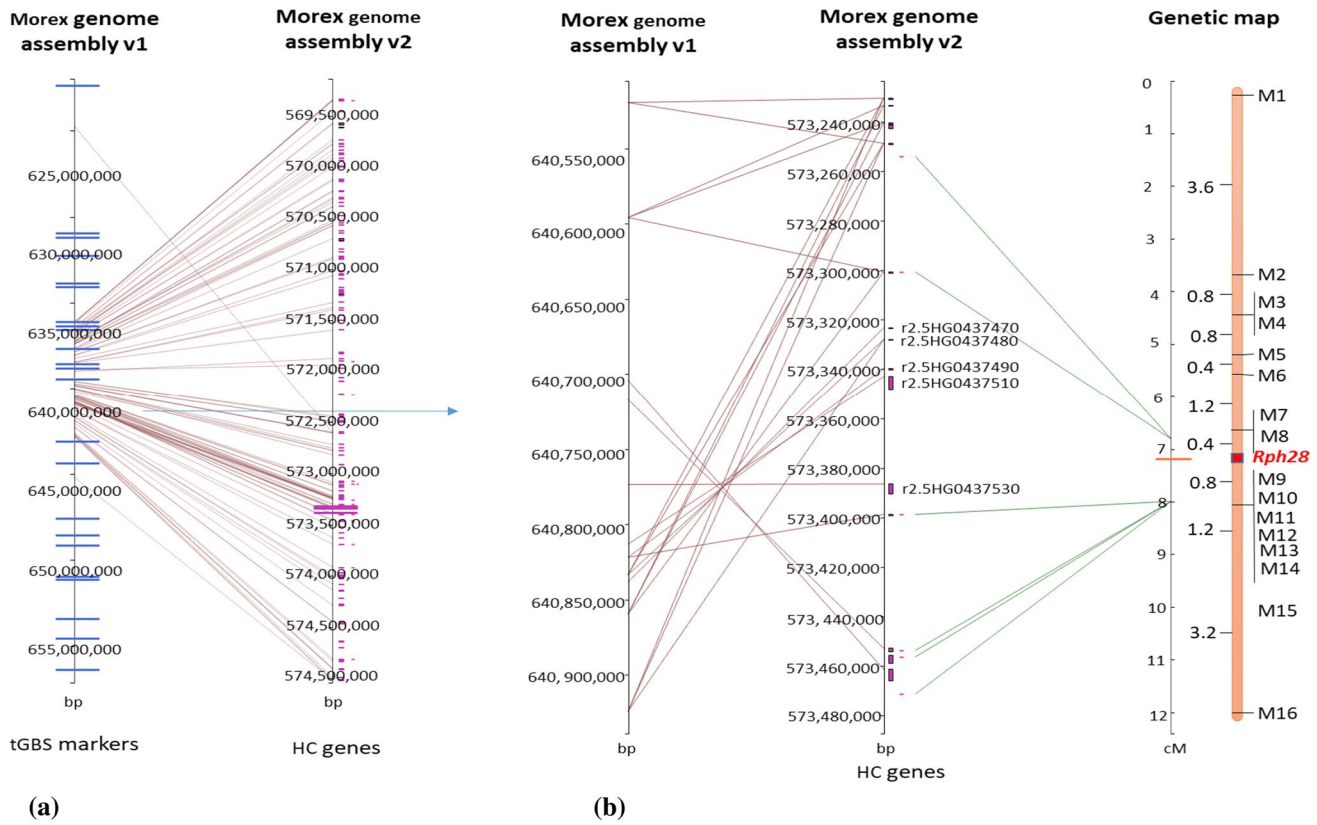

Fig. 5 Physical and genetic map for *Rph28* based on Morex genome assembly v1 and v2. **a** Barley chromosome 5HL showing physical window of 47 Mb (622–669) in Morex genome assembly v1 based on tGBS markers. Figure illustrates that a region 634–642 Mb of v1 corresponds to 569–574 Mb in Morex assembly v2. **b** Detail representation of 640 Mb (v1) and 573 Mb (v2) linked regions and *Rph28* fine mapping based on 16 polymorphic markers. Figure shows *Rph28* is fine-mapped in a physical region of 98.6 kb between M8 (0.4 cM) and M9 (0.8 cM) at 573.30 and 573.39 Mb, respectively. Five HC genes including two disease resistant genes highlighted in purple are shown between flanking markers. Diagram generated using the software Pretzel (Keeble-Gagnere et al. 2019) and Mapchart 2.32 (Voorrips 2002)

Table 6 Five high confidence (HC) genes between flanking markers M8 and M9 and their functions based on v2.0 2019 Morex genome assembly

Gene ID	Chr	Physical position*	Confidence class	Annotation
HORVU.MOREX.r2.5HG0437470	5HL	573,323,162–573,323,632	HC	Zinc finger protein
HORVU.MOREX.r2.5HG0437480	5HL	573,327,967–573,328,389	HC	Zinc finger protein
HORVU.MOREX.r2.5HG0437490	5HL	573,339,696–573,340,403	HC	Actin depolymerizing factor
HORVU.MOREX. r2.5HG0437510	5HL	573,343,017–573,348,147	HC	NLR disease resistance protein
HORVU.MOREX. r2.5HG0437530	5HL	573,386,447–573,390,561	HC	HC_HC1lNLR resistance-like protein

*Physical position of five HC genes in base pairs (bp) based on Morex reference v2.0

Discussion

Several previous studies (Russell et al. 2004; Jakob et al. 2014; Wang et al. 2015) have demonstrated that *Hvs* has a greater genetic diversity than cultivated barley *H. vulgare* because of its long co-evolution with various pathogens in nature. At the same time, genetic diversity of cultivated barley has been reduced over time as a consequence of domestication, selective breeding, or both (Badr and El-Shazly 2012). This highlights the need to explore untapped diversity in wild barley progenitors to broaden the genetic diversity in cultivated barley. This is especially so in the case of resistance to biotic stress, given that several studies have established that wild barley is a rich source of

resistance genes to rust pathogens (Moseman et al. 1990; Fetch et al. 2003; Steffenson et al. 2007).

Maurer et al. (2015) developed a NAM population-'Halle Exotic Barley 25' (HEB-25) from initial crosses between the spring barley elite cultivar Barke (*H. vulgare*) and 25 highly divergent exotic barley accessions (24 wild barley accessions of *Hvs* and one Tibetan *H. vulgare* ssp. *agriocrithon* accession). The HEB-25 population was introduced from Germany to Australia to assess its value in barley breeding. Preliminary rust screening of 1420 HEB lines identified over 100 that were highly resistant in both the greenhouse and the field. The present study reports on the characterization and fine mapping of a new seedling *Rph* gene (tentatively designated as *RphHEB*) in one of these lines, HEB-04-101, which was shown to be effective to a wide array of Australian *P. hordei* pathotypes. Using a selective genotyping approach, *RphHEB* was localized to the telomeric region of the long arm of chromosome 5H within an interval of 47 Mb.

Of all the *Rph* genes identified to date, *Rph2* (Borovkova et al. 1997; Franckowiak et al. 1997) and *Rph20* (an adult plant resistance gene reported by Hickey et al. 2011) are located on chromosome 5HS, while the alleles *Rph9*, *Rph9.am*, *Rph12* (Borovkova et al. 1998; Dracatos et al. 2014) and *Rph25* (Kavanagh et al. 2017) are located on chromosome 5HL. The pathotype used in this study (5457 P+) is virulent on all five alleles located on chromosome 5H that confer seedling resistance to *P. hordei* (viz. *Rph2, Rph9/Rph9.am/Rph12* and *Rph25*), demonstrating that the leaf rust resistance in HEB-04-101 is distinct from them. Previous genome wide association studies by Vatter et al. (2018) identified a QTL for leaf rust resistance (QPh.5H-1) in the same HEB-25 population on chromosome 5HL; a single, highly significant SNP marker- i_SCRI_RS_212784 positioned QPh.5H-1 at a physical location of 534,723,802 bp based on the Morex v1.0 assembly. The physical position of QPh.5H-1 does not overlap with the 47 Mb physical region for the *RphHEB* locus identified in the present study, indicating that Qph.5H-1 and *RphHEB* are distinct. While Jin et al. (1996) reported a very distant linkage ($30.4 \pm 4.5\%$) between *Rph13* in PI531849 and *Rph9* (on chromosome 5H) in Hor2596, two very recent studies confirmed that *Rph13* actually maps to the long arm of chromosome 3H and not 5H (Jost et al. 2020; Martin et al. 2020).

Morex references v1.0 and v2.0 were used to design markers in a 47 Mb region identified through a next-generation sequencing approach. The results of this study revealed the usefulness of the Morex reference for molecular marker development and fine mapping of resistance genes. Recombination-based mapping approach was used to localize *RphHEB* to the telomeric region of chromosome 5HL. A high number of recombination events was observed in the region carrying *RphHEB*, which facilitated narrowing the interval to 98.6 kb in a population size of 125 individuals. Although F_2 population was screened with the flanking markers M8 and M9 and three recombinants were detected, the target interval could not be reduced further as the markers developed between M8 and M9 did not amplify using DNA from the *RphHEB* parent. Despite the availability of the *Hvs* WB1 sequence (Liu et al. 2020), a lack of sequence information for the resistant parent HEB-04-101 precluded narrowing the region further.

In the present study, the final interval of 98.6 kb carried five high confidence candidate genes of which r2.5HG0437470 and r2.5HG0437480 were annotated as zinc finger proteins and a third gene r2.5HG0437490 was predicted to encode an actin depolymerizing factor (ADF). The remaining two genes-r2.5HG0437510 and r2.5HG0437530 annotated in the interval belong to the NLR gene family, which is the largest class of resistance genes characterized in plants so far (Grant et al. 1998; Jones et al. 2016). Most rust resistance genes isolated from cereals to date belong to the NLR family, including genes *Rph1* (Dracatos et al. 2019) and *Rph15* (Chen et al. 2020) in barley, and *Lr21* (Huang et al. 2003) and *Sr33* (Periyannan et al. 2013) in wheat. The two NLR genes identified in our target interval are the most likely candidates to underlie the leaf rust resistance in HEB-04–101. A third gene encoding for ADF is also a possible candidate for *RphHEB*. ADFs are encoded by genes that play a crucial role in defence related mechanisms in plants (Huang et al. 2020), for example the stem rust resistance gene *Rpg4* (resistance to *Puccinia graminis*) in barley encodes an ADF protein (Kleinhofs et al. 2009).

Unsuccessful PCR amplification of HEB-04-101 with 14 markers (developed between M8 and M9) within two NLR genes suggests a possible variation between sequence of these resistance genes in Morex and wild barley as the same markers within these high confidence genes were successfully amplified on Morex DNA. Several previous studies suggest that repeated sequences within NLR genes can give rise to structural variations that lead to the evolution of new resistance genes (Hulbert 1997; Ellis and Jones 1998; Tamborski and Krasileva 2020). On comparing sequences from the two NLR genes in the target region with the recently sequenced wild barley reference genome-WB1, a sequence variation (912 bp deletion) was detected in gene r2.5HG0437510.1, suggesting the possibility of sequence variation at *RphHEB* locus that may have contributed to the amplification failure of the NLR gene markers on the resistant parent. This led to the conclusion that the *RphHEB* locus may not be present in Morex. It is also possible that genes other than those that were annotated in the interval of the resistant parent -HEB-04-101, are present. A lack of sequence information for HEB-04-101 and variation between the wild barley and

Morex reference genomes impeded further marker development in the 98.6 kb interval. The future availability of sequence information for HEB-04-101 resistant accession will play an important role in identifying the gene conferring *RphHEB* mediated resistance. Various rapid cloning strategies such as MutChromSeq (Sandnchez-Martin et al. 2016) which has been recently used to clone *Rph1* (Dracatos et al. 2019) and MutRenSeq (Steuernagel et al. 2017) can be employed to fully understand the nature and structure of the *RphHEB* locus.

Various *Rph* genes have been identified and characterized from wild relatives of barley, but very few have been effectively utilized in breeding programmes due to the problem of linkage drag (Summers and Brown 2013). Introgressing *RphHEB* in barley breeding programmes will be more efficient as the donor line HEB-04-101 has been crossed with the barley cultivar Barke during the development of the HEB-25 population (Vatter et al. 2018). Furthermore, to develop the mapping population used to characterize the *RphHEB* locus, the Australian barley cultivar Flagship was intentionally used as a leaf rust susceptible parent. Flagship is an early- to mid-season Australian malting variety carrying the APR gene *Rph20*, which is also resistant to several other barley diseases such as cereal cyst nematode, spot- and net-form of blotch, and scald. Several lines have been isolated from the mapping population carrying both the *RphHEB* and *Rph20* resistance genes in the Flagship background. It is likely that several of these lines will have reduced linkage drag from the wild progenitor donor of the *RphHEB* resistance and will therefore have tremendous potential for increased diversification of Australian and global barley breeding programmes. Although *RphHEB* is widely effective to Australian pathotypes of *P. hordei*, its effectiveness to populations of *P. hordei* outside of Australia is unknown. It will be therefore very useful in future to test *RphHEB* stocks generated in this study with an array of global *P. hordei* pathotypes to predict value of this gene in resistance breeding at a continental scale.

In conclusion, this study characterized a new gene (*RphHEB*) conferring seedling resistance to leaf rust, mapped it to the long arm of chromosome 5H using targeted genotype by sequencing approach and demonstrated its distinctiveness from all other *Rph* genes mapped to the same chromosome. Using the recently available Morex reference genome and a recombination-based mapping approach, *RphHEB* locus was fine-mapped to a 98.6 kb physical interval and in the process closely linked CAPS marker M9 was identified that has potential for marker assisted selection (MAS) of the gene. Although M9 is the marker closely linked to *RphHEB*, yet it is not a co-segregating marker and hence may provide false positive or negative results. Marker M9 was validated on 80 Australian barley cultivars, and based on M9-marker genotyping, *RphHEB* was present in 12 cultivars out of 80 (data not presented). As these cultivars are susceptible and unlikely carry *RphHEB*, it is concluded that M9 is likely producing some false positives and the marker has about 85 percent accuracy for MAS. We are currently developing a chemically induced mutant population to identify knockout mutants to functionally verify the candidates identified in the present study and facilitate the cloning of the *RphHEB*. As *RphHEB* is a new and distinct leaf rust resistance locus, the gene symbol *Rph28* is recommended for *RphHEB* in accordance with the rules and cataloguing system of barley gene nomenclature.

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Author contribution MM led the studies and DS designed the studies. DS, PD and RFP supervised the studies. TM, AP, AM and KP provided critical material/germplasm. MM, PD, TK, KF and MP performed recombination and/or tGBS analysis. MM and DS performed phenotyping and data analysis. MM and DS wrote the MS, and all authors contributed to the MS.

Declarations

Conflict of interest The authors declare no conflict of interest.

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