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Mining Middle Eastern and Central Asian Barley Germplasm to Understand Diversity for Resistance to *Puccinia hordei*, Causal Agent of Leaf Rust

Mehnaz Mehnaz, Peter M. Dracatos, Robert F. Park and Davinder Singh *

Plant Breeding Institute Cobbitty, School of Life and Environmental Sciences, University of Sydney, Narellan, NSW 2567, Australia; mehnaz.mehnaz@sydney.edu.au (M.M.); peter.dracatos@sydney.edu.au (P.M.D.); robert.park@sydney.edu.au (R.F.P.)

* Correspondence: davinder.singh@sydney.edu.au; Tel.: +61-293518828

Abstract: Vast collections of barley germplasm have been established and conserved in various global gene banks. These collections hold tremendous genetic diversity for resistance genes to *Puccinia hordei*, a causal agent of barley leaf rust. This study was undertaken to discover, characterize and postulate the known *Rph* genes (resistance to *Puccinia hordei*) and identify novel sources of ASR (all-stage resistance) and APR (adult plant resistance) to *P. hordei*. A core set of 315 barley lines were rust-tested as seedlings for their response to eight Australian pathotypes of *P. hordei* and genotyped with molecular markers linked to the known characterised ASR and APR genes. These tests led to the postulation of ASR leaf rust resistance genes *Rph1*, *Rph2*, *Rph3*, *Rph9*.am, *Rph12*, *Rph15*, *Rph19* and *Rph25* singly or in combination. Field tests revealed that the vast majority of lines (84%) carried APR. Genotyping of the APR-carrying lines with markers bPb-0837, Ebmac0603 and sun43-44 identified lines that likely carry the known APR genes *Rph20*, *Rph23* and *Rph24* singly or in combination. Thirty-nine per cent of the lines were negative for all the three markers and were thus postulated to carry uncharacterized APR. The sources of resistance identified in this study provide a valuable resource to breeders for further utilization and diversifying the genetic basis of leaf rust resistance in barley.

Keywords: barley; leaf rust; *Puccinia hordei*; resistance; *Rph*; gene postulation; germplasm; PCR; genetic diversity

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1. Introduction

Barley (*Hordeum* species), a founder crop of the Old World Neolithic food production, was first domesticated in the Fertile Crescent ~8000 years ago. The genus *Hordeum* is extremely diverse, comprising ca. 32 species (diploid and polyploid), and is distributed widely in most temperate areas and grown throughout annual winter cropping systems of the world [1]. With the renaissance of Mendelian work in early 1900s and the sustained efforts of breeding, cultivated barley (*Hordeum vulgare* L.) is adapted to marginal environments and today ranks as the fourth most important cereal crop after wheat, maize and rice with a global production of more than 150 million tonnes produced from about 60 million hectares [2].

Several biotic and abiotic stresses hamper barley production worldwide. Among the biotic stresses that threaten barley, rust diseases are of significant concern. Leaf rust, caused by the fungal pathogen *Puccinia hordei*, is considered to be the most widespread and devastating of the rusts affecting barley [3]. Yield losses due to leaf rust as high as 60% have been reported throughout barley growing regions in Africa, Asia, Australia, Europe, New Zealand, North America and South America [4,5].

The deployment of genetic resistance is considered the preferred approach of long-term protection against leaf rust epidemics because it is more economical and eco-friendly

than fungicides. Although leaf rust resistance genes are common in *Hordeum* spp., durability and mechanistic diversity are increasingly important for the effective management of the disease. Resistance to *P. hordei* can be broadly categorized as "all-stage resistance" (ASR) and "adult plant resistance" (APR); the former typically is monogenically inherited, race-specific and considered to be non-durable, and the latter in many instances is polygenic and race-nonspecific and reputed for its durability [6–8]. In barley, 25 ASR resistance loci (*Rph1–Rph19, Rph21–Rph22* [4], *Rph25–Rph28* [9–12]) and three APR genes (*Rph20* [13], *Rph23* [14] and *Rph24* [15]) have been catalogued and mapped to chromosomes.

The emergence of new pathotypes of *P. hordei* has rendered many of the ASR *Rph* genes ineffective, leaving few resistance genes effective globally [4]. Identification of novel sources of ASR as well as APR are crucial to diversify the genetic base of resistance [16] as they can be used in gene pyramiding with other resistance genes and hence protect important varieties from new pathotypes. At the same time, understanding the effectiveness of resistance genes is vital for durability and ensuring diversity of resistance [4].

The need to conserve and utilize plant genetic resources in different crop species, including barley, has been well-recognized. Vast collections of barley germplasm have been established over the last 100 years and conserved in various gene banks around the world. These collections hold tremendous genetic diversity for resistance to various pathogens and pests, including *P. hordei.* To effectively utilize leaf rust resistance genes from these genetic resources, it is important to conduct detailed phenotypic screening and evaluation of the germplasm for disease response. The aims of this study were (1) to identify and characterize the genes conferring ASR and APR to *P. hordei* in the barley germplasm derived from the Middle East and Central Asia using multi-pathotype greenhouse rust tests and field-based phenotypic screening and (2) to genotype the accessions with the diagnostic molecular markers linked to the APR and ASR genes conferring resistance to *P. hordei*.

2. Materials and Methods

2.1. Plant Materials

The germplasm used in this research comprised a collection of 1855 barley accessions originating from Central Asia and the Middle East (Afghanistan, Cyprus, Iran, Iraq, Israel, Jordan, Kazakhstan, Lebanon, Palestine, Syria, Tajikistan, Turkmenistan, Turkey and Uzbekistan) (Figure 1) sourced from the Australian Grains Genebank (AGG) and currently maintained at the Plant Breeding Institute Cobbitty (PBIC). Initially, 1855 lines were phenotyped in the greenhouse and field in 2017 with *P. hordei* pathotype 5457 P+ (PBI culture No. 612), which is virulent on the ASR genes *Rph1*, *Rph2, Rph3*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*, *Rph19*, *Rph27* and avirulent for *Rph5*, *Rph7*, *Rph8*, *Rph11*, *Rph13*, *Rph14*, *Rph15*, *Rph17*, *Rph18*, *Rph21* and *Rph28*. Based on the initial field screening of the 1855 lines, only the resistant to moderately susceptible lines were selected for further testing. The lines that were prone to lodging in the field and those with poor germination and with segregating responses to individual pathotypes in greenhouse tests were also excluded, establishing a core set of 315 lines (Figure 2) for further multi-pathotype tests. The passport data for the core set including origin, AGG number, taxonomy, pedigree information (where available) and phenotypic data for greenhouse with eight *P. hordei* pathotypes are provided in Supplementary Table S1, while the data for field screening with *P. hordei* pathotype 5457 P+ for the years 2017, 2018 and 2019 are provided in Supplementary Table S2.

Figure 1. Map showing the countries of origin of the barley lines used in this study and the number of lines (in brackets) from each country. Asia Minor and Palestine (328 and 21 lines, respectively) are not shown in the map.

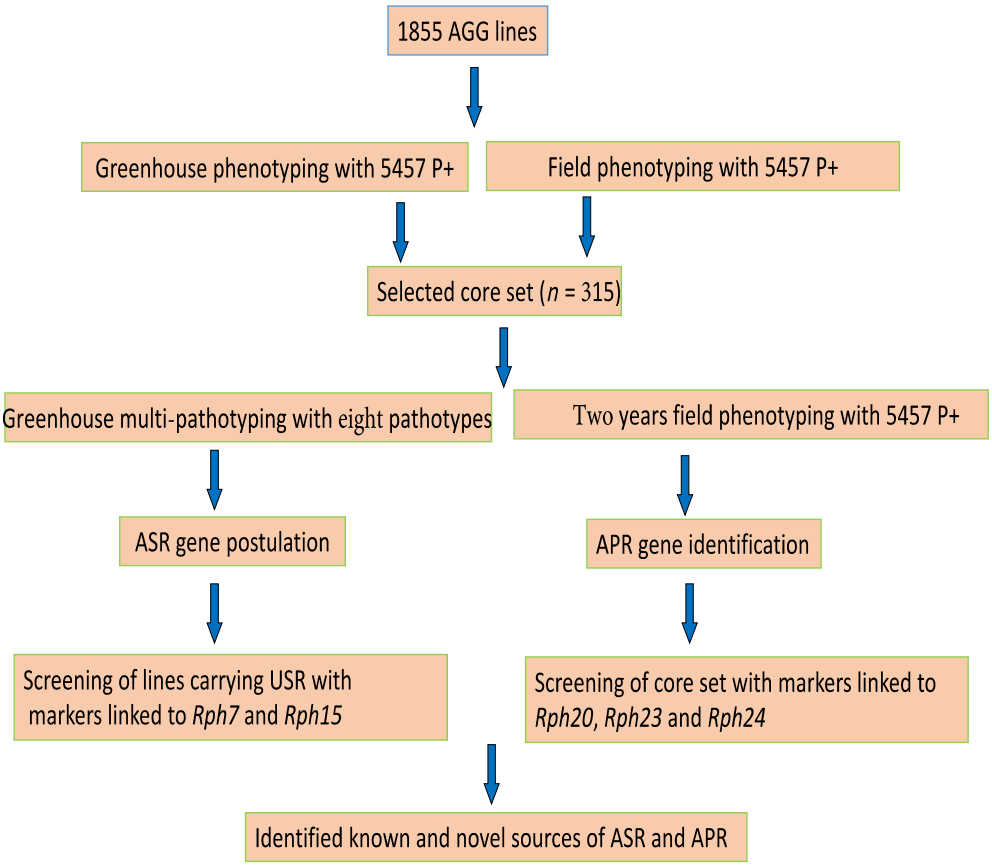

Figure 2. Schematic diagram summarizing identification and postulation of the ASR and APR genes via phenotyping and genotyping of 1855 AGG lines and the core set (n = 315) in the greenhouse and the Plant Breeding Institute Cobbitty, NSW, Australia, fields.

2.2. Pathogen Isolates

All the lines of the core set were evaluated at the seedling growth stages under controlled conditions in the greenhouse with eight Australian pathotypes (pts.) of *P. hordei*: 200 P− (PBI culture No. 518), 220 P+ +*Rph13* (577), 253 P− (490), 5652 P+ (561), 5610 P+ (520), 5453 P− (560), 5457 P− (626) and 5457 P+ (612). All the pathotypes used in this study for greenhouse screening originated from annual pathogenicity surveys of *P. hordei* conducted in Australia and are preserved in liquid nitrogen at the Plant Breeding Institute, University of Sydney. Pathotype designation is based on the virulence/avirulence pattern of an isolate on the differential set using the octal notation system proposed by Gilmour [17]. The symbol P− or P+ was used to specify avirulence and virulence, respectively, on barley cultivar Prior carrying *Rph19* [18].

2.3. Seedling Tests in the Greenhouse

All the lines were sown and raised as clumps in 90 mm plastic pots at three lines per pot. The pots were filled with potting media containing composted pine bark and sand (4:1) and fertilized with soluble fertilizer Aquasol (Hortico Pty Ltd., Revesby, NSW, Australia) at 25 g/10 L of water. A set of differential lines [18] planted at five lines per pot were included. After sowing, the pots were shifted to seedling raising rooms with a temperature of ±25 °C. Ten-day-old seedlings with a fully expanded first leaf were inoculated with each *P. hordei* pathotype. A suspension was prepared by adding 10 mg urediniospores/10 mL of oil for 200 pots. The mixture was then homogenously sprayed over the top of the seedlings with a mist atomizer. The inoculation kit was washed with 70% ethanol and then rinsed with tap water after each inoculation. To avoid contamination, the inoculation chamber was also washed down with tap water for five minutes between successive inoculations.

Following inoculation, the seedlings were incubated at ambient temperatures in a dark chamber for 24 h. An ultrasonic humidifier was used to create mist in the chamber. After 24 h incubation, the seedlings were shifted to microclimate rooms with natural lighting and an automated irrigation system. Temperatures within microclimate rooms were maintained within the range of 22–24 °C.

Disease Scoring

The disease data were recorded 10–12 days after inoculation using a modified infection type (IT) scale of 0–4 as outlined in [4]. Various infection type (IT) patterns were observed and recorded in this study (Figure 3). ITs 0, ;, 1 and 2 were used to indicate a resistant host response while ITs 3 or higher were used to indicate a susceptible host response. Variations in IT patterns were recorded using the symbols "−" = less than average for the class, "+" = more than average for the class, "C" = chlorosis and "N" = necrosis. For gene postulation, low and high ITs produced by the test lines were compared with those produced by the reference genotypes in the differential set. The reference genotypes used in this study included Gus (susceptible), Sudan (*Rph1*), Peruvian (*Rph2*), Reka I (*Rph2* + *Rph19*), Ricardo (*Rph2* + *Rph21*), Estate (*Rph3*), Gold (*Rph4*), Quinn (*Rph2* + *Rph5*), Magnif 104 (*Rph5*), Bolivia (*Rph2* + *Rph6*), Cebada Capa (*Rph7*), Egypt 4 (*Rph8*), Abyssinian (*Rph9*), Cantala (*Rph9.am*), Clipper BC8 (*Rph10*), Clipper BC67 (*Rph11*), Triumph (*Rph12*), PI 531,849 (*Rph13*), PI 584,760 (*Rph14*), Bowman + *Rph15* (*Rph15*), Prior (*Rph19*) and Fong Tien (*Rph25*).

2.4. Adult Plant Tests in the Field

All the lines were tested for their adult plant response to leaf rust over three consecutive years (2017–2019) at the Horse Unit field site of the Plant Breeding Institute Cobbitty (34°02′60.00″ S, 150 41′59.99″ E; average annual precipitation of 834 mm), NSW, Australia. All the 1855 lines were planted in the field in one replication in 2017, while in 2018 and 2019, only the core set was sown with two randomized replications (Supplementary Table S2). For field sowings, 20–30 seeds of each line were sown in 0.7 m long rows with a distance of 0.3 m between the rows. The universal leaf rust susceptible barley line Gus was used as

a susceptible control. Gus was also used as a disease spreader and was sown after every five test lines to ensure uniform disease spread. Lines Flagship, Yerong and ND24260 were sown as comparative controls for APR genes *Rph20*, *Rph23* and *Rph24*. For field epidemics, inoculations were carried out using the procedures highlighted in [19]. Ultralow volume applicator (Microfitâ, Micron Sprayer Ltd., Bromyard, Herefordshire, UK) was used to spray the suspension (urediniospores of pathotype 5457 P+ in light mineral oil (Isopar L Univar®, Ingleburn, NSW, Australia) at 30 mg spores/1.5 L of oil) on susceptible spreader Gus on evenings when there was strong prediction of overnight dew. For weed management, plots were sprayed with a herbicide. Plots were fertilized with urea (*w/w* 46% nitrogen) at 100 kg/ha 3–4 weeks after sowing and irrigated when required using fixed sprinklers.

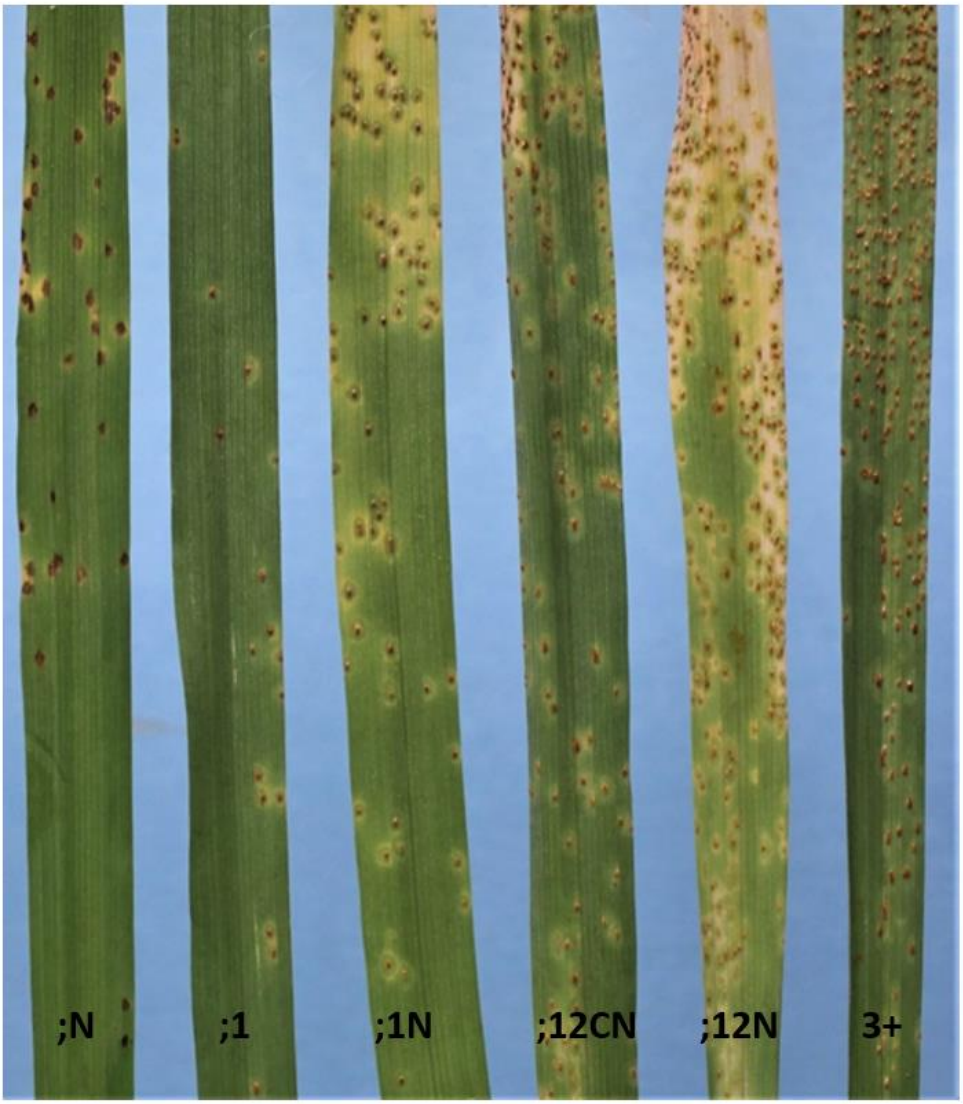

Figure 3. Range of seedling infection types recorded in tested barley lines with different *P. hordei* pathotypes in the greenhouse. Infection types are based on the 0–4 scale [4].

Disease Scoring

For field disease assessment, all the lines were phenotyped in the field using the 1–9 scale modified from McNeal [20]. Disease assessment was made at two stages of crop development, firstly approximately upon appearance of the first spikelet and secondly approximately upon completion of anthesis when the susceptible standard Gus reached

a scale of 9 (VS = very susceptible). Details of the disease scale used to assess the field phenotype is given in Table 1.

Table 1. Description of the infection type scale used to assess adult plant response of barley lines to *Puccinia hordei* in the field.

Host Response	Abbreviation	Infection Type	Disease Symptoms/Description
Resistant	R	1	No uredinia or flecking
Resistant or moderately resistant	RMR	2	No uredinia but flecking may be present
Moderately resistant	MR	3	Traces of uredinia without sporulation
Moderately resistant or moderately susceptible	MRMS	4	Small uredinia with restricted sporulation
Moderately susceptible	MS	5	Small or medium-sized uredinia with moderate sporulation
Moderately susceptible or susceptible	MSS	6	Medium-sized uredinia with heavy sporulation
Susceptible	S	7	Large-sized uredinia with abundant sporulation
Susceptible or very susceptible	SVS	8	Large-sized coalesced uredinia with abundant sporulation
Very susceptible	VS	9	Large-sized coalesced uredinia with abundant sporulation and lesions

2.5. Marker Genotyping

2.5.1. DNA Extraction

For DNA extractions, the seedlings were raised for 10–12 days in the greenhouse and samples were collected from individual leaves. Leaf tissues were dried using silica gel beads. DNA was extracted using the CTAB protocol [21]. All the samples were quantified using a spectrophotometer (NanodropTM, Biolab, Melbourne, VIC, Australia). The quality of extracted DNA was determined by running all the samples on a 0.8% agarose gel. DNA was diluted to 50 ng/µL for use in all the PCR reactions.

2.5.2. Genotyping with Markers for the APR Genes *Rph20*, *Rph23* and *Rph24*

A total of 265 of the 315 core set lines (50 lines that were resistant to the field pathotype at seedling stages were excluded) were genotyped with molecular markers bPb-0837 (linked to *Rph20*) [13], Ebmac0603 (linked to *Rph23*) [14] and sun43-44 (linked to *Rph24*) [22]. PCR products were separated using gel electrophoresis (2% agarose) for 90 min at 110 volts and visualised under UV light using a Gel Doc IT imaging System (Model M-26, Bioimaging Systems, San Diego, CA, USA). For *Rph20*, PCRs were performed using a 10 µL reaction mixture containing 100 ng of genomic DNA, 2 µL MyFi buffer, 0.1 µL MyFi DNA Taq polymerase (Bioline Alexandria, NSW, Australia), 1 µL of 10 µM each of forward and reverse primers and 3.9 µL double-distilled water. All the reactions were conducted in a 96-well plate using an automated thermocycler with the initial denaturation step at 95 °C for 1 min followed by 35 cycles at 94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s and the final extension for 5 min at 72 °C [13]. Flagship was used as the positive control and Gus was used as the negative control for screens with the *Rph20*-linked marker. For *Rph23*, PCR reactions were performed with the same reagents as described above with the initial denaturation at 95 °C for 1 min followed by one cycle at 94 °C for 2 min, 58 °C for 45 s,

72 °C for 40 s and 30 cycles at 94 °C for 20 s, 56 °C for 20 s and 72 °C for 15 s with the final extension for 5 min at 72 °C [14]. Yerong and Franklin were used as the positive and negative controls, respectively, for *Rph23*. The *Rph24* marker sun43-44 [22] was also applied using the same procedure as described above with the initial denaturation at 95 °C for 1 min followed by 35 cycles at 94 °C for 20 s, 65 °C for 20 s and 72 °C for 30 s with the final extension step of 72 °C for 5 min. Barley line ND24260 was used as the positive control while Flagship was used as the negative control for all the *Rph24* PCRs.

2.5.3. Genotyping with the *Rph7* and *Rph15* Markers

Twenty-seven lines that were resistant to all the pathotypes at the seedling growth stages were screened with markers linked to the ASR genes *Rph7* (Dracatos et al., unpublished) and *Rph15* [23]. Bowman + *Rph7* was used as the positive control while Gus was used as the negative control for assays using the *Rph7*-linked marker. For genotyping with the *Rph15* marker, Bowman + *Rph15* and Gus were used as the positive and negative controls, respectively. Marker assays for both *Rph7* and *Rph15* were performed with the initial denaturation step of 95 °C for 1 min, 35 cycles with 94 °C for 15 s, 60 °C for 30 s and the final extension step of 72 °C for 5 min. Detailed information of all the molecular markers used in this study is provided in Table 2.

Table 2. Names and sequence information of primer pairs associated with molecular markers used to genotype the selected barley lines.

Markers	Gene	Forward Primer Sequence 5′–3′	Reverse Primer Sequence 5′–3′	Reference
bPb-0837	*Rph20*	GACACTTCGTGCCAGTTTG	CCTCCCTCCCTCTTCTCAAC	[13]
Ebmac0603	*Rph23*	ACCGAAACTAAATGAACTACTTCG	TGCAAACTGTGCTATTAAGGG	[14]
sun43-44	*Rph24*	CTAGACACCACCACCACACC	ATACCAGAGTTTGCGTCCGG	[22]
Unknown	*Rph7*	GAGATAAAAGCATTACCAAAGGCTCAT	GCGCGCGCAACAGCAAACGGC	Unpublished
Unknown	*Rph15*	TGAAGAAGCTGGAAGGTCACC	AGCCAAAAACCCTTCTGGCT	[23]

3. Results

3.1. ASR Gene Postulation and Marker Analysis

A range of infection types (ITs) was observed across the 315 lines and reference differentials when tested with eight pathotypes. Based on the ITs and resistance genes postulated, the genotypes were divided into 11 groups (1–11; Supplementary Table S1; Figure 4). One hundred fifty-four lines were seedling-susceptible (IT from 33+ to 3+) to all the eight pathotypes and therefore lacked ASR genes that could be detected with the array of the pathotypes used.

Eight lines (AGG-3, AGG-45, AGG-624, AGG-662, AGG-663, AGG-1104, AGG-1124 and AGG-1724) were postulated to carry *Rph1* based on the IT patterns that matched with the differential line Sudan. A possible combination of *Rph1* + *Rph2* cannot be ruled out in these lines because all the test pathotypes that were virulent or avirulent on *Rph1* had the same virulence/avirulence on the combination of *Rph1* + *Rph2*. Therefore, to discriminate *Rph1* from *Rph1* + *Rph2*, these lines were further screened with pathotype 211 P+ (avirulent on *Rph2* and virulent on *Rph1*), to which all produced a susceptible response (IT 3+) indicating the presence of *Rph1* alone. It was also noted that line AGG-45 did not show a fully compatible IT with pathotypes 253 P− and 5457 P+, raising the possibility of additional uncharacterised resistance in this line (Table 3).

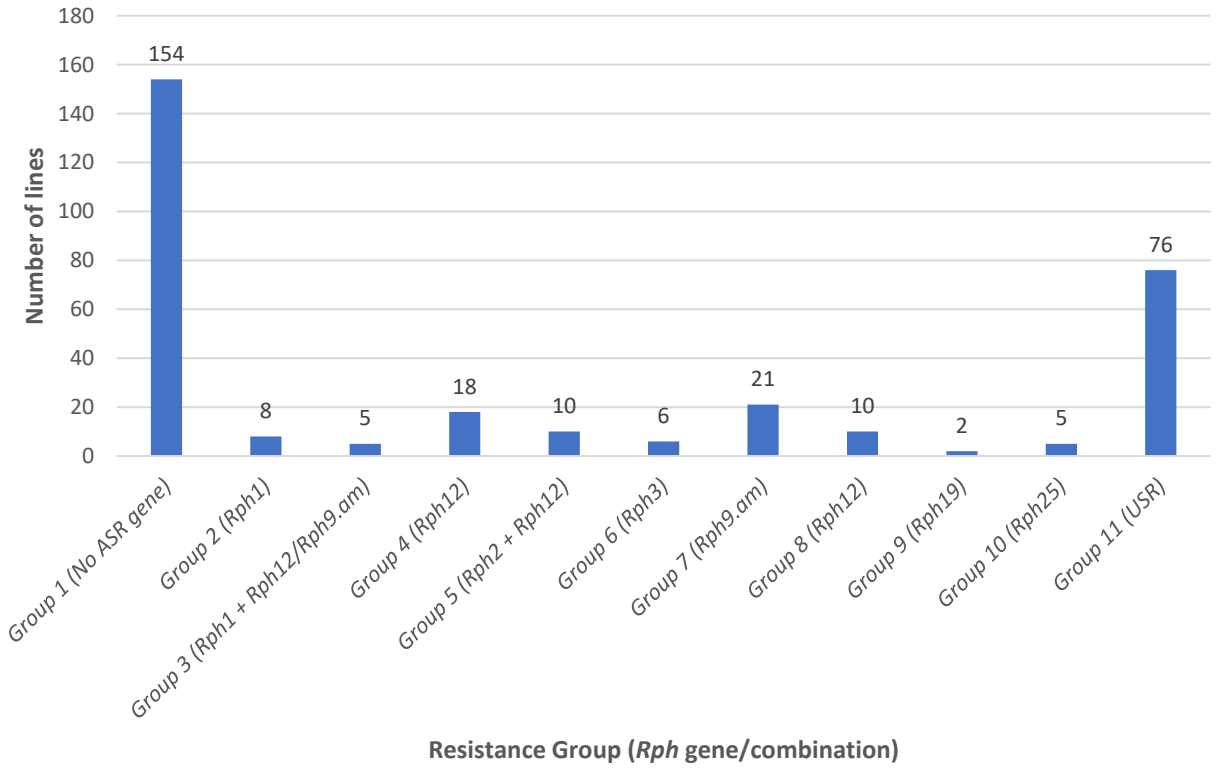

Figure 4. Distribution of barley lines from eleven groups (groups 1–11) postulated to carry various all-stage resistance (ASR) *Rph* genes when tested with eight Australian *Puccinia hordei* pathotypes (USR = uncharacterised seedling resistance; ASR = all-stage resistance).

Low ITs were recorded for AGG-8, AGG-29, AGG-30, AGG-492, AGG-595, AGG-1056, AGG-1060, AGG-1130, AGG-1707 and AGG-1737 with pts 200 P−, 220 P+ and 253 P−, similarly to the *Rph12*-carrying differential line Triumph. Additionally, these lines also exhibited low ITs with pt 5610 P+. These lines were therefore postulated to carry *Rph2* and *Rph12* in combination (Table 3). Eighteen lines (viz. AGG-11, AGG-123, AGG-129, AGG-1052, AGG-1061, AGG-1089, AGG-1303, AGG-1691, AGG-1710, AGG-1735, AGG-1738, AGG-1744, AGG-1783, AGG-1786, AGG-1796, AGG-1802, AGG-1818 and AGG-1824) were postulated to carry *Rph2*. These lines displayed low ITs to pts 200 P−, 220 P+ and 5610 P+ (avirulent on *Rph2*) and high ITs to all the other pathotypes (virulent on *Rph2*), similarly to the *Rph2*-carrying differential line Peruvian (Table 3).

The barley differential line Estate carries *Rph3* and produced very low ITs with all the pathotypes except for 5457 P− and 5457 P+. IT patterns similar to Estate were recorded for AGG-6, AGG-36, AGG-53, AGG-497, AGG-556 and AGG-694 with all the pathotypes tested leading to the postulation of *Rph3* in these lines (Table 3).

Rph12 was postulated in AGG-125, AGG-128, AGG-130, AGG-140, AGG-150, AGG-199, AGG-216, AGG-682, AGG-683 and AGG-1076. These lines showed low ITs with pathotypes 200P−, 220P+ and 253P− (avirulent on *Rph12*) and high ITs with all the other tested pathotypes (virulent on *Rph12*), a response pattern that matched differential line Triumph carrying *Rph12* (Table 3). Twenty-one lines (AGG-100, AGG-138, AGG-181, AGG-214, AG-223, AGG-225, AGG-349, AGG-351, AGG-389, AGG-394, AGG-500, AGG-505, AGG-520, AGG-617, AGG-622, AGG-623, AGG-651, AGG-772, AGG-1054, AGG-1126 and AGG-1638) showed a low IT to pt 253 P− but high ITs to all the other pathotypes. This IT pattern is typical of *Rph9.am* (formerly *RphCantala*; allele of *Rph9* and *Rph12*), carried by the differential line Cantala, and hence the presence of *Rph9.am* was postulated in these 21 lines (Table 3).

Table 3. Infection types recorded for barley lines when tested against eight *Puccinia hordei* pathotypes in the greenhouse.

Groups	*Rph* Genes	Pathotypes							
		200 P−	220 P+	253 P−	5652 P+	5610 P+	5453 P−	5457 P−	5457 P+
1	No R genes	3+	3+	3+	3+	3+	3+	3+	3+
2	*Rph1*	;N to ;+CN	;N to ;N=C	;1CN to 3+	;CN to ;=C	;N to ;1=CN	3+	3+	23- to 3+
3	*Rph1* + *Rph9.am*	;N to ;1+CN	;1-CN to ;12CN	;1-CN to ;12C	;1-CN to 23-CN	;N to ;1-CN	33-C to 3+	23C to 3+	23-C to 3+
4	*Rph2*	;1+N to ;12C	;N to ;12C	3+	3 to 3+	;1 to ;12CN	33+ to 3+	3+	3+
5	*Rph2* + *Rph12*	;N to ;12C	;1 to ;12C	;1C to ;12+C	3+	;1N to ;12CN	3+	3+	3+
6	*Rph3*	0; to ;1+CN	0; to ;1-CN	0; to ;1+CN	0; to ;1-CN	;C to ;1+CN	;C to ;1CN	3+C to 3+	3+C to 3+
7	*Rph9.am*	3+	3+	;1C to ;12C	3+	3+	3C to 3+	3C to 3+	3+
8	*Rph12*	;1C to ;12+C	;1C to ;12+C	;1C to 12+C	3 to 3+	33+C to 3+	3- to 3+	3 to 3+	3 to 3+
9	*Rph19*	;1 to ;12C	3+	;1+CN to ;12	3+	3+	;1+C to ;12CN	;12 to;12+	3+
10	*Rph25*	33+ to 3+	;1-CN to ;12+C	3+	3+	3+	3+	3+	3+
11	USR [#]	0; to 2-C	0; to 3-C	;C to 2C	0; to 23-C	;N to 2C	; to 3-C	0; to 23C	;N to 2+3+C
[1] Gus	No R genes	3+	3+	3+	3+	3+	3+	3+	3+
[2] Sudan	*Rph1*	;N	;+N	3+	;+N	;-N	3+	3+	3+
[3] Peruvian	*Rph2*	;1-N	;1-N	3-	3+	;1+CN	3+	3+C	3+C
[4] Estate	*Rph3*	;C	0;	0;	0;C	;-CN	;1-	3+C	3+C
[5] Cantala	*Rph9.am*	3+	3+	12-C	3+	3+	3c	3+c	3+c
[6] Triumph	*Rph12*	;+N	;+N	;1CN	3+	3	3+	3+	3+
[7] Prior	*Rph19*	;1	33+	;1-CN	3+	33+	12-	12-	3+
[8] Fong Tien	*Rph25*	3+	12=CN	3+	3+	3+	3+	3+	3+

Virulence on specific *Rph* genes for each pathotype is shown in parenthesis: 200 P− (*Rph8*), 220 P+ (*Rph8*, *Rph5*, *Rph19*), 253 P− (*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph8*), 5652 P+ (*Rph2*, *Rph4*, *Rph6*, *Rph8*, *Rph9*, *Rph10*, *Rph12*, *Rph19*), 5610 P+ (*Rph4*, *Rph8*, *Rph9*, *Rph10*, *Rph12*, *Rph19*), 5453 P+ (*Rph1*, *Rph2*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*, *Rph19*), 5457 P− (*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*), 5457 P+ (*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph6*, *Rph9*, *Rph10*, *Rph12*, *Rph19*). Infection types are based on the 0–4 scale [4], where 0 = no visible symptoms, ; = flecks, 1 = minute uredinia enclosed by necrotic tissue, 2 = small or medium-sized uredinia enclosed by chlorotic and/or necrotic tissue, 3 = medium-sized or large uredinia with or without chlorosis. The letters C and N indicate chlorosis or necrosis, respectively; "+" and "–" indicate higher and lower infection types than normal, respectively. Infection types of 3+ or higher were considered to indicate host susceptibility. [1–8] are differential genotypes carrying the reference *Rph* genes identified in this study. [#] USR = uncharacterised seedling resistance.

Rph19 was detected in two lines, AGG-311 and AGG-582, because these lines showed low ITs with *Rph19* avirulent pathotypes (with the P− designation) and high ITs with *Rph19* virulent pathotypes (with the P+ designation) (Table 3). *Rph25* is only effective with one of the eight *P. hordei* pathotypes used, viz. pt 220 P+ (also virulent on *Rph13*). Of the 315 lines tested, five (AGG-554, AGG-1074, AGG-1105, AGG-1659 and AGG-1660) were resistant only to 220 P+ +*Rph13*, leading to the postulation of *Rph25* in these lines.

Seventy-seven lines produced IT patterns that did not allow postulation of any catalogued *Rph* gene. Among this set, 27 lines showed resistance to all the eight pathotypes (Supplementary Table S1). Apart from AGG-157, AGG-249 and AGG-1125 which produced intermediate ITs, all the lines produced very low ITs to all the pathotypes used. These lines may carry gene *Rph7* or *Rph15*, for which none of the test pathotypes used are virulent. As virulence for *Rph7* and *Rph15* has not been detected in Australia [24], these lines were screened with markers closely linked to both genes. None of the lines were positive for the *Rph7* marker, while only one line (AGG-514) was positive for the *Rph15* marker indicating

the presence of *Rph15* in this line. The resistance gene(s) in the remaining 50 lines (resistant to one or more of the pathotypes used) could not be postulated with the array of pathotypes used, and it is likely that they carry either uncharacterised resistance genes or combinations of unknown resistance genes.

3.2. Characterization of APR and Marker Analysis

The core set was phenotyped in the field for three consecutive years (2017, 2018 and 2019; Supplementary Table S2). All the lines were also screened with molecular markers bPb-0837, Ebmac0603 and sun43–44 linked to *Rph20*, *Rph23* and *Rph24*, respectively (Figure 5). Based on the phenotypic and genotypic data, the lines were divided into two groups:

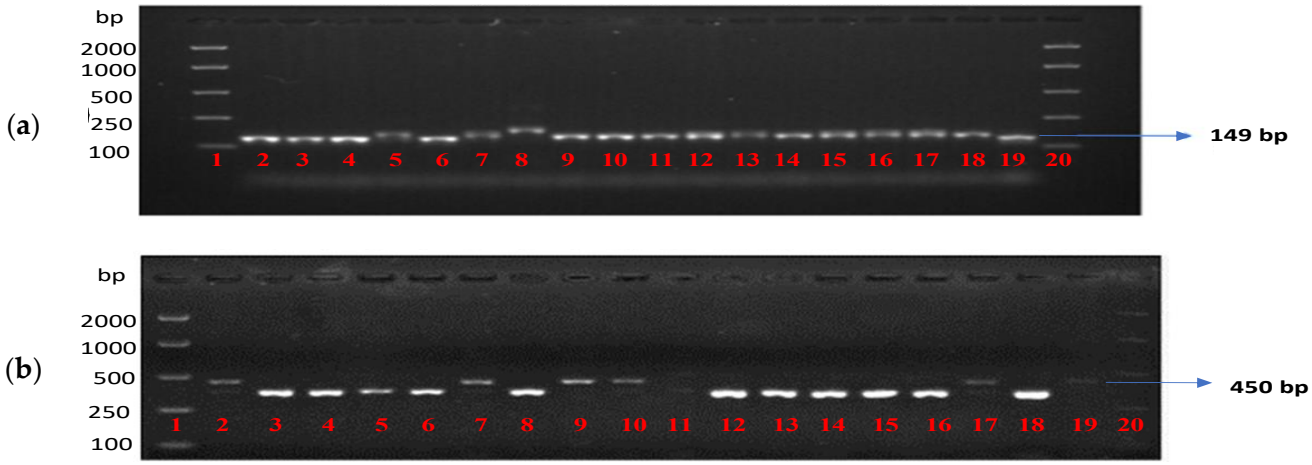

Figure 5. Gel images showing PCR amplification of the product size of (**a**) 149 bp in the lines carrying the Yerong allele linked to APR gene *Rph23*. From left to right, 2–17 = AGG lines, 18 = negative control Franklin and 19 = positive control Yerong; 1 and 20 = Easy Ladder (Bioline). The lines were scored as positive and negative with reference to Yerong and Franklin. (**b**) Product size of 450 bp in the AGG lines (well Nos. 2, 7, 9, 10 and 17) carrying the ND24260 allele linked to APR gene *Rph24*. From left to right, 2–17 = AGG lines, 18 = negative control Flagship, 19 = positive control ND24260; 1 and 20 = Easy Ladder (Bioline).

3.2.1. Group A

This group comprised the 154 lines that lacked any detectable ASR gene. Nine lines in this group were highly resistant and categorized as R. Five of these lines were positive for both the bPb-0837 and Ebmac0603 markers and hence the APR in these lines is likely due to the combination of *Rph20* and *Rph23*. One line was positive for both Ebmac0603 and sun43–44, indicating the presence of *Rph23* and *Rph24*. Two lines (AGG-5 and AGG-2) carried *Rph20* and *Rph23*, respectively, while one line was negative to all the three test markers and therefore likely lacked any of the three known APR genes (Supplementary File 2). Twenty-one lines were rated RMR, of which eight were positive for bPb-0837, three—positive for Ebmac0603 and one—positive for sun43–44. Nine lines in the RMR category were negative to all the three markers. Fifty-six lines that showed an MR response carried either bPb-0837 (22 lines), Ebmac0603 (five lines) or sun43–44 (two lines) singly or the combination of bPb-0837 + Ebmac0603 (two lines) or Ebmac0603 + sun43–44 (one line), while 24 lines were negative to all the three markers. Forty-four lines were rated as MRMS, eight of which were positive for Ebmac0603, five—positive for sun43–44, while the remaining 31 lines were negative for all the three markers. Twenty-four lines were recorded as MS, of which only two were positive for *Rph20*, six were positive for *Rph23*, one—positive for *Rph24* and one carried both *Rph23* and *Rph24*. All the other lines (14) with the MS phenotypic reaction in this group were found negative for all the three tested APR markers (Supplementary Table S2, Figure 6).

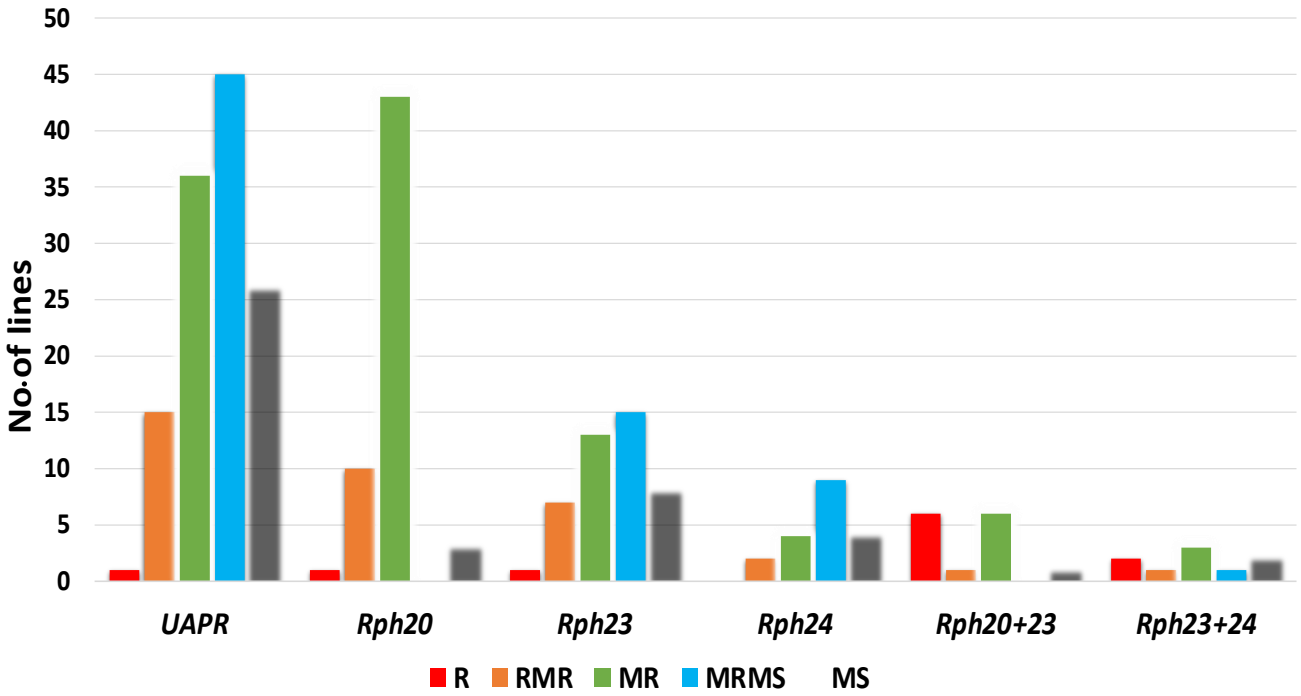

Figure 6. Graphical representation of the barley lines (from groups A and B) carrying UAPR (Uncharacterised adult plant resistance), *Rph20*, *Rph23*, *Rph24*, *Rph20* + *Rph23* and *Rph23* + *Rph24* genes and their various resistance responses recorded in the field.

3.2.2. Group B

This collection comprised 161 lines that were resistant to one or more pathotypes at the seedling growth stages and were therefore postulated to carry various ASR genes. Of these, 50 were resistant to the field pathotype at the seedling stages and hence they could not be assessed for the presence of APR. The remaining 111 lines in this group were seedling-susceptible to pt 5457 P+ used for field inoculations, and hence it was possible to assess the presence of APR in these lines. The application of the bPb-0837, Ebmac0603 and sun43–44 markers within each resistance category of the Group B lines indicated presence of either *Rph20* (24 lines), *Rph23* (21 lines) or *Rph24* (11 lines) singly or the combination of *Rph20* + *23* (seven lines) or *Rph23* + *Rph24* (four lines). Forty-four lines were negative for all the three APR markers and potentially carry uncharacterised APR distinct from the three known APR genes (Supplementary Table S2; Figure 6).

4. Discussion

This study focused on the discovery and characterization of novel sources of resistance (ASR and APR) to *P. hordei* that can be effectively utilized by barley breeders to diversify the genetic basis of leaf rust resistance deployed in agriculture. Sourced from the Australian Grains Genebank, the germplasm evaluated in these studies originated from Afghanistan, Asia Minor, Cyprus, Iran, Iraq, Israel, Jordan, Kyrgyzstan, Lebanon, Palestine, Syria, Turkmenistan, Turkey and Uzbekistan, countries representing the Middle East and Central Asia. Given that these regions are the centre of origin of barley domestication, it was anticipated that the germplasm selected might contain high levels of genetic diversity for resistance due to the coevolution of barley species/landraces with several pathogens in these regions as previously hypothesised by [25].

This study investigated and evaluated a vast collection comprising 1855 barley lines. Based on the preliminary seedling greenhouse and adult plant field tests with the same *P. hordei* pathotype (5457 P+), an elite core set of 315 lines was selected that were less prone to lodging and possessed high or moderate levels of field resistance to the most virulent *P. hordei* pathotype prevalent in Australia. By integrating multi-pathotype seedling tests

conducted in the greenhouse, replicating artificially inoculated field trials over multiple years, genotyping with molecular markers linked to important leaf rust resistance genes, putative new and potentially important sources of ASR and APR to *P. hordei* were identified.

Multi-pathotype testing of the core set with eight *P. hordei* pathotypes in the greenhouse allowed the postulation of various known (*Rph1*, *Rph2*, *Rph3*, *Rph9.am*, *Rph12*, *Rph19* and *Rph25*) and unknown ASR genes. The seven known ASR genes detected in this study were also common in several other germplasm collections [26–29]. In our studies, the ASR genes *Rph2* (8.8% of the lines) and *Rph9.am* (8.2% of the lines) were the most frequently postulated genes, followed by *Rph12* (4.7% of the lines). Elmansour et al. [29] also reported *Rph2* and *Rph12* as the most frequent ASR genes in African barley accessions using the same collection of pathotypes. Virulence for these genes is common within Australian *P. hordei* populations [4], and consequently they are of little value in resistance breeding. In this study, we did not find any correlation between the country of origin and the ASR gene postulated. However, the Syrian germplasm was the most susceptible (42%), although this may be explained by its high representation within the total lines assessed in this study (190/315). The highest frequency of resistant lines (68%) was observed within the germplasm from Cyprus and Israel.

The identification of uncharacterized seedling resistance in 76 (24%) lines, of which 27 were resistant to all the eight pathotypes (an array broadly covering the virulence spectrum of Australian *Puccinia hordei* pathotypes), indicates that either virulence for the ASR genes in these lines is not present in Australia or that the lines could carry novel ASR genes. Virulence for genes *Rph7*, *Rph14*, *Rph15* and *Rph21* has not been detected in Australia [4]. Highly predictive codominant genetic markers were recently developed for ASR genes *Rph7* (Dracatos et al. unpublished) and *Rph15* [23], for which virulent pathotypes are not available in Australia. Assays of 27 lines that were resistant to all the test pathotypes with markers linked to *Rph7* and *Rph15* indicated the presence of *Rph15* in one line only (viz. AGG-402), while *Rph7* was not detected in any of the lines, suggesting that the remaining lines carry sources of resistance distinct from *Rph7* and *Rph15*. Genes *Rph15* and *Rph16* were derived from *H. vulgare* ssp. *spontaneum* [4], and recently both were shown to be one and the same [23]. The presence of *Rph15*/*Rph16* in line AGG-402 indicates the likely possibility of *H. spontaneum* origin/derivation of this line. Genetic studies of the remaining 26 lines carrying uncharacterised ASR will be important to assess their value in protecting barley crops from leaf rust.

Multiyear (2017–2019) field phenotyping of the core set with 5457 P+ revealed varying levels of resistance ranging from resistant (R) to moderately susceptible (MS). Marker genotyping of the core set with markers bPb-0837, Ebmac0603 and sun43–44 indicated the presence of all the three APR genes either singly or in combination in 265 of the 315 lines. Both marker data and field response for these lines were strongly associated. Lines carrying *Rph20* singly exhibited an RMR or MR response in the field. These findings correspond with the previous findings [11,13–16] which reported MR–MS responses for barley genotypes carrying *Rph20* alone.

The lines that carried *Rph23* only (based on the presence of marker Ebmac0603) produced variable responses in the field that ranged from MR to MS. However, higher levels of field resistance (R or MR) were noticed in the lines carrying the combination of *Rph20* + *Rph23* (bPb-0837 + Ebmac0603). *Rph23* itself is a minor-effect gene that provides low levels of protection when present alone but is additive with other minor effect genes and can enhance the level of field resistance [14]. Singh et al. [30] further revealed the additive nature and high levels of protection conferred by the combination of *Rph20* and *Rph23* in a panel of international lines. Similarly, Dracatos et al. [31] also reported MS and S responses in several Australian barley lines carrying the marker Ebmac0603 and detected the highest levels of field resistance in the lines positive for both the bPb-0837 and Ebmac0603 markers. Our studies provide further confirmation that the combination of *Rph20* and *Rph23* confers high levels of resistance to leaf rust under high disease pressure in the field. Although an MS response was recorded for the lines carrying *Rph23* in this

study, some lines that were positive for the *Rph23* marker only displayed MR responses, indicating the presence of additional uncharacterised APR in these lines.

Rph24 is known to provide low levels of protection in the field when present alone. However, this gene is also reported to be highly additive when present with *Rph20* and *Rph23* [15,32]. While most of the lines carrying *Rph24* in this study displayed MRMS or MS reactions in the field, three (*viz*. AGG-104, AGG-1713 and AGG-1818) produced R and MR responses, strongly suggesting the presence of additional uncharacterized APR in these lines. The lines in which both *Rph23* and *Rph24* were postulated produced R–MRMS responses in the field, once again demonstrating the additive nature of both these genes as well as the possible presence of additional uncharacterized resistance. All the lines carrying *Rph23* and *Rph24* that were highly resistant in the field should be further investigated as they may carry additional APR gene(s). Based on the markers, none of the lines in this study carried the combination of *Rph20* + *Rph24*. Although this combination was detected in previous studies of diverse barley germplasm [22] and in the Australian germplasm [32], the results of this study clearly demonstrate that it did not occur in the Middle Eastern and Central Asian germplasm we assessed. The absence of pedigree information for most of the lines examined in this study makes it difficult to draw conclusions regarding the sources of the resistance genes found.

This study did not find any lines carrying a combination of the three catalogued APR genes (*Rph20* + *Rph23* + *Rph24*) but detected 124 lines that carried none of these three genes based on the linked marker alleles. The lines that displayed APR responses ranging from RMR to MS were deemed as sources of uncharacterised putative new APR genes. These lines represent an important resource for further genetic characterisation and a means of further diversifying the genetic basis of leaf rust resistance in barley.

5. Conclusions

This study focused on the discovery and characterization of novel sources of resistance (ASR and APR) to *P. hordei*. By systematically conducting integrated seedling greenhouse tests and field evaluations and applying linked molecular markers, we successfully characterized resistance to *P. hordei* in a large set of lines from Central Asia and the Middle East. In our studies, we identified eight known ASR genes (*Rph1*, *Rph2*, *Rph3*, *Rph9*.am, *Rph12*, *Rph15*, *Rph19* and *Rph25*) and three APR genes (*Rph20*, *Rph23* and *Rph24*) that confer resistance to *P. hordei*. Significantly, we also found several sources of resistance that appear potentially novel. Further characterisation, genetic studies and mapping are recommended on lines carrying distinct novel resistance sources. The studies also highlighted the effective utilization of predictive molecular markers for APR genes *Rph20*, *Rph23* and *Rph24* and ASR genes *Rph7* and *Rph15* and their utility for genotyping large germplasm collections and ultimately marker-assisted selection. The information and the leaf rust-resistant germplasm identified in this study represent a useful resource for breeders to further diversify the genetic basis of leaf rust resistance in barley.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112146/s1, Table S1: Groupwise seedling leaf rust response of 315 lines when tested in the greenhouse with eight Australian Puccinia hordei pathotypes and postulation of Rph genes based on multi-pathotype tests; Table S2: Leaf rust response of the 315 barley lines tested in the field for three years (2017–2019) and prediction of the APR genes based on the linked molecular markers.

Author Contributions: M.M. executed the study and D.S. designed the research. M.M., D.S. and R.F.P. analysed the greenhouse and field data. P.M.D. assisted with marker screening. D.S., P.M.D. and R.F.P. supervised the studies. M.M. and D.S. wrote the manuscript and all the authors contributed to the manuscript. All authors have read and agreed to the published version of the manuscript.

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