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Identification of new sources of adult plant resistance to *Puccinia hordei* in international barley (*Hordeum vulgare* L.) germplasm

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Abstract Barley leaf rust, caused by *Puccinia hordei*, is one of the most destructive foliar pathogens of cultivated barley, causing significant yield losses in many regions throughout the world. In this study, 521 seedling-susceptible barley germplasm obtained from diverse breeding material sourced from the Australian Winter Cereals Collection (AWCC), the University of Western Australia (UWA), and international collections from Australia, China, Germany, Spain, and Uruguay were assessed for adult plant resistance (APR) in field nurseries over multiple growing seasons. Lines (213) that consistently showed APR over multiple seasons were screened with PCR-based markers closely linked to *Rph20* (*bPb0837*) and *Rph23* (*EBmac0603*). About 93 % of the lines that were resistant in the field carried one or more uncharacterised APR genes with or without *Rph20* and *Rph23*. There was high variability for APR within specific international germplasm collections. The presence of *bPb0837* was strongly correlated with high APR (TR-MR) responses in the field, while lines that were positive for the *EBmac0603* allele had intermediate resistance (MRMS). Both *EBmac0603* and *bPb0837* were present in three German lines (Lenka, Line 17 and Volla) and in the Australian variety Macquarie, all were thus were postulated to carry both *Rph20* and *Rph23* and had TR-20MR responses in the field. Molecular markers closely linked to *Rph20* and *Rph23* provide a valuable resource that can be used to assist the incorporation of these genes into new cultivars and identify uncharacterised APR.

Keywords Adult plant resistance · Leaf rust · International germplasm · *Rph20* · *Rph23*

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Introduction

Barley (*Hordeum vulgare* L.) is a valuable grain crop in all global cereal growing areas. It is used predominately for malting and for ruminant feed, whilst a small percentage is used for human consumption in low rainfall areas with poor fertility. Barley leaf rust, caused by *Puccinia hordei*, is one of the most destructive foliar diseases and has caused significant yield losses in many regions where barley is grown (Cotterill et al. 1992; Woldeab et al. 2007; Murray and Brennan 2009). Yield reductions of up to 32 % have been reported in certain susceptible barley cultivars in both Australia and North America (Park and Karakousis 2002). Due to the adverse environmental effect of fungicides, the most preferable and cost-effective means of controlling barley leaf rust is through the deployment of durable host resistance genes (Park 2008).

Two major types of resistance have been described for cereal rust pathogens, seedling/all-stage resistance (ASR) and adult plant resistance (APR). ASR genes are usually effective at all stages of crop development and are often characterised by hypersensitive reactions. Numerous ASR genes for resistance to *P. hordei* (*Rph*) have been identified (*Rph1-Rph19*, Golegaonkar et al.

2009; *Rph21*, Sandhu et al. 2012; *Rph22*, Johnson et al. 2013), but most have been rendered ineffective by mutational changes in *P. hordei*. In some regions including Australia, the presence of the alternate host *Ornithogalum umbellatum* ('Star of Bethlehem') can facilitate sexual recombination and increase the likelihood of new virulent pathotypes developing (Clifford 1985; Wallwork et al. 1992; Steffenson et al. 1993; Park 2003). APR in barley has been reported to provide incomplete resistance phenotypes that are often additive (Carlborg and Haley 2004; Golegaonkar et al. 2009; Singh et al. 2014 *in press*). Genes conferring resistance to leaf rust (*P. triticina*), stripe rust (*P. striiformis* f. sp. *tritici*) and stem rust (*P. graminis* f. sp. *tritici*) at adult plant growth stages are often additive and more durable (Singh 1992). Furthermore, some durable APR genes in wheat have been shown to be pleiotropic, providing effective resistance against multiple rust pathogens (i.e. *Lr34*/*Yr18*/*Sr57*, *Lr46*/*Yr29*/*Sr58* and *Lr67*/*Yr46*/*Sr55*) (Singh 1992; Singh et al. 2000, 2011).

In previous studies, 13 quantitative trait loci (QTL) associated with partial leaf rust resistance to leaf rust (designated *Rphq1* to *Rphq13*) were reported at both seedling and adult plant growth stages (Qi et al. 2000). A recent study also reported a total of 29 genomic regions that confer quantitative resistance to leaf rust in barley (Marcel et al. 2007). Among these QTL, 21 *Rphq* genes were identified and 19 were located on a high-density consensus map, including four loci effective only at the adult plant stage. Within barley, only two APR loci have been designated, however numerous other sources of APR have been identified although not yet characterized (Golegaonkar et al. 2009, 2010; Hickey et al. 2011; Singh et al. 2014 *in press*). Gene *Rph20*, originally from European descent, was sourced from the variety Pompadour and mapped to chromosome 5HS (Golegaonkar et al. 2009). A dominant marker has been developed from a DArT marker *bPb0837* that maps 0.7 cM away from the *Rph20* QTL (Liu et al. 2011). This marker has been found to be highly diagnostic for the presence of *Rph20*-derived APR resistance, but is not completely linked based on the presence of rare false positives (Hickey et al. 2011). Gene *Rph23*, which was found in the Australian barley variety 'Yerong', was recently designated and mapped to chromosome 7H (Singh et al. 2014 *in press*). A co-dominant SSR marker *EBmac0603* was closely linked to the APR allele present in 'Yerong' (Singh et al. 2014 *in press*). Interestingly, *Rph23* on its own displays a 60–70 moderately susceptible (MS) reaction in the field. However, when present with another minor QTL on chromosome 6HL (*RphYer*-6HL), the infection type decreases to 40–60 MRMS, indicating that these genes are additive (Singh et al. 2014 *in press*).

Diagnostic PCR-based markers for APR in barley facilitate screening of both advanced breeding lines and international germplasm collections to identify known APR genes for further genetic analysis and use. In this study, we used PCR-based markers linked to *Rph20* and to *Rph23* to screen a diverse set of international germplasm comprising lines carrying varying levels of APR based on the consensus of up to 3 years of phenotypic field data. We also report on the presence uncharacterised APR sources and discuss implications for barley germplasm improvement.

Materials and methods

Plant material

International barley germplasm (521 lines) was sourced from the Australian Winter Cereals Collection (AWCC) (92 lines), the University of Western Australia (UWA) (113 lines) and international collections from Australia (107 lines), China (20 lines), Germany (58 lines), Spain (80 lines), and Uruguay (20 lines).

Field assessment of APR to leaf rust in international germplasm

A total of 521 diverse international barley lines that were identified in previous studies (Golegaonkar et al. 2009; Sandhu 2011; Derevnina et al. 2013; Park pers. Comm.) to be seedling susceptible to *P. hordei* pt. 5457P+were assessed in field leaf rust nurseries either during 2011, 2012, 2013 or all 3 years at the Horse Unit field experimental site at the University of Sydney Plant Breeding Institute, Cobbitty (PBIC). Approximately 20–30 seeds of each line were sown in 0.7 m rows at 0.3 m spacing. The leaf rust susceptible barley genotype Gus was used in disease spreader rows sown after every five plots of test lines to assist uniform inoculum increase and spread across the experimental areas.

Pathogen isolates

Gene postulation was previously carried out on all lines using multipathotype testing (Derevnina et al. 2013; Sandhu 2011; Golegaonkar et al. 2009; Park pers. comm.). Pathotype designation was based on virulence/avirulence on the standard differential genotypes using the octal notation system (Gilmour 1973). The addition of 'P+' or 'P-' was used to indicate virulence or avirulence, respectively, on resistance gene *Rph19*, present in the cultivar 'Prior' (Park 2003). Field inoculations were carried out using pathotype 5457P+(accession number 090017), which is virulent on all postulated ASR genes previously found within the international germplasm screened in this study (*Rph1*, *Rph2*, *Rph3*, *Rph4*, *Rph12* and *Rph19*) and *Rph6*, *Rph9* and *Rph10*.

Inoculation procedures

Field inoculations were performed according to the technique outlined by McIntosh et al. (1995). Epidemics were produced in the field using a urediniospore–mineral oil suspension (30 mg of spores in 1.5 l of Isopar L mineral oil), which was misted over spreader rows using an ultra-low-volume applicator (Microfit; Micron sprayer Ltd. Bromyard, Herefordshire, UK). Four successive inoculations were carried out on afternoons when there was a high possibility of overnight dew.

Phenotypic analysis of field infection to barley leaf rust

A modified Cobb scale (Peterson et al. 1948) was used in the field to assess disease severity (percentage leaf area affected). Host response (R, no uredinia present; TR, trace or minute uredinia on leaves without sporulation; MR, small uredinia with slight sporulation; MR-MS, small-to-medium-sized uredinia with moderate sporulation; MS-S, medium-sized uredinia with moderate to heavy sporulation; S, large uredinia with abundant sporulation, uredinia often coalesced to form lesions) was assessed using scale outlined by Roelfs et al. (1992). Disease severity and host response were combined to represent field response for each line. The data on each line was collected either for 1, 2 or 3 years. Where lines were scored over multiple years, a range of lower and higher field response score was presented. Plants were scored when the susceptible check Gus reached 90–100S.

DNA extraction

Genomic DNA was extracted using the CTAB method (Fulton et al. 1995). The concentration and purity of each sample was measured using a NanoDrop 1000 Spectrophotometer (Thermoscientific, Scoresby, Victoria, Australia) and each sample was diluted to 50 ng μl^{-1} for PCR analysis as previously described by Singh et al. (2014) *in press*.

PCR marker screening analysis

All barley lines carrying APR used in this study were screened using markers *bPb-0837* and *Ebmac060*3, which are linked to *Rph20* and to *Rph23*, respectively. PCR analysis was performed for *bPb-0837* as described by Hickey et al. (2011). Varieties Pompadour and Baronesse were used as positive controls for *bPb-0837*. Published primer pairs for *Ebmac0603* were optimized to develop an assay permitting PCR–based genotypic screening for *Rph23* (Singh et al. 2014 *in press*; Hayden et al. 2008). A 149-bp fragment containing 10× CA dinucleotide repeats was amplified from a 10 μl reaction containing 40 μM dNTP, 10x Immolase Buffer (Bioline), 2.5 mM $MgCl_2$, 1 μM of each primer (*Ebmac0603F* 5'ACCGAAACTAAATGAACTACTT CG3'; *Ebmac0603R* 5'TGCAAACTGTGCTATTAA GGG3'), 0.2U of Immolase Taq polymerase (Bioline), and approximately 40 ng of genomic DNA. The PCR conditions involved an initial denaturation step of 10 min at 95 °C followed by an initial cycle of 3 min at 94 °C, 1 min at 58 °C, 1 min at 72 °C and 30 cycles with the profile 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C. Different allele sizes were separated on a 2 % agarose gel against a 25 bp DNA ladder (Bioline) as described in Singh et al. (2014) *in press*.

Results

Adult plant resistance in international germplasm collections

A total of 521 seedling susceptible barley lines from diverse origin were screened for leaf rust field resistance at PBIC using the *P. hordei* pt. 5457P+. The 213 lines

that consistently carried APR over multiple seasons are listed with their pedigree information in Table 1. On average, 41 % of lines across the collections carried APR and 80–90 % of these lines carried one or more uncharacterised APR genes either in the presence or absence of *Rph20* or *Rph23*. The lowest frequency of APR was observed within Chinese germplasm, whilst over 50 % of both Spanish and Uruguayan lines carried APR (>90 % uncharacterised) and all were highly resistant in the field (TR-30MRMS) (Fig. 1). All 213 barley with APR were analyzed by different groupings based on factors such as the presence of uncharacterised APR, characterized plus uncharacterised APR genes, and geographic origin (Table 1).

APR gene postulation

Postulation of APR genes was performed by screening molecular markers *bPb-0837* and *Ebmac0603* linked to APR genes *Rph20* and *Rph23*, respectively, across all 213 lines that carried APR compared to known field responses for both *Rph20* (30-40MRMS) and *Rph23* (60-70MS) respectively (Table 1). Approximately 48 % carried one of more uncharacterised APR gene and were negative for both *bPb-0837* and *Ebmac0603*, 38 % were positive for *bPb-0837*, 12 % were positive for *Ebmac0603* and 2 % carried both *bPb-0837* and *Ebmac0603* (Table 1, Fig. 2). In all cases, positive controls for *bPb-0837* (Pompadour and Baronesse) and for *Ebmac0603* (Yerong and Onslow) generated positive bands indicating the presence of their respective alleles (Table 1). Rarely were both *bPb-0837* and *Ebmac0603* found in combination, with the exceptions of German (Lenka, Line 17, and Volla) and Australian (Macquarie) lines that were all highly resistant (TR-20MR) under field conditions (Table 1). This suggests that both *Rph20* and *Rph23* are additive.

The APR response was classified into three groups: (High <20MR, Moderate 20MRMS - 50MS, and Low 50MS - 70MSS). Across the 213 lines with APR, 137 (64 %) were TR-20MR but many were negative for either the *bPb-0837* or *Ebmac0603* markers (Table 1). This suggests that there must also be uncharacterized additive APR genes present in much of the germplasm tested. From a total of 51 (23 %) lines that were rated MRMS, only a relatively small proportion carried *bPb-0837* (7, 14 %) or *Ebmac0603* (6, 12 %) markers. The remaining 27 (12 %) lines carried low levels of APR, three of which were positive for *Ebmac0603* and likely carried *Rph23* only (Table 1).

The frequency of *Rph20* and *Rph23* in international germplasm collections

Both *bPb-0837* and *Ebmac0603* marker alleles were present in five out of the seven (71 %) germplasm collections tested (Fig. 2). The highest frequency of *Ebmac0603* was 20 % within Chinese lines and 18 % in Spanish lines, while the *bPb-0837* allele was not present within any of the Chinese or Spanish germplasm tested, but was found at high frequencies within German, Uruguayan, Australian, UWA, and AWCC breeding lines (Table 1).

There was significant variation between Australian lines in terms of their APR. A smaller frequency of lines were TR-20MR, while this collection had the highest frequency of MS lines. All lines but one that carried *Rph23* were MS to S, suggesting that they carry only *Rph23*. Macquarie was positive for both *Rph20* and *Rph23* markers and was TR, which may indicate a third APR gene yet to be characterised (Table 1). The majority of genotypes (76 %) within the AWCC breeding collection were TR-MR and this was likely to be attributed to the presence of the *Rph20* (*bPb-0837* marker allele at high frequencies) (Table 1, Fig. 3). From a total of 37 genotypes, 32 (86 %) had very high levels of APR and of these, 23 (62 %) were postulated to carried *Rph20* and none carried *Rph23* based on the presence of both markers suggesting there may be some uncharacterized APRs in these lines (Fig. 3). Furthermore, many of the lines were scored as TR suggesting the presence of additive genes other than *Rph20* that are yet to be characterized. Lines within the UWA collection were mostly TR-20MR which was largely attributed to the presence of *Rph20* and to a lesser extent *Rph23*. Two lines in particular contained *Rph23* and had low APR, whilst there were many lines with high APR that contained neither known APR gene (Table 1, Fig. 3).

A total of 20 and 44 lines from Germany, and Spain, respectively, formed the European collection. Most German lines were rated TR-20MR and this correlated strongly with the presence of *bPb-0837*, which was more frequent (70 %) in German lines than in any other collection. Volla (0-1R), Lenka (TR-15MR), and Line 17 (5MR-20MR) displayed positive bands that supported the presence of both *Rph20* and *Rph23*. Interestingly,

Table 1 List of information (source, identifier name, pedigree information, field response and the presence of known and uncharacterised APR sourced based on marker data) for 213 barley lines that carried adult plant resistance in response to *Puccinia hordei* (pathotype 5457P+) from 2011 to 2013

Source	Name[a]	Pedigree	*Rph23*	*Rph20*	Field response[b]	APR genes
Australia	Buloke	Franklin/VB9104//VB9104	-	-	60-70 MSS	*Unknown APR*
Australia	Cask	SCRI-8313/Fleet//Regatta	-	-	50MS-80S	*Unknown APR*
Australia	Chieftan	Brittania/Prisma	-	-	10R/MR-40MS	*Unknown APR*
Australia	Fairview	Unknown	-	-	70-80MSS	*Unknown APR*
Australia	Galaxy	24719DB/Robin SIB	-	-	TR-5R/MR	*Unknown APR*
Australia	Galleon	Clipper/Hiproly//3*Proctor/ CI3576	-	-	20MR	*Unknown APR*
Australia	Grimmett	Bussel/Zepher	-	-	70S	*Unknown APR*
Australia	Hindmarsh	Dash/VB9409	-	-	30MRMS	*Unknown APR*
Australia	Molloy	Golden Promise/WI2395 (WARI2-38)/4/(72S:267) XBVT210/3/	-	-	50MS-70S	*Unknown APR*
Australia	Moondyne	Dampier//(A14) Prior/Ymer/3/ Kristina/	-	-	50MS-70MSS	*Unknown APR*
Australia	Mundah	O'Connor/Yagan	-	-	70S	*Unknown APR*
Australia	Navigator	Unknown	-	-	60-70MSS	*Unknown APR*
Australia	Tantangara	AB6/Skiff (AB6 is Hordeum spontaneum CPI71283/ 4*Clipper)	-	-	50MS-75S	*Unknown APR*
Australia	Tulla	Skiff/FM437	-	-	60S	*Unknown APR*
Australia	Ulandra	Warboys/Alpha	-	-	40MS-60MS	*Unknown APR*
Australia	Urambie	Yagan/Ulandra//Ulandra	-	-	30MRMS-60MRMS	*Unknown APR*
Australia	Wimmera	Unknown	-	-	70MSS	*Unknown APR*
Australia	**Beecher**	Atlas/Vaughan	+	-	70-80S	*Rph23*
Australia	**Dictator**	reselection of USDA accession CI2204	+	-	80S	*Rph23*
Australia	**Gairdner**	Onslow/Tas 83-587	+	-	-60-70MSS	*Rph23*
Australia	**Kaputar**	5604/1025/3/Emir/Shabet// CM67/4/F3 Bulk Hip	+	-	70MS-80S	*Rph23*
Australia	**Malebo**	Selection from CPI11083 (Palladium WWB 18)	+	-	70MSS	*Rph23*
Australia	**Onslow**	Forrest/Aapo	+	-	70MSS	*Rph23*
Australia	Yerong	M22/Malebo	+	-	40–50MRMS	*Rph23*+?
Australia	***Capstan***	Waveney/WI2875//Chariot/ Chebec	-	+	40MRMS-70S	*Rph20*
Australia	Cosmic	Unknown	-	+	TR-10RMR	*Rph20*+?
Australia	Dash	Chad/Joline//Cask	-	+	TR	*Rph20*+?
Australia	Fathom	Unknown	-	+	TR-20MR	*Rph20*+?
Australia	Finniss	Unknown	-	+	MR20	*Rph20*+?
Australia	***Flagship***	Chieftan/Barque//Manley/ VB9104	-	+	30MRMS	*Rph20*
Australia	Fleet	Mundah/Keel//Barque	-	+	10MR	*Rph20*+?
Australia	Flinders	Unknown	-	+	TR	*Rph20*+?
Australia	***Gilbert***	Reselection 0f Mx (Q21517)	-	+	20MR-50MS	*Rph20*
Australia	Grange	Unknown	-	+	TR	*Rph20*+?
Australia	Henley	Unknown	-	+	5MR-20MR	*Rph20*+?
Australia	Mackay	Cameo/Koru	-	+	TR-10MR	*Rph20*+?
Australia	Oxford	Unknown	-	+	10MR	*Rph20*+?

Table 1 (continued)

Source	Name[a]	Pedigree	*Rph23*	*Rph20*	Field response[b]	APR genes
Australia	Quasar	Chalice/NFC breeding line	-	+	TR-5R	*Rph20*+?
Australia	Quickstar	Unknown	-	+	5R-20MR	*Rph20*+?
Australia	Shepherd	Baronesse/Cheri	-	+	TR-20MRMS	*Rph20*+?
Australia	Starmalt	Unknown	-	+	TR-10MR	*Rph20*+?
Australia	Vertess	Franklin/Cooper	-	+	TR-5R	*Rph20*+?
Australia	Westminster	Unknown	-	+	TR	*Rph20*+?
Australia	***Macquarie***	Unknown	+	+	TR	*Rph20*+*Rph23*
Australian	Franklin	Shannon/Triumph	-	-	70MS-80S	*Unknown APR*
AWCCDiverse	Abacus	Vada/Zephyr	-	-	TR-10MR	*Unknown APR*
AWCCDiverse	Arrow	Lignee 39/Vada//Emir/Zephyr	-	-	5R-TR	*Unknown APR*
AWCCDiverse	Atem	L 92/Minerva//Emir/3/Zephyr	-	-	20MRMS-30MS	*Unknown APR*
AWCCDiverse	Egmont	Maris Yak/W 1001//Vada	-	-	15MRMS-20MS	*Unknown APR*
AWCCDiverse	Golf	Armelle/Lud//Luke	-	-	10MR-40MSS	*Unknown APR*
AWCCDiverse	Hart	Egmont/Atem	-	-	10MR-20MRMS	*Unknown APR*
AWCCDiverse	Iban	Aramir//LW 64192/Zephyr/ Sultan	-	-	5MR-20MRMS	*Unknown APR*
AWCCDiverse	Klimek	Klimek BC RphCantala Unknown	-	-	10MR-40MS	*Unknown APR*
AWCCDiverse	Minerva	Minerva 401434 Nil H. laevigatum/Gull	-	-	TR-15MR	*Unknown APR*
AWCCDiverse	Optic	Optic BC Rph12 Chad// Corniche/Force	-	-	30MS-40MSS	*Unknown APR*
AWCCDiverse	Simba	Simba 401956 Nil Herta/ BYG 191//Minerva	-	-	TR-10MR	*Unknown APR*
AWCCDiverse	Toddy	Toddy 407579 Rph12 Optic/ Chariot	-	-	TR-10MR	*Unknown APR*
AWCCDiverse	Universe	Universe 402169 Nil Abed 3371/Vada	-	-	10MR-40MS	*Unknown APR*
AWCCDiverse	Uta	Uta 402175 Nil Emir/Quantum	-	-	TR-20MSS	*Unknown APR*
AWCCDiverse	Aramir	Volla/Emir	-	+	TR-5R	*Rph20*+?
AWCCDiverse	Baronesse	Mentor/Minerva//Vada mutant/ 4/Carlsberg/Union//	-	+	TR-10MR	*Rph20*+?
AWCCDiverse	***Barque***	Opavsky/Salle/3/Ricardo/5/ Oriol/6153P0	-	+	20MR-40MRMS	*Rph20*
AWCCDiverse	Chariot	Dera//Carnival/Atem	-	+	TR	*Rph20*+?
AWCCDiverse	Cornel	Volla//Emir/Cebeco 6010	-	+	TR-10MR	*Rph20*+?
AWCCDiverse	Corniche	Diamant/14029/64//Emir/HOR 3270/46132/68	-	+	0-TR	*Rph20*+?
AWCCDiverse	Cygnet	Target/Patty	-	+	TR-20MR	*Rph20*+?
AWCCDiverse	Derkado	Lada/Salome	-	+	0-TR	*Rph20*+?
AWCCDiverse	Draught	Platoon/Chariot	-	+	TR-5MR	*Rph20*+?
AWCCDiverse	Emir	Delta//Agio/Kenia/2/Arabian Variety	-	+	TR	*Rph20*+?
AWCCDiverse	Georgie	Vada/Zephyr	-	+	TR	*Rph20*+?
AWCCDiverse	Javelin	Athos/Trumpf	-	+	TR	*Rph20*+?
AWCCDiverse	Lada	Lada 404731 Rph12 St 49619/ 68//	-	+	TR-10MR	*Rph20*+?
AWCCDiverse	Landlord	Landlord 407578 Rph12 Platoon/NFC86/60//Chariot	-	+	TR-5R	*Rph20*+?
AWCCDiverse	Miranda	Miranda 402838 Nil Volla/Vada	-	+	10MR-20MR	*Rph20*+?
AWCCDiverse	Pompadour	Pompadour BC Nil FDO192/ Patty	-	+	TR	*Rph20*+?

Table 1 (continued)

Source	Name[a]	Pedigree	*Rph23*	*Rph20*	Field response[b]	APR genes
AWCCDiverse	Porthos	Porthos 401762 Nil 207/Emir	-	+	TR-10MR	*Rph20*+?
AWCCDiverse	RAH-1995	RAH-1995 BC Nil Unknown	-	+	TR-10MR	*Rph20*+?
AWCCDiverse	Ramona	Ramona 401814 Nil Cambrinus/Emir	-	+	TR	*Rph20*+?
AWCCDiverse	Tintern	Tintern 402083 Rph4 Sebarlis/ Zephyr/2/Emir//Zephyr	-	+	TR	*Rph20*+?
AWCCDiverse	Trinity	Trinity 407398 Nil Platoon/ Chariot	-	+	TR-10MR	*Rph20*+?
AWCCDiverse	Tweed	Tweed 403017 Nil Akka/ Maris Mink//Maris Mink	-	+	TR	*Rph20*+?
AWCCDiverse	WI 3407	Unknown	-	+	TR	*Rph20*+?
Chinese	Aiganqi	Unknown	-	-	40MS-60MS	*Unknown APR*
Chinese	Fumai 8	76-22///Zaoshu 3//Humai 1/ 8-2	-	-	40MS-60MSS	*Unknown APR*
Chinese	Yan90260	Unknown	-	-	20MRMS-50MS	*Unknown APR*
Chinese	Zhoungdamei	Chinese Landrace	-	-	20MR-40MS	*Unknown APR*
Chinese	ZUG161	Zhejiang University	-	-	30MRMS-40MRMS	*Unknown APR*
Chinese	ZUG25	Zhejiang University	-	-	30MRMS-40MRMS	*Unknown APR*
Chinese	ZUG31	Zhejiang University	-	-	20MRMS-30MRMS	*Unknown APR*
Chinese	Aiganqi 3	Unknown	+	-	20MS-50MS	*Rph23*+?
Chinese	CI 9422	Unknown	+	-	20MR-40MRMS	*Rph23*+?
Chinese	ZUG9	Zhejiang University	miss	-	50MRMS-60MRMS	*Unknown APR*
Germany	Belana (b)	Unknown	-	-	TR-5MR	*Unknown APR*
Germany	Extract	Cask/Chariot//Amber	-	-	10MR-20MR	*Unknown APR*
Germany	Fergie	Athos/Hood//Marion/ Goldmarker	-	-	20MR-40MS	*Unknown APR*
Germany	Halla	Unknown	-	-	20MRMS-40MSS	*Unknown APR*
Germany	Luna	Tanja//Weihenstephan-1937/ W-8-89	-	-	10MRMS-40MRMS	*Unknown APR*
Germany	Marthe	Unknown	-	-	TR-20MR	*Unknown APR*
Germany	Aura	Wisa/Carlsberg//Breuns-212-A-20/3/Villa	-	+	TR-5MR	*Rph20*+?
Germany	Auriga	Viskosa/Krona//Annabell	-	+	TR-20MR	*Rph20*+?
Germany	Belana (a)	Unknown	-	+	TR-10MR	*Rph20*+?
Germany	Cellar	NFC-94-20/Cork//NFC-94-11	-	+	5MR-10MR	*Rph20*+?
Germany	Ditta	Apex/76-1754-6	-	+	TR-5RMR	*Rph20*+?
Germany	Eunova	Unknown	-	+	0-1R	*Rph20*+?
Germany	Line15	(BR.3546	-	+	TR-20MR	*Rph20*+?
Germany	Line8	STNG 1970 x Manon x Brenda	-	+	TR	*Rph20*+?
Germany	Line9	BRGD 1977 x EBS 1601 x Scarlett	-	+	TR-20MR	*Rph20*+?
Germany	Margret	Unknown	-	+	TR-30MRMS	*Rph20*+?
Germany	Union	Unknown	-	+	TR-5RMR	*Rph20*+?
Germany	***Lenka***	HVS-5013-74/Q-496-72	+	+	TR-15MR	*Rph20+Rph23*
Germany	***Line17***	(Steffi x 11258 W) x (Krona x Chariot) x ACK 1916	+	+	5MR-20MR	*Rph20+Rph23*
Germany	***Volla***	Wisa/Haisa-I	+	+	0-1R	*Rph20+Rph23*
Spanish	BNG7944	Unknown	-	-	10MR	*Unknown APR*

Table 1 (continued)

Source	Name[a]	Pedigree	*Rph23*	*Rph20*	Field response[b]	APR genes
Spanish	BNG9104	Unknown	-	-	10MR	*Unknown APR*
Spanish	BNG9143	Unknown	-	-	TR	*Unknown APR*
Spanish	BNG9334	Unknown	-	-	10MR	*Unknown APR*
Spanish	BNG9362	Unknown	-	-	TR	*Unknown APR*
Spanish	BNG11379	Unknown	-	-	20MR	*Unknown APR*
Spanish	BNG19826	Unknown	-	-	20MR	*Unknown APR*
Spanish	BNG7898	Unknown	-	-	20MR	*Unknown APR*
Spanish	BNG7978	Unknown	-	-	TR	*Unknown APR*
Spanish	Beka	Unknown	-	-	10MR	*Unknown APR*
Spanish	BNG8778	Unknown	-	-	10MR	*Unknown APR*
Spanish	BNG9309	Unknown	-	-	TR	*Unknown APR*
Spanish	Monlón	Unknown	-	-	10MR	*Unknown APR*
Spanish	Albacete	Unknown	-	-	20MR	*Unknown APR*
Spanish	BNG7608	Unknown	-	-	TR	*Unknown APR*
Spanish	BNG7631	Unknown	-	-	20MR	*Unknown APR*
Spanish	BNG9130	Unknown	-	-	5MR	*Unknown APR*
Spanish	BNG9311	Unknown	-	-	TR	*Unknown APR*
Spanish	BNG9322	Unknown	-	-	10-20MR	*Unknown APR*
Spanish	BNG8929	Unknown	-	-	10-20MR	*Unknown APR*
Spanish	BNG9178	Unknown	-	-	10MR	*Unknown APR*
Spanish	BNG11216	Unknown	-	-	TR	*Unknown APR*
Spanish	BNG9355	Unknown	-	-	10MR	*Unknown APR*
Spanish	BNG9266	Unknown	-	-	50MSS	*Unknown APR*
Spanish	BNG7840	Unknown	-	-	40MRMS	*Unknown APR*
Spanish	BNG9279	Unknown	-	-	70MSS	*Unknown APR*
Spanish	BNG26979	Unknown	-	-	60MSS	*Unknown APR*
Spanish	BNG7492	Unknown	-	-	30-40MRMS	*Unknown APR*
Spanish	BNG7888	Unknown	-	-	40MRMS	*Unknown APR*
Spanish	BNG8805	Unknown	-	-	60MSS	*Unknown APR*
Spanish	BNG9182	Unknown	-	-	60MSS	*Unknown APR*
Spanish	BNG11152	Unknown	-	-	40MRMS	*Unknown APR*
Spanish	BNG11306	Unknown	-	-	40MRMS	*Unknown APR*
Spanish	BNG7835	Unknown	-	-	40MRMS	*Unknown APR*
Spanish	BNG7839	Unknown	-	-	40-50MRMS	*Unknown APR*
Spanish	BNG9058	Unknown	-	-	30MRMS	*Unknown APR*
Spanish	BNG7894	Unknown	+	-	20MR	*Rph23*+?
Spanish	BNG9276	Unknown	+	-	TR	*Rph23*+?
Spanish	BNG7516	Unknown	+	-	TR	*Rph23*+?
Spanish	BNG7551	Unknown	+	-	10MR	*Rph23*+?
Spanish	BNG8030	Unknown	+	-	10MR	*Rph23*+?
Spanish	BNG19828	Unknown	+	-	10-20MR	*Rph23*+?
Spanish	Hatif de Grignon	Unknown	+	-	10MR	*Rph23*+?
Spanish	BNG9077	Unknown	+	-	10MR	*Rph23*+?
Uruguay	620	Unknown	-	-	10MR-15MR	*Unknown APR*
Uruguay	621	Unknown	-	-	50MS-70MS	*Unknown APR*
Uruguay	623	Unknown	-	-	10MR-20MR	*Unknown APR*

Table 1 (continued)

Source	Name[a]	Pedigree	*Rph23*	*Rph20*	Field response[b]	APR genes
Uruguay	624	Unknown	-	-	10MR-20MR	*Unknown APR*
Uruguay	626	Unknown	-	+	10MR-20MR	*Rph20*+?
Uruguay	ISR 912.1	Unknown	-	+	TR-10MR	*Rph20*+?
Uruguay	ISR 912.2	Unknown	-	+	10MR-20MR	*Rph20*+?
Uruguay	ISR 912.6	Unknown	-	+	10MR-20MR	*Rph20*+?
Uruguay	ISR 912.9	Unknown	-	+	10MR-20MRMS	*Rph20*+?
Uruguay	***ISR 912.3***	Unknown	-	+	30MRMS-40MRMS	*Rph20*
UWA Diverse	Caravela	Ribeka/Union	-	-	40MS-50MSS	*Unknown APR*
UWA Diverse	CPI 36396 A	Unknown	-	-	20MS-30MS	*Unknown APR*
UWA Diverse	Farmington	WA-7190-86/Maresi	-	-	30MS-40MS	*Unknown APR*
UWA Diverse	GSHO 1436	Unknown	-	-	10-20MR	*Unknown APR*
UWA Diverse	HOR 2410	Unknown	-	-	40MS-60MSS	*Unknown APR*
UWA Diverse	HOR 9696	Unknown	-	-	40MS-50MS	*Unknown APR*
UWA Diverse	ICB94-0705-AP-4AP	Unknown	-	-	40MS-50MS	*Unknown APR*
UWA Diverse	LAURA	Pallas/Herta	-	-	30MS-40MSS	*Unknown APR*
UWA Diverse	ORGE 403-9	Unknown	-	-	30MS-40MSS	*Unknown APR*
UWA Diverse	SB02420	TR251/TR970	-	-	20MS-40MS	*Unknown APR*
UWA Diverse	TENNESSEE WINTER COAST SELN.	Unknown	-	-	10MR-20MR	*Unknown APR*
UWA Diverse	TR03273	TR251/TR253	-	-	40MS-50MSS	*Unknown APR*
UWA Diverse	TR03274	TR253/BM9216-4	-	-	30MRMS-50MSS	*Unknown APR*
UWA Diverse	UWA late seln4887	Selection from 91HBSN 4	-	-	30MS-40MS	*Unknown APR*
UWA Diverse	UWA lg late 6R seln8951	Selection from CI5791/Ca1607//Shyri/5	-	-	30MS-40MS	*Unknown APR*
UWA Diverse	UWA short awned husked seln4886	Selection from 91NBGP 50	-	-	30MRMS-40MS	*Unknown APR*
UWA Diverse	Andapi 3	Unknown	+	-	TR-10MR	*Rph23*+?
UWA Diverse	GLACIER / TITAN	Glacier/Titan	+	-	10MR-30MRMS	*Rph23*+?
UWA Diverse	GSHO 1452	Unknown	+	-	10-20MR	*Rph23*+?
UWA Diverse	ICB82-0114-6AP-0AP	Unknown	+	-	30MS-40MSS	*Rph23*+?
UWA Diverse	UWA bright small seed late seln8939	Selection from SLB 66-050	+	-	30MRMS-40MS	*Rph23*+?
UWA Diverse	UWA late husked seln4900	Selection from 91HBSN 4	+	-	40MS-50MSS	*Rph23*+?
UWA Diverse	**UWA late seln3849**	Selection from WA3849	+	-	40MS-60MSS	*Rph23*
UWA Diverse	YALE	Unknown	+	-	10MR-20MR	*Rph23*+?
UWA Diverse	74043	Kenia/Erectoides 16	-	+	TR	*Rph20*+?
UWA Diverse	115-9505-B	Unknown	-	+	TR-10MR	*Rph20*+?
UWA Diverse	Astoria	Nevada/STA 13817	-	+	TR-10MR	*Rph20*+?
UWA Diverse	Brenda	Nebi/11827-80//Gimpel	-	+	10MR-20MR	*Rph20*+?
UWA Diverse	***Chalice***	Cooper/NFC514-5/2/Chariot	-	+	20MS-40MSS	*Rph20*
UWA Diverse	Corvette	Bonus/CI 3576	-	+	TR	*Rph20*+?
UWA Diverse	Decanter	Heron/Dallas	-	+	TR-5R	*Rph20*+?
UWA Diverse	Esperance	(S) LV-Northern-Africa	-	+	5MR-10MR	*Rph20*+?
UWA Diverse	Expres	SK 3455/Akcent	-	+	TR-5R	*Rph20*+?
UWA Diverse	Giza 127	Unknown	-	+	TR	*Rph20*+?
UWA Diverse	Giza 128	Unknown	-	+	TR	*Rph20*+?
UWA Diverse	Harriot	Hanka/Nordus//Annabell	-	+	10MR-20MR	*Rph20*+?

Table 1 (continued)

Source	Name[a]	Pedigree	*Rph23*	*Rph20*	Field response[b]	APR genes
UWA Diverse	Hassan	[Arabische/Kenia)///Agio]/Delta	-	+	TR-5MR	*Rph20*+?
UWA Diverse	ICB83-0157-10AP-0TR-0AP-7AP-1APH-0AP	Unknown	-	+	TR-5MR	*Rph20*+?
UWA Diverse	***M-Q-54***	Unknown	-	+	20MS-30MS	*Rph20*
UWA Diverse	Nord GS1749	Unknown	-	+	TR-20MRMS	*Rph20*+?
UWA Diverse	Nordus	845/Krona	-	+	TR	*Rph20*+?
UWA Diverse	***Olbran***	Unknown	-	+	30MS-40MSS	*Rph20*
UWA Diverse	Pewter	NFC94-20/NFC94-11	-	+	TR-5MR	*Rph20*+?
UWA Diverse	SB01675	4176 N/cdc mCgWIRE	-	+	TR-10MRMS	*Rph20*+?
UWA Diverse	SE627.02	Unknown	-	+	0-TR	*Rph20*+?
UWA Diverse	Ursa	Thuringa/Hanka//Annabell	-	+	TR-10MR	*Rph20*+?
UWA Diverse	***UWA late seln8861***	Selection from Tipper//WI-2291/WI-2269	-	+	20MS-30MS	*Rph20*
Australia	Gus	Unknown	-	-	90-100S	*Nil*

[a] Names of the barley genotypes that are likely to be single gene lines for *Rph23* (*bold*), *Rph20* (*italics*/*bold*) and *Rph20* and *Rph23* in combination (*bold*/*italics*/*underlined*)

[b] A modified Cobb scale (Peterson et al. 1948) was used in the field to assess disease severity (percentage leaf area affected). Host response (*R*, no uredinia present; *TR*, trace or minute uredinia on leaves without sporulation; *MR*, small uredinia with slight sporulation; *MR-MS*, small-to-medium-sized uredinia with moderate sporulation; *MS-S*, medium-sized uredinia with moderate to heavy sporulation; *S*, large uredinia with abundant sporulation, uredinia often coalesced to form lesions) was assessed using scale outlined by Roelfs et al. (1992)

most (70 %) Spanish lines tested were also TR-20MR, but there was no evidence of *Rph20* in any of these lines. *Ebmac0603* was present in eight lines (18 %), all of which were TR-20MR. These lines, in addition to most

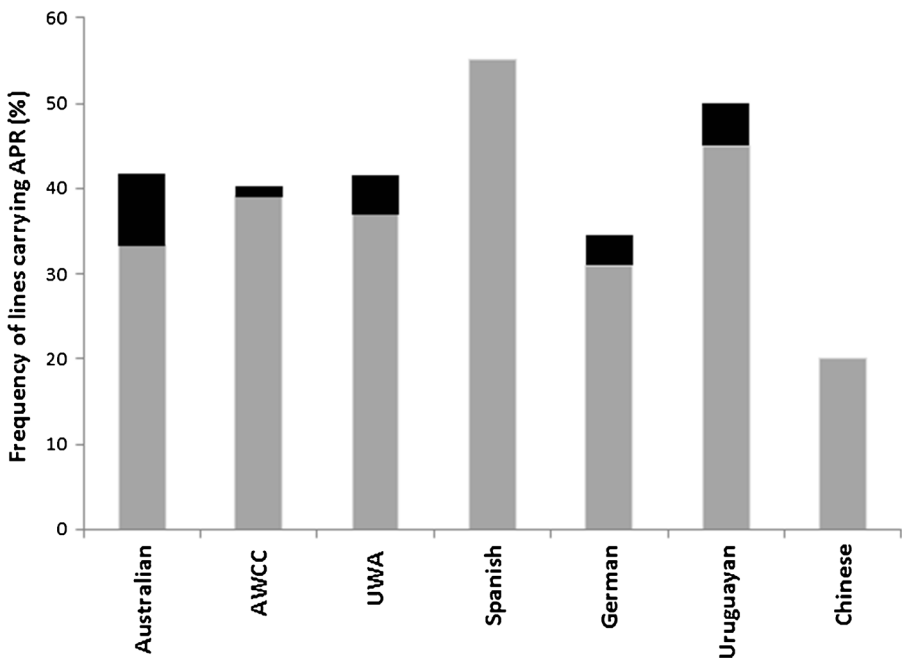

Fig. 1 Graphical representation of the frequency of lines across all seven International germplasm collections that carried APR from both known (*black*) and uncharacterised (*grey*) sources when assessed across three separate field seasons with the *P. hordei* pathotype 5457P+for APR and screened with markers *bPb-0837* and *Ebmac0603* that are closely linked to *Rph20* and *Rph23* respectively

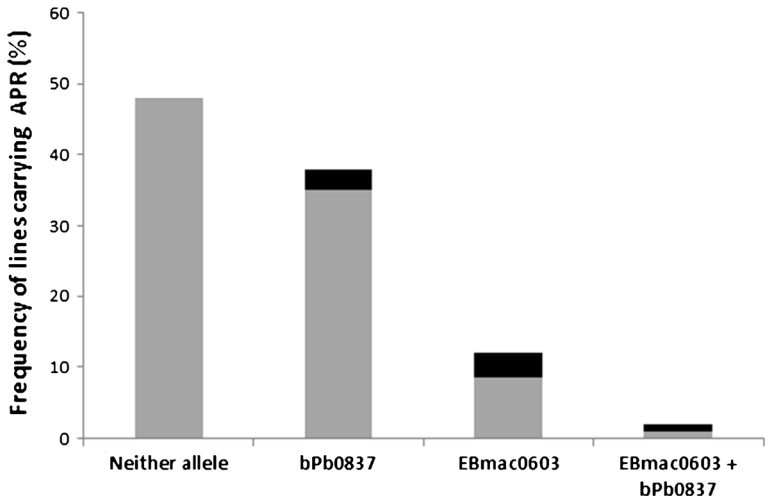

Fig. 2 Graphical representation of the frequency of 213 APR lines within specific germplasm collections derived from Australia, AWCC, China, UWA, Germany, Spain and Uruguay that carry varying levels of APR [low-MS-S (*grey*), medium-MRMS (*light grey*) and high-TR-20MR (*black*)]

lines in the Spanish collection, contain at least one or more uncharacterized APR genes (Table 1, Fig. 3).

Lines from two separate and geographically diverse barley-growing locations (China and Uruguay), all carried varying levels of APR. Uruguayan lines had high levels of APR, which correlated strongly with the presence of *bPb-0837*. The *bPb-0837* allele was not found in any of the lines originating from China, and the Chinese collection had no lines with high APR. *Ebmac0603* was present at relatively high frequencies within the Chinese germplasm and absent from Uruguayan lines (Table 1, Fig. 3).

Discussion

The objective of this study was to characterize and provide a comprehensive understanding of the diversity

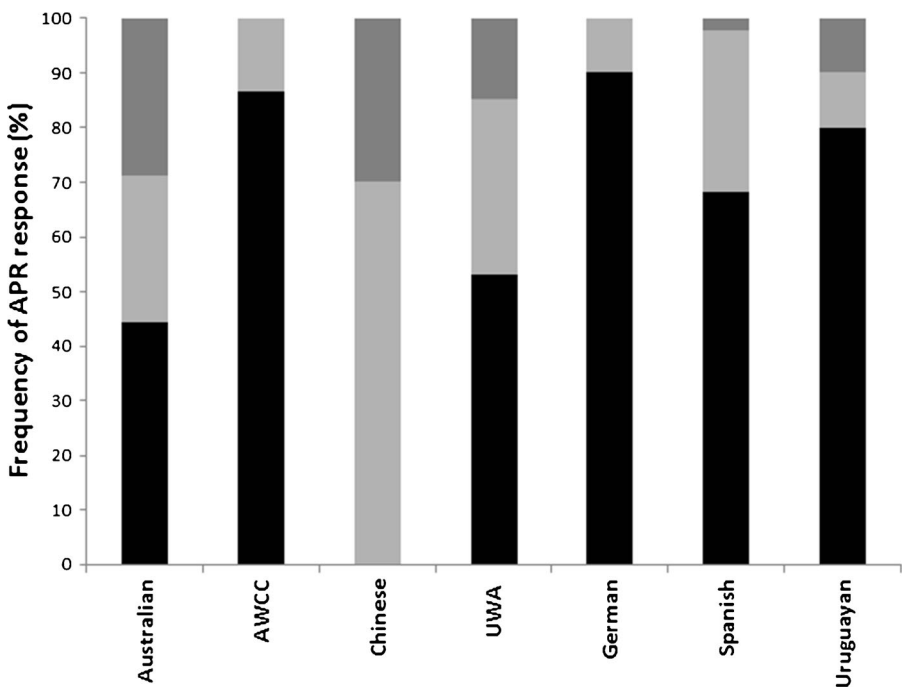

Fig. 3 Graphical representation of the frequency of 213 APR lines that carried neither *bPb-0837* or *Ebmac0603* alleles, *bPb-0837* allele only, *Ebmac0603* allele only or both *bPb-0837* and *Ebmac0603* alleles. Shading denotes the proportion of lines that are postulated to carry only known APR (*Rph20* or *Rph23*) (*black*) and unknown APR (*grey*)

of APR within international germplasm collections from the major barley regions of the world. We assessed 521 seedling susceptible barley genotypes derived from elite breeding material, landraces, and germplasm collections for APR over multiple field seasons using a highly virulent pathotype of *P. hordei* (5457P+) . Those lines (213) that carried APR were subsequently screened for the presence of known genes (*Rph20* and *Rph23*) based on marker assays. This marker data and disease rating for each line was then compared to known field infection types expected for the presence of *Rph20* and *Rph23* alone to postulate the likely presence of known and uncharacterised APR. Varying levels of APR were found based on 3 years of phenotypic data from field tests, with any differences in disease severity across years being attributed to environmental factors and /or growth stages at the time of disease scoring. Previous studies on the expression of APR to leaf rust in both wheat (*Lr34*) and barley (*Rph20*) suggest that these genes are most effective at cooler temperatures (Singh et al. 1998, 2013). This could also account for variation of APR observed over different growing seasons, especially within Australian lines in this study.

The APR response within international germplasm collections was associated with the presence of *Rph20*, and to a lesser extent to the presence of *Rph23*. Lines that were positive for *bPb-0837* almost always had high levels of APR. Lines that carried *Rph23* as determined by marker *EBmac0603*, showed greater variation in their APR response, which can be explained by the MS to S field reaction contributed by this gene. Previous reports found *Rph20* provided moderate protection (20MR - 40MS) and *Rph23* provided low (60MS - 70MS) protection, respectively (Derevnina et al. 2013; Sandhu 2011; Golegaonkar et al. 2009; Singh et al. 2014 *in press*). The presence of *Ebmac0603* within Chinese and especially Australian lines was correlated with low APR in the field across multiple test years. Lines with low levels of APR (60-70MSS) that were positive for the *Rph23* marker allele may represent single gene lines that can be used for further genetic analysis and selection of *Rph23* for varietal improvement. Recent genetic studies of *Rph23* from the Australian barley cultivar Yerong suggests that it is a highly additive gene (Singh et al. 2014 *in press*). Future characterization and molecular marker development for newly identified additive APR sources will enable breeders to pyramid such resistance.

The additively of uncharacterised APR genes with either *Rph20* or *Rph23* resulted in lines that showed TR-20MR responses in the field. Additionally, there were many lines showing high APR in this study that lack the markers linked to *Rph20* and *Rph23*. These lines likely carry other uncharacterized APR genes that may provide more protection in the field than either *Rph20* or *Rph23*, as none carried any seedling resistance that is effective against the *P. hordei* 5457P+pathotype. Further genetic analysis and characterization of such uncharacterised APR is required for marker development and varietal improvement.

There was no relationship between geographic location and the presence of known APR genes. However, in some cases the presence of particular APR and ASR genes appeared to be associated with human-mediated movement of germplasm. The *Rph20* marker *bPb-0837* was present at almost equal frequencies within collections from Uruguay and Germany. Interestingly, previous studies determined that lines from both these countries carried the ASR gene *Rph3*, and none of the lines carried *Rph23*. Historical evidence suggests that two German scientists established the first barley breeding program in Uruguay (Newman and Newman 2008). It is possible that some of the lines derived from both collections may share the same or similar pedigrees, but there was no information available on pedigrees derived from Uruguay. Additionally, *bPb-0837* was not present within any of the Spanish barley lines, which is somewhat unusual given the previously reported European origin of this APR gene (Hickey et al. 2011, 2012). One likely explanation is that the Spanish lines tested in this study were all landraces, which may pre-date intercrossing of cultivated barley with *Hordeum laevigatum* reported to be the original source of *Rph20*, in cultivated barley (Hickey et al. 2012).

It was not possible to pinpoint the ancestry of all *Rph23*-positive lines detected in this study because apart from the Australian lines investigated, pedigree information is not available. The pedigrees of Australian lines that were positive for *Ebmac0603* were highly related. Singh et al. (2014) *in press* predicted variety Lion (C.I. 923; a selection from black six-rowed barley from Taganrog) as a likely progenitor of *Rph23* in Australian barleys including lines that were positive for *Ebmac0603* in this study. Lion was introduced into Australian barley germplasm via Forrest through Onslow. Forrest (C.I. 9187) is a North America barley released in the 1940s from the original cross Newal/Peatland. Lion and Forrest are genetically related through Newal. The Australian variety Beecher

(positive for *Ebmac0603*) and one of the parents (Glacier) of the North American line Glacier/Titan (a sib of variety Unitan; also positive for *Rph23* marker) are both derived from the same cross, Atlas/Vaughn. Vaughn was used widely in North American breeding programs for high yield. Interestingly, Vaughn is derived from the cross Lion/Club Mariout, which further suggests that Lion is the likely progenitor of *Rph23* in Australian and North American barleys.

In summary, here we report on APR gene postulation and the discovery of numerous new APR sources within seven international germplasm collections based on screening with markers closely linked to known APR genes *Rph20* and *Rph23*. Markers *bPb0837* and *Ebmac0603* were highly diagnostic for the presence of *Rph20* and *Rph23* and illustrates their suitability for MAS. This study also identified a large diversity in APR response and the likely combination of both known and uncharacterised APR genes within germplasm collections that can be further implemented within barley breeding programs. Further research is required to identify single gene donors of each uncharacterised APR source for trait dissection and marker development to pyramid multiple APR sources into barley varieties to achieve durable resistance. Further characterization of unknown APR genes and marker development will be important in future efforts to develop cultivars with durable resistance to *P. hordei*.

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