Inheritance and Characterization of *Rph27*: A Third Race-Specific Resistance Gene in the Barley Cultivar Quinn

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ABSTRACT

The barley cultivar Quinn was previously reported to carry two genes for resistance to *Puccinia hordei*, viz. *Rph2* and *Rph5*. In this study, we characterized and mapped a third resistance gene (*RphCRQ3*) in cultivar Quinn. Multipathotype testing in the greenhouse on a panel of barley genotypes previously postulated to carry *Rph2* revealed rare race specificity in four genotypes in response to *P. hordei* pathotype (pt.) 222 P+ (virulent on *Rph2* and *Rph5*). This suggested either the presence of a race-specific allele variant of *Rph2* or the presence of an independent uncharacterized leaf rust resistance locus. A test of allelism on 1,271 F₂ Peruvian (*Rph2*)/Quinn (*Rph2* + *Rph5*) derived seedlings with *P. hordei* pt. 220 P+ (avirulent on *Rph2* and virulent on *Rph5*) revealed no susceptible segregants. To determine whether the race-specific resistance

in Quinn was due to an allele of *Rph2* on chromosome 5H or a third uncharacterized resistance gene, we tested the Peruvian/Quinn F₃ population with 222 P+ and observed monogenic inheritance. Subsequent bulked segregant analysis indicated the presence of complete in-phase marker fixation near the telomere on the short arm of chromosome 4H, confirming the presence of a third resistance locus in Quinn in addition to *Rph2* and *Rph5*. In accordance with the rules and numbering system of barley gene nomenclature, *RphCRQ3* has been designated *Rph27*.

Keywords: barley, bulked segregant analysis, genetics and resistance, leaf rust, race specificity, *RphCRQ3*

Barley is a major cereal crop produced globally for food, feed, and malting purposes. It is the fourth most important cereal crop in terms of production globally (FAOSTAT 2017). Cultivated barley worldwide is affected by four rust diseases; stripe rust (caused by *Puccinia striiformis* f. sp. *hordei*), crown rust (*P. coronata* var. *hordei*), stem rust (*P. graminis* either f. sp. *secalis* or *tritici*), and leaf rust (*P. hordei*) (Park 2003). *P. hordei* is an important foliar pathogen of barley both in Australia and globally (Clifford 1985; Park 2003), causing yield losses of up to 62% in susceptible cultivars and damage to Australian barley crops was estimated in 2009 at \$21 million per annum (Cotterill et al. 1992; Murray and Brennan 2009). The impact of this disease and use of fungicides can be reduced by deploying genetic resistance (Park et al. 2015).

At present, 26 *Rph* (reaction to *P. hordei*) loci have been mapped and formally designated. A majority of these were found in landraces and commercially grown barley cultivars. All stage resistance (ASR) is by far the most commonly deployed resistance in barley. To date in barley, 23 (*Rph1* to *Rph19*, *Rph21*, *Rph22*, *Rph25*, and *Rph26*) of the 26 loci designated for leaf rust resistance are ASR genes. However, a majority of them have been rendered ineffective due to ongoing rapid evolution in prevailing *P. hordei* pathogen populations (Park et al. 2015). Recent studies reporting on the screening of international germplasm collections suggests the effective available sources of leaf rust resistance are limited, emphasizing the critical need to further characterize newly identified resistance sources (Derevnina et al. 2013; Golegaonkar et al. 2009; Sandhu et al. 2014; Singh et al. 2015).

To best utilize available sources of rust resistance, an understanding of the underlying genetic basis is required. A number of

previous studies have demonstrated allelic relationships between *Rph* genes: *Rph5/Rph6* (Zhong et al. 2003), *Rph9/Rph12* (Borovkova et al. 1998), *Rph12/RphCantala* (Dracatos et al. 2014), and *Rph15/Rph16* (Weerasena et al. 2004). The resistance locus *Rph2* was first designated as *Pa* in cultivar Weider (Henderson 1945). The locus was designated as *Pa2* under a unified nomenclature for *P. hordei* resistance (Roane and Starling, 1967), and then later as *Rph2* (Moseman 1972). A series of crosses between Quinn, Bolivia, and Reka 1 previously generated by Roane (1962) and assessed at the F₂ and F₃ stages also determined the presence of *Rph2* in all three differentials. Further tests of allelism were performed by Miah (2004) by crossing Peruvian with genotypes postulated to carry *Rph2* including Namoi, Ulandra, Yerong, Mimosa, Skiff, Clarine, Torrent, and Ricardo. The lack of segregation in F₃ families in response to the Australian *P. hordei* pathotype (pt.) 4610 P+ (900380 = 491) confirmed the presence of *Rph2* in all. Virulence for the barley differential genotypes Peruvian (*Rph2*), Bolivia (*Rph2*, *Rph6*) and Reka I (*Rph2*, *Rph19*) and Q21861 (*RphQ*) is common in the Australian *P. hordei* population, and all show the same specificity to pathotypes virulent for *Rph6*, *Rph19*, and/or *RphQ* (Park et al. 2015) (R. Park, unpublished data). Assessments of numerous *Rph2* donors in multipathotype tests in other studies have found varying resistance levels (Levine and Cherewick 1952; Reinhold and Sharp 1982; Steffenson and Jin 1997; Tan 1977). On the basis of these studies, Franckowiak et al. (1997) concluded that *Rph2* is a complex locus comprising multiple alleles.

The *P. hordei* differential cultivar Quinn was first identified as the original donor for the *Rph5* resistance (Levine and Cherewick 1952). Historical data collected during routine race typing at the Plant Breeding Institute (PBI), Cobbitty in Australia determined that Quinn carried race-specific resistance in response to rare *P. hordei* pathotypes carrying combined virulence on *Rph2* and *Rph5* (R. Park, unpublished data). The novel race-specific locus in Quinn will hereafter be referred to as *RphCRQ3*, as it is the third resistance gene identified in this donor background. In this study, we aimed to (i) determine the presence of race-specific resistance in panel of international *Rph2* donors, (ii) determine the mode of inheritance of *RphCRQ3* and the genetic relationship between

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RphCRQ3 and the *Rph2* allele in Peruvian (*Rph2.b*), and (iii) perform genetic mapping to determine the chromosomal location of *RphCRQ3*.

MATERIALS AND METHODS

Plant material. A panel of known and putative *Rph2* donor lines from the literature was assembled (Table 1). Additional lines potentially carrying *Rph2* from unpublished testing at the PBI were also included (R. Park, *unpublished data*). Seed for these genotypes was sourced either from the PBI germplasm collection, or from the Australian Grains Genebank (AGG) (Clarine, Kwan, and Mimosa). A Peruvian (*Rph2*) × Quinn (*Rph2* + *Rph5*) F₂ mapping population was developed and used for both inheritance and allelism studies and advanced to the F₃ generation. The Sloop (*Rph19*) × Halcyon (*Rph2*+) doubled haploid (DH) population previously reported on by Park et al. (2003) was used to refine the map location of *Rph2*. The Australian barley leaf rust differential set (Park 2003) was used to confirm the virulence phenotypes of the eight *P. hordei* pathotypes used in this study.

Pathogen isolates. Eight Australian *P. hordei* pathotypes were used for phenotyping (Table 2). Three of these pathotypes (*viz.* pts. 220 P+, 222 P+, and 5457 P+) were also used for genetic studies. All isolates were originally collected and characterized in national cereal rust surveys (Park, 2003) and are maintained in liquid nitrogen at PBI, the University of Sydney. Australian *P. hordei* pathotypes are designated according to the octal notation proposed by Gilmour (1973). A P+ or P– suffix indicates either virulence or avirulence with respect to the barley leaf rust resistance gene *Rph19* in cultivar Prior (not included in the octal system). Only one P– pathotype was used during phenotyping, therefore we could not postulate the presence of *Rph19* in the present study accurately.

Genetic studies. Populations were developed by crossing cultivar Peruvian with cultivar Quinn. The F₁ seeds were advanced to F₂, transplanted, raised in a poly-house, harvested as single plant F₃ families, and then stored in a dehumidified and temperature controlled seed room until further use. F₃ families (16 to 20 individuals per family) from the Peruvian/Quinn population were assessed with three different *P. hordei* pathotypes including: pt. 5457 P+ (*Rph2* virulent), pt. 220 P+ (*Rph5* virulent), and pt. 222 P+ (*Rph2* and *Rph5* virulent). Each family was scored as either nonsegregating resistant (NSR), segregating (Seg), or nonsegregating susceptible (NSS). Fit for theoretical segregation ratios to

observed segregation for allelism and inheritance testing was assessed using Pearson's χ^2 test at significance level $\alpha = 0.05$. For the test of allelism, 1,271 F₂ seedlings from the Peruvian/Quinn population were assessed phenotypically in the greenhouse with an *Rph2*-avirulent *P. hordei* pt. (220 P+). The allelism test was repeated using ~256 F₃ progeny again from the Peruvian/Quinn cross.

Phenotyping rust response in the greenhouse. Seedlings were raised in a mixture of bark fines and coarse sand, fertilized at sowing using Aquasol (Hortico, Revesby, NSW, Australia) (100 g/10 liters of H₂O). Seedlings were maintained in a rust-free growth room at 17 to 20°C until inoculated. Inoculation was conducted in an enclosed inoculation chamber at ambient temperature. Urediniospores (10 mg/10 ml) were suspended in Isopar L, a light mineral oil (Univar, Ingleburn, NSW, Australia), and sprayed above

TABLE 2. Virulence profiles of Australian *Puccinia hordei* isolates used during this study

Pathotype	Culture number	Virulence/avirulence
200 P–	518	<i>Rph8/Rph1, Rph2, Rph3, Rph4, Rph5, Rph6, Rph7, Rph9, Rph11, Rph12, Rph13, Rph14, Rph15, Rph19</i>
220 P+	485	<i>Rph5, Rph8, Rph19/Rph1, Rph2, Rph3, Rph6, Rph7, Rph9, Rph10, Rph11, Rph12, Rph13, Rph14, Rph15</i>
222 P+	545	<i>Rph2, Rph5, Rph8, Rph19/Rph1, Rph3, Rph7, Rph9, Rph10, Rph11, Rph12, Rph13, Rph14, Rph15</i>
4610 P+	491	<i>Rph4, Rph8, Rph9, Rph12, Rph19/Rph1, Rph2, Rph3, Rph5, Rph6, Rph7, Rph10, Rph11, Rph13, Rph14, Rph15</i>
5457 P+	612	<i>Rph1, Rph2, Rph3, Rph4, Rph6, Rph9, Rph10, Rph12, Rph19/Rph7, Rph11, Rph13, Rph14, Rph15</i>
5611 P+	660	<i>Rph1, Rph4, Rph8, Rph9, Rph10, Rph12, Rph19/Rph2, Rph3, Rph5, Rph6, Rph7, Rph11, Rph13, Rph14, Rph15</i>
5631 P+	640	<i>Rph1, Rph4, Rph5, Rph8, Rph9, Rph10, Rph12, Rph19/Rph2, Rph3, Rph6, Rph7, Rph11, Rph13, Rph14, Rph15</i>
5672 P+	627	<i>Rph2, Rph4, Rph5, Rph6, Rph8, Rph9, Rph10, Rph12, Rph19/Rph1, Rph3, Rph7, Rph11, Rph13, Rph14, Rph15</i>

TABLE 1. Summary of previously postulated barley leaf rust all stage resistance (ASR) gene complement and pedigree for germplasm panel

Cultivar	Previously postulated ASR gene(s)	Identifier ^a	Pedigree	Reference
Ariana	<i>Rph2</i>	CIho 2524	Landrace	(Moseman and Greeley 1965)
Batna	<i>Rph2</i>	CIho 3391	Harlan/4/Trebi(ms)/(MS)California-Mariout/Mars/3/Harlan/5/Arivat	(Reinhold and Sharp 1982)
Bolivia	<i>Rph2</i> + <i>Rph6</i>	PI 36360	Landrace	(Henderson 1945)
Clarine	<i>Rph2</i> + ?	PI 584869	Igri/Mogador	(Miah 2004)
Grout	<i>Rph2</i> + ?	NRB 01001	Kaputar/Cameo	R. Park, <i>unpublished data</i>
Halcyon	<i>Rph2</i>	PI 531062	Warboys/Maris-Otter	(Park et al. 2003)
Kaputar	<i>Rph2</i>	PI 591928	5604/1025/3/Emir/Shabet/CM67/4*F3 Bulk HIP	(Park et al. 2003)
Kwan	<i>Rph2</i>	PI 39367	Landrace	(Zloten 1952)
Mimosa	<i>Rph2</i> + ?	EBDB 25876	Alpha/Galion/Sonja/Maris-Trojan	(Miah 2004)
Namoi	<i>Rph2</i> + ?	PI 591929	Sultan/Nackta/RM1508/Godiva	(Miah 2004)
Peruvian	<i>Rph2</i>	CIho 935	NA (Landrace)	(Starling 1955)
PI 531841	<i>Rph2</i>	PI 531841	HJ 198*3/HS 2310	(Jin et al. 1995)
Quasar	<i>Rph2</i> + ?	SYN 6921-23	Chalice/NFC breeding line	R. Park, <i>unpublished data</i>
Quinn	<i>Rph2</i> + <i>Rph5</i>	PI 39401	NA (Landrace)	(Roane and Starling 1967)
Reka 1	<i>Rph2</i> + <i>Rph19</i>	CIho 5051	–	(Roane and Starling 1967)
Ricardo	<i>Rph2</i> + <i>Rph21</i>	PI 45492	NA (Landrace)	(Henderson 1945)
SY Rattler	<i>Rph2</i> + ?	SYN 937-5	498-50/498-46/Colston	R. Park/D. Singh, <i>unpublished data</i>
Torrent	<i>Rph2</i> + ?	PI 506314	Fenella/Tri 685	(Miah 2004)
Weider	<i>Rph2</i>	PI 39398	–	(Henderson 1945)
Yerong	<i>Rph2</i> + <i>Rph23</i>	PI 573730	M22/Malebo	(Miah 2004)

^a Genotype identifier prefix: CIho, Cereal Investigation number *Hordeum*; EBDB, European Barley Database; NRB, Australian Plant Breeder's Rights Breeder code; PI, Plant ID (USDA); and SYN, Syngenta Seed Breeder's code.

seedlings using an atomizer with a hydrocarbon propellant pressure pack. After five minutes, seedlings were moved from the inoculation room to a dark incubation chamber maintained at 100% humidity by an ultrasonic humidifier for 12 to 18 h at room temperature. The plants were then moved to temperature controlled microclimate room (20 to 23°C) to allow for development of the disease and phenotypically assessed nine days after inoculation.

DNA extraction and genotyping using DArT-Seq analysis. Genomic DNA was extracted from the leaf tissues of seedlings of the Sloop/Halcyon DH population and resistant and susceptible bulks prepared from the Peruvian/Quinn F₃ population using a modified CTAB method following Doyle and Doyle (1987). Instead of a mortar and pestle, 2 × 5 mm sterile steel ball bearings were added to each tube then ground using a bead mill. The concentration of extracted DNA was determined using a spectrophotometer and adjusted to 100 ng/μl and sent to Diversity Arrays Technology Pty Ltd., Australia (DArTs; <https://www.diversityarrays.com/>) on a fee for service basis for whole genome profiling using DArT-Seq (>10 K markers). The physical position of each marker loci

was depicted as part of the fee-for-service by Diversity Arrays Technology based on the 2017 Morex sequence assembly (Mascher et al. 2017). Markers that could not be anchored to the Morex genome assembly were excluded from bulked segregant analysis (BSA) and genetic map construction.

Bulked segregant analysis. BSA was conducted on the Peruvian × Quinn F₃ population. A total of nine pooled DNA bulks of susceptible material and nine bulks of resistant material were genotyped in addition to the parental lines. Each bulk comprised three F₃ confirmed homozygous susceptible or resistant families. Using a 6 mm plier hole-punch, three leaf discs per plant from three plants in each family were collected in a 1.5-ml Eppendorf tube and dried over silica beads for 3 days. DNA from resistant and susceptible bulks plus the parents were prepared and genotyped using the DArT-Seq platform on a fee for service basis as described on the website (<https://www.diversityarrays.com/>). Following genotyping using the DArT-Seq platform, markers that were monomorphic across the parents were excluded. The finalized dataset was then assessed for regions of in-phase marker fixation.

TABLE 3. Multipathotype testing phenotypes for 20 barley lines postulated to carry *Rph2*

Pathotype	5672 P+	222 P+	200 P–	220 P+	5631 P+	5611 P+	4610 P+	5457 P+	Postulated genes ^a
Ariana	3-c	;1+c	;c	0;;1–	;12-c	;1-cn	;12-c	3+	<i>Rph2</i> + <i>RphCRQ3</i>
Batna	;12+c	;1-c	0;	0;;1–	;12-c	;1cn	;12-cn	3+	<i>Rph2</i> + <i>Rph6</i>
Bolivia	;12-c	;cn	0;c	0;	;12-c	;1+c	;0;;1-c	3+	<i>Rph2</i> + <i>Rph6</i>
Clarine	2+	2–	;1-c	;1–	;12-c	;1-c	;1-c	2-c	R
Grout	3-c	3+	0;cn	0;n	;12+c	;12-cn	;12-c	3c	<i>Rph2</i>
Halcyon	2-cn	;12-n	;cn	0;	;12-cn	;1-cn	;1-cn	2cn	<i>Rph2</i> + <i>RphCRQ3</i>
Kaputar	3	3	;cn	;1	;12-c	;12-c	;12-c	3c	<i>Rph2</i>
Kwan	;	;	;	;1–	;1+c	2c	;1	2, 3-c	R
Mimosa	3c	3	0;c	;1-c	;12-c	;1cn	;12-cn	3+	<i>Rph2</i>
Namoi	3+	3–	0;cn	0;;1–	;1+c	;1c	;12-c	3–	<i>Rph2</i> + <i>Rph19?</i>
Peruvian	3	3-c	0;cn	0;;1–	;12+cn	;1-cn	;1-cn	3+	<i>Rph2</i>
PI 531841	–	;12-c	0;	0;	;1+c	;12-c	;12-c	;12+c	R
Quasar	;1–	2–	0;	0;	;1–	0;;1	;1	;1–	R
Quinn	3+	;12-c	0;n	;12–	;12-cn	;n	;cn	;n	<i>Rph2</i> + <i>Rph5</i> + <i>RphCRQ3</i>
Reka 1	3+	3–	;cn	;12–	;12+c	;12-c	;12+c	3+	<i>Rph2</i> + <i>Rph19</i>
Ricardo	;12-c	;1-c	;cn	0;;1	;1+c	;1+c	;1+c	;1+c	<i>Rph2</i> + <i>Rph21^b</i>
SY Rattler	;1-cn	;cn	;1-cn	;1	;1-c	;1-c	;1-c	;1+c	R
Torrent	3+	2	0;	;12-c	;12c	;1	;12-c	3+	<i>Rph2</i> + <i>Rph4</i>
Weider	2+c	;1-c	;cn	;1-c	;12-c	;1+c	;1-c	;1	<i>Rph2</i> + <i>RphCRQ3</i>
Yerong	3+	3+	;1c	;12-c	;12+c	;12-c	;12c	3+	<i>Rph2</i>

^a Lines with resistance to all tested pathotypes are labeled as R. Lines with additional unknown genes are indicated with a question mark.

^b Ricardo is known to carry *Rph21* (Sandhu et al. 2012).

TABLE 4. Virulence phenotypes for *Rph2* donors with race specificity for *Puccinia hordei* pathotype 222 P+

Pathotype	5672 P+	222 P+	200 P–	220 P+	5631 P+	5611 P+	4610 P+	5457 P+	Postulated genes
Ariana	S	R	R	R	R	R	R	S	<i>Rph2</i> + <i>RphCRQ3</i>
Grout	S	S	R	R	R	R	R	S	<i>Rph2</i>
Halcyon	R	R	R	R	R	R	R	S	<i>Rph2</i> + <i>RphCRQ3</i>
Kaputar	S	S	R	R	R	R	R	S	<i>Rph2</i>
Mimosa	S	S	R	R	R	R	R	S	<i>Rph2</i>
Namoi	S	S	R	R	R	R	R	S	<i>Rph2</i> + <i>Rph19?</i>
Peruvian	S	S	R	R	R	R	R	S	<i>Rph2</i>
Quinn	S	R	R	R	R	R	R	S	<i>Rph2</i> + <i>Rph5</i> + <i>RphCRQ3</i>
Reka 1	S	S	R	R	R	R	R	S	<i>Rph2</i> + <i>Rph19</i>
Weider	R	R	R	R	R	R	R	R	<i>Rph2</i> + <i>RphCRQ3</i>

TABLE 5. Segregation ratios for inheritance and allelism testing in the Peruvian/Quinn population

Generation	Pathotype	NSR ^a	Segregating	NSS ^a	Expected segregation ratio	χ ² value ^b
F ₂	220 P+	1,279	NA	0	None	NA
F ₃	220 P+	154	0	0	None	NA
F ₃	222 P+	68	134	56	1:2:1 (1 gene)	1.50 (2 df)*
F ₃	5457 P+	60	142	74	1:2:1 (1 gene)	1.65 (2 df)*

^a NSR, nonsegregating resistant; NSS, nonsegregating susceptible.

^b χ² critical values at *P* = 0.05 are 3.84 (1 df) and 5.99 (2 df). An asterisk indicates that the χ² value < χ² critical value and thus fits the expected segregation ratio at *P* = 0.05.

Mapping *Rph2* in the Sloop/Halcyon DH population.

Genetic map construction was performed as described by Dracatos et al. (2019) using 80 DH lines from the Sloop/Halcyon mapping population. The population was phenotyped at the seedling stage using the *Rph2*-avirulent *P. hordei* pt. 4610 P+ and the infection type (IT) of each DH line was classified as either resistant or susceptible. The *Rph2* phenotypes were converted to binary scores of resistant = A and susceptible = B and integrated into the genetic map as a polymorphic marker.

RESULTS

Multipathotype rust testing of *Rph2* donors using Australian *P. hordei* pathotypes. We used eight diverse Australian *P. hordei* pathotypes to postulate the presence of leaf

Fig. 1. Seedling leaves of (left to right): Peruvian (*Rph2*, *rph5*, and *RphCRQ3*), Quinn (*Rph2*, *Rph5*, and *RphCRQ3*), Peruvian/Quinn F₃ (*RphCRQ3*), Peruvian/Quinn F₃ line (*RphCRQ3*), and Gus (susceptible control). Seedlings were inoculated with *Puccinia hordei* pathotype 222 P+.

rust resistance genes among a panel of 20 barley genotypes postulated to carry *Rph2* and in some instances additional resistance (Table 3). Postulation of resistance gene complement was conducted on the basis of the resistant or susceptible IT. Additional phenotypes from previous multipathotype testing (R. Park, unpublished data) were used to supplement postulation (Supplementary Table S2 and S3). Of the 20 donor lines tested, 15 were clearly postulated to carry *Rph2*, six of which (Ariana, Grout, Kaputar, Mimosa, Peruvian, and Yerong) were postulated to carry *Rph2* alone. The remaining nine lines were postulated to carry either *Rph4*, *Rph5*, or *Rph6* in combination with *Rph2*. Of the 20 lines tested, five (Clarine, Kwan, PI 531841, Quasar, and SY Rattler) were resistant to all pathotypes, indicating that they likely carried uncharacterized resistance or resistance that could not be postulated based on the pathotypes used.

Race specificity of resistance in response to *P. hordei* pts.

222 P+ and 220 P+. Race specificity for resistance to *P. hordei* pt. 222 P+ (virulent to *Rph2*, *Rph5*, *Rph8*, and *Rph19*) was observed across the *Rph2* donor lines tested (Table 4). Despite this pathotype being virulent on the *Rph2* allele(s) present in the differential genotypes Peruvian, Bolivia, and Reka I, four lines (Ariana, Halcyon, Quinn, and Weider) postulated to carry *Rph2* based on the multipathotype testing in this study exhibited race-specific resistance to pt. 222 P+. Of the four lines that were resistant to pt. 222 P+, Quinn was selected for further investigation of the race-specific response. Due to the combined virulence for *Rph2* and *Rph5* in pt. 222 P+, the observed resistance in Quinn was hypothesized to be an allele of *Rph2* with different specificity, or a third unidentified gene. To confirm the presence of *Rph2* in Quinn, an allelism test was conducted by rust testing a Peruvian/Quinn F₂ population with *P. hordei* pt. 220 P+ (virulent for *Rph5*, avirulent for *Rph2*) (Table 5). No susceptible plants were recovered from 1,271 seedlings, confirming either that Quinn carries *Rph2* and another uncharacterized race specific resistance gene or a different race-specific allele of *Rph2*. Additional rust testing at the F₃ stage again with pt. 220 P+ confirmed the F₂ result. The resistance of Quinn to pt. 222 P+ was therefore concluded to be due to the presence of a third race-specific resistance gene or an allele of *Rph2*.

Fig. 2. Peruvian/Quinn bulked segregant analysis was conducted using nine resistant and nine susceptible bulks collected after scoring with pathotype 222 P+. Each chromosome is indicated in a different color. Linkage is calculated as a percentage of in-phase linkage with the parental genotype across the resistant and susceptible bulks. Regions of fixation at 100% indicate association with the target trait, *RphCRQ3*.

Inheritance studies. The Peruvian/Quinn population was tested at the F₃ generation using pts. 5457 P+ (virulent for *Rph2* and avirulent for *Rph5*) and 222 P+ (virulent for both genes) to examine the inheritance of the resistance in these two genotypes more critically. Single gene segregation was observed using pt. 5457 P+, demonstrating that the population segregated for a single dominant gene, *Rph5*, present in the Quinn parent (Table 5). Segregation for a single gene was also observed using pt. 222 P+, indicating that a single dominant gene (temporarily designated as *RphCRQ3*) is responsible for the observed race-specific resistance (Fig. 1).

Genetic mapping of *RphCRQ3* versus *Rph2*. We genotyped DNA pools bulked from NSR and NSS families using the DArT-Seq platform and performed a BSA to map the race-specific resistance in Quinn to a chromosome arm to resolve either the presence of a new *Rph2* allele on chromosome 5H or the presence of a third independent uncharacterized resistance locus. The percentage of in-phase linkage was calculated for each marker across the genome (Fig. 2). Complete linkage was observed at two locations in the genome. The first region of linkage was at the extreme telomeric end of chromosome 4HS. A total of 20 markers displayed complete, in-phase linkage, surrounded by a region of increased linkage. This result indicated that this chromosomal region is associated with *RphCRQ3*. A partial consensus map was generated covering this region of fixation (Fig. 3). To confirm the map location of *RphQ3*, we designed a polymorphic insertion-deletion marker to gene HORVU4Hr1G000050.3 within the Morex reference on chromosome 4HS within the target interval using the BARLEX website (https://apex.ipk-gatersleben.de/apex/f?p=284:41:::NO:RP:P41_GENE_CHOICE:2). We genotyped the resistant and susceptible bulk samples sent to DArT and compared the band size to the parents (Supplementary Fig. S1).

A single linked marker was also observed at the telomeric end of chromosome 7HL. This marker was not surrounded by a region of increased linkage, indicating that it is likely a result of mis-assembly in the reference sequence. The marker was associated with 2012 Morex sequence assembly locus AK250035.1 (Sato et al. 2009). AK250035.1 sequence was retrieved from the GrainGenes online database (<https://wheat.pw.usda.gov/GG3/>), and the sequence blasted onto the Morex 2.0 reference sequence pseudomolecule using the IPK Barley ViroBLAST server (https://webblast.ipk-gatersleben.de/barley_ibsc/) (Deng et al. 2007). The AK250035.1 sequence blasted to five of the seven Morex chromosomes with an E value of 0 (Table 6). The highest sequence match (95%) was to 4H (0.67 cM), with the lower percentage (77 to 78%) matches on chromosomes 2H, 3H, 5H, and 7H. Higher E-value matches were found for chromosomes 1H (1 e⁻¹³¹), 6H (5 e⁻¹⁰), and the unassigned sequence pool (0.018). This indicates that the 7H marker associated locus has highly similar sequences across barley chromosomes. The BSA demonstrated that *RphCRQ3* is due a single, uncharacterized locus on 4HS rather than a variant allele of *Rph2*.

Genetic mapping of *Rph2* was also performed in this study using the Sloop/Halcyon (S/H) DH population as a positive control for the previously determined 5H map location reported by Park et al. (2003). The S/H population was also genotyped using the DArT-Seq platform and genetic map construction using 1,989 markers (SNPs and in-silico DArTs) resulted in 11 different linkage groups (LGs) spanning 3,057 cM, with chromosomes 1H, 2H, 3H, and 5H having multiple LGs. We delimited the *Rph2* locus to a 3.2 cM region, flanked by DArT markers (3264599-126.6 cM and 4013955-128.8 cM), on LG 5H.1.

DISCUSSION

It has been suggested previously that the *Rph2* locus comprises various alleles that may display different race-specificities (Franckowiak et al. 1997). Small panels of putative *Rph2* donors subjected to multipathotype testing have shown variable responses

(Levine and Cherewick 1952; Reinhold and Sharp 1982; Steffenson and Jin 1997; Tan 1977). We assessed a panel of 20 *Rph2* donors using Australian pathotypes of *P. hordei* to identify potential race-specific alleles and confirm the *Rph2* postulation from previous studies (Table 1). A highly variable response to the pathotypes

Fig. 3. Partial consensus map of chromosome 4HS. Bulk segregant analysis derived polymorphic markers covering *RphCRQ3* locus based on physical locations depicted in the Morex reference sequence assembly (Mascher et al. 2017). Region of complete, in-phase linkage indicated by shaded quantitative trait loci bar.

tested was observed in the assembled *Rph2* donor panel in the present study.

Cultivar Kwan was initially postulated to carry *Rph2* by Henderson (1945) using North American isolates of *P. hordei*. F₂ and F₃ of Ricardo/Kwan intercross populations were tested by Zloten (1952), who identified segregation conforming to a three-factor inheritance in the population. Ricardo is now known to carry *Rph2* and *Rph21* (Sandhu et al. 2012), suggesting the third factor to be an unknown gene in Kwan. The phenotype observed in the present study indicates that Kwan likely does not carry *Rph2* but an uncharacterized resistance gene that confers resistance to the Australian pathotypes used in this study. Our study included pt. 5457 P+, a pathotype that was not available during previous testing. It is also possible that the additional resistance observed in the five resistant lines (Clarine, Kwan, PI 531841, Quasar, and SY Rattler) is due to the presence of one or more genes that are effective to 5457 P+ (viz. *Rph7*, *Rph11*, *Rph14*, and/or *Rph15*), however the involvement of *Rph11* and *Rph15* is unlikely because both are derived from *Hordeum vulgare* subsp. *spontaneum* and neither have been deployed in cultivated barley. The broadly resistant lines identified in this study are possible targets for new sources of leaf rust resistance.

Race specificity was observed in a subset of lines when tested with pt. 222 P+. Several previous studies have identified similar examples of variable response in lines postulated to carry *Rph2*. Levine and Cherewick (1952) assessed a panel that included Reka 1, Bolivia, and Quinn with 52 North American races of *P. hordei*. Variable responses were observed among the three lines, however, as they carry genes in addition to *Rph2*, clear separation of potential race specificity from the additional genes was not possible. A similar study was conducted by Reinhold and Sharp (1982), who observed variable responses for Batna and Bolivia (*Rph2* + *Rph6*). As these cultivars were postulated to carry identical genes, the variable response may be due to race-specific alleles or additional genes not identified in the present study. A later study aimed to investigate potential allelism at the *Rph2* locus that assessed seven *Rph2* donors postulated to carry *Rph2* singly (Austral, Batna, Peruvian, PI 531841, Kwan, Q21861, and Weider) (Steffenson and Jin 1997). Variable responses were observed, indicating potential race-specificity. Of this panel however, only one line (Peruvian) was found to carry *Rph2* singly in the present study, rendering direct comparison impossible probably due to differences in pathogen virulence between countries.

An improved understanding of the barley-*P. hordei* pathosystem requires an in-depth knowledge of the standard differential genotypes used for race analysis. The identification of race-specific resistance in genetic stocks, however, can be masked by the presence of other effective resistance genes. Our data confirm that Quinn carries *Rph5* and the same *Rph2* allele as Peruvian, and reveal the presence of a third race-specific resistance locus in Quinn that is effective against pt. 222 P+, which we temporarily designated as *RphCRQ3*. The broad resistance provided by the combination of

both *Rph2* and *Rph5*, and the ineffectiveness of *RphCRQ3* to many pathotypes of *P. hordei*, likely explains why *RphCRQ3* has not been identified previously. A similar situation was observed during the discovery of *Rph25*; a race-specific *Rph* gene for which avirulence appears to be rare. *Rph25* was identified in cultivar Fong Tien and is only effective against a single pathotype, viz. 220 P+ (+*Rph13*) (Kavanagh et al. 2017).

A BSA approach was chosen to investigate *RphCRQ3* as it is a localization agnostic approach that requires no previous knowledge of the nature of the gene class involved (Michelmore et al. 1991). The *RphCRQ3* locus was identified at the telomeric end of 4HS based on complete in-phase fixation of 20 DArT-Seq markers. To date, only one *Rph* gene has been mapped to chromosome 4H. *Rph21* was identified in cultivar Ricardo (*Rph2* + *Rph21*) and was mapped to the long arm of chromosome 4H in a Ricardo/Gus F₃ population (Sandhu et al. 2012). A second single marker with complete in-phase fixation was also located on chromosome 7HL, however further investigation determined that the gene underlying the 7HL marker had the best match to chromosome 4H likely suggesting an error in the Morex reference assembly. Based on our BSA data and genotypic analysis of the bulks using an indel marker, *RphCRQ3* is located in the telomeric region of 4HS, confirming that *RphCRQ3* and *Rph21* are independent. *Rph2* was previously mapped to chromosome 5H in the Sloop/Halcyon doubled haploid (DH) population (Park et al. 2003). In a very recent study Martin and colleagues also mapped *Rph2* also to chromosome 5H using introgression mapping (Martin et al. 2020). We genotyped the Sloop/Halcyon DH population using the DArT-seq platform and further refined the map location of the *Rph2* locus from the Halcyon parent to a 3.2 cM interval flanked by two DArT-seq markers near the centromeric region on chromosome 5H. Our data confirmed the previous mapping of *Rph2* and also clearly demonstrated that *RphCRQ3* represents an uncharacterized resistance locus on chromosome 4HS. The second known resistance gene in Quinn, *Rph5*, has been mapped to a separate chromosome, 3HS (Mammadov et al. 2003). This gene was also excluded during testing by utilizing the *Rph5* virulent pathotype 222 P+.

We successfully resolved the race-specific resistance in Quinn as third uncharacterized resistance locus on the short arm of chromosome 4H independent of *Rph2*. Consequently, *RphCRQ3* was cataloged as *Rph27* according to the barley leaf rust resistance gene nomenclature (Moseman 1972). *Rph27* is of limited breeding value because it is effective to only two *P. hordei* Australian pathotypes (222 P+ [980207 = 545]; 232 P+ [920401 = 506]); nevertheless, it may enhance resistance when used in combination with other *Rph* genes as part of gene pyramiding efforts. We aim to further validate the indel marker designed in this study to determine the frequency of the *Rph27* resistance allele within both Australian and International barley germplasm.

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TABLE 6. Morex 2.0 reference sequence pseudomolecule sequence similarity output for locus AK250035.1

Chromosome	Identities (query length)	Percentage match	Expect (E) value ^a
4H	894/1154 (1379)	95	0
3H	880/1133 (1379)	77	0
7H	883/1142 (1379)	78	0
2H	874/1129 (1379)	77	0
5H	687/967 (1379)	77	0
1H	687/967 (1379)	71	1 e ⁻¹³¹
6H	61/72 (1379)	85	5 e ⁻¹⁰
Unknown	32/36 (1379)	89	0.018

^a The expected value (E value) is a measure of the likelihood a hit could be observed by chance, relative to the size of the database in use.

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