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ORIGINAL PAPER

Mapping *Rph20*: a gene conferring adult plant resistance to *Puccinia hordei* in barley

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Abstract A doubled haploid (DH) barley (*Hordeum vulgare* L.) population of 334 lines (ND24260 × Flagship) genotyped with DArT markers was used to map genes for adult plant resistance (APR) to leaf rust (*Puccinia hordei* Otth) under field conditions in Australia and Uruguay. The Australian barley cultivar Flagship carries an APR gene (*qRphFlag*) derived from the cultivar Vada. Association analysis and composite interval mapping identified two genes conferring APR in this DH population. *qRphFlag* was mapped to the short arm of chromosome 5H (5HS), accounting for 64–85% of the phenotypic variation across four field environments and 56% under controlled environmental conditions (CEC). A second quantitative trait locus (QTL) from ND24260 (*qRphND*) with smaller effect was mapped to chromosome 6HL. In the absence of *qRphFlag*, *qRphND* conferred only a low level of resistance. DH lines displaying the highest level of APR carried both genes. Sequence information for the critical DArT marker bPb-0837 (positioned at 21.2 cM on chromosome 5HS) was used to develop *bPb-0837-PCR*, a simple PCR-based marker for *qRphFlag*. The 245 bp fragment for *bPb-0837-PCR* was detected in a range of barley cultivars known to possess APR, which was consistent with previous tests of allelism, demonstrating that the *qRphFlag* resistant allele is common in leaf rust resistant cultivars derived from Vada and Emir. *qRphFlag* has been designated *Rph20*, the first gene conferring APR to *P. hordei* to be characterised in barley. The PCR marker will likely be effective in marker-assisted selection for *Rph20*.

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Introduction

The pathogen *Puccinia hordei* causes leaf rust in barley crops around the world, including Australasia, Europe, North America and South America (Clifford 1985; Golegaonkar et al. 2009). The disease has been commonly reported to cause yield losses up to 32% in Australia and North America (Park and Karakousis 2002) and as high as 62% in very susceptible cultivars (Cotterill et al. 1992; Castro et al. 2006).

Sustainable control of rust pathogens is possible through the use and manipulation of resistance genes (Hong and Singh 1996). Barley breeders around the world have relied

primarily on seedling resistance genes for protection against *P. hordei*. Whilst some of the characterised seedling genes conferring resistance to *P. hordei* (i.e. *Rph1–19*) have been exploited in developing new barley cultivars, adult plant resistance (APR) has not to date been intentionally deployed to control leaf rust. Studies of rust resistance in wheat have shown that APR usually involves the additive and/or epistatic effects of multiple genes (partial resistance) and so is thought to be a more durable form of resistance than seedling (major) gene resistance, which is often pathotype specific and may be overcome by mutations within the pathogen population (McIntosh 1992; Hong and Singh 1996; Lagudah et al. 2006; Pretorius et al. 2007). However, there are some major genes for APR to leaf rust in wheat (*Lr12*, *Lr13*, *Lr22a* and *Lr37*) that are race specific. Earlier work conducted by Parlevliet (1977) suggested that genes conveying partial resistance or slow rusting to *P. hordei* exist in barley. In the current study, this type of resistance is referred to as APR.

Golegaonkar et al. (2009) identified 57 barley genotypes that carry uncharacterised APR genes to *P. hordei* from a broad range of germplasm, some of which have been reported by Cotterill and Rees (1993) to display partial resistance in Australian environments. Allelism tests performed by Golegaonkar et al. (2010) demonstrated that a number of these barley genotypes share a common gene for APR and preliminary mapping located the gene on chromosome 5HS. This location was confirmed by Liu et al. (2010). The gene provides variable levels of APR across a wide range of germplasm and is thought to be derived from Vada, a barley cultivar from the Netherlands released in the 1950s (Dros 1957). Additional genes with smaller effects were also detected in some populations under some environments (Qi et al. 1998; Golegaonkar et al. 2010).

Selection for APR is often conducted using specialised disease nurseries or opportunistic observations from field trials. The accuracy of selection can be compromised by environmental factors that influence the expression of APR, such as weather patterns, inoculum pressure and sequential infections, differences in plant maturity and the presence of other diseases. For this reason, molecular markers closely linked to the APR genes are a highly desirable means of improving the reliability of selection in breeding populations. Diagnostic molecular markers for some APR genes in wheat (*Triticum aestivum*) have been developed, for instance *Lr34/Yr18* (Lagudah et al. 2006) and *Lr46/Yr29* (personal communication Dr. Evans Lagudah), which provide valuable selection tools in wheat breeding programs.

The current study is designed to characterise genetic components and map genes for APR to *P. hordei* through assessment of a doubled haploid (DH) population (ND24260 × Flagship) genotyped with DArT (Diversity Array Technology Pty Inc.) molecular markers. Flagship is an Australian barley cultivar with APR to *P. hordei* and is believed to carry an APR gene inherited from Vada. Single-marker association analysis and composite interval mapping (CIM) were employed to estimate the position of this QTL using data collected across five environments in Australia and Uruguay. Published sequence information for the DArT markers was then used to develop a PCR-based marker for the APR gene on chromosome 5HS, referred to as *qRphFlag*. This study also validates assessment of APR to *P. hordei* using multiple disease assessments within and across trials. Accelerated plant development under controlled environmental conditions (CEC) was investigated as an alternative to field screening for APR.

Materials and methods

Plant material

A DH population consisting of 334 lines was derived from the cross ND24260 × Flagship using the F_1 anther culture method performed by the Cereal Doubled Haploid Program, at the Department of Agriculture and Food, Western Australia. ND24260 (ND19869-1//ND17274/ND19119) is an advanced breeding line from the barley breeding programme at North Dakota State University, Fargo, ND, USA, which is susceptible to Australian pathotypes of *P. hordei* at the seedling stage (R.F. Park, unpublished). However, ND24260 displays low levels of resistance (i.e. susceptibility) at the adult plant stage in the field. Flagship (Chieftain/Barque//Manley/VB9104) is an Australian barley cultivar selected from a doubled haploid population as having APR to *P. hordei*. The ND24260 × Flagship DH population was developed to combine desirable traits from Australian and North Dakota germplasm. Neither Flagship nor ND24260 carry any known seedling resistance genes for *P. hordei* (R.F. Park, unpublished). Flagship is a sister line of WI3407, an advanced breeding line reported by Golegaonkar et al. (2010), to carry an APR gene that is also present in the cultivar Vada. Therefore, it is highly likely that Flagship also carries this gene.

Pathogen material

The DH population was screened in Australia with *P. hordei* pathotypes 5453P+ (virulent for *Rph1,2,4,6,9,10,12,19*) and 5457P+ (virulent for *Rph1,2,3,4,6,9,10,12,19*). Pathotypes were isolated from single pustules on a susceptible breeding line. Pathotype 5453P+ was used in a nursery grown at the Leslie Research Centre (LRC), Toowoomba, Queensland in 2009 and in screening trials grown under CEC at St Lucia (Brisbane, QLD, Australia) in 2010,

whereas the pathotype 5457P+ was used in disease screening nurseries grown in 2010 in Toowoomba and Cobbitty, New South Wales. In Uruguay, UPh3 (Uruguay, *P. hordei*, number 3; virulent for *Rph1,2,3,4,6,9,10,11,12,16*) was used, or was naturally present, in field assessment of the DH population at La Estanzuela (Colonia, Uruguay) in 2010. UPh3 was initially isolated from a single pustule and increased on the susceptible cultivar Morex for this study.

Assessment for adult plant resistance in the field

The DH population was assessed for APR to *P. hordei* across four field environments over a 2-year period: Toowoomba (Queensland, Australia) in 2009 and 2010, Cobbitty (New South Wales, Australia) in 2010 and three separate nurseries at La Estanzuela (Colonia, Uruguay) in 2010. DH lines along with disease standards were sown as hill plots (15–20 seeds) or 0.5-m rows (La Estanzuela only), according to a partially replicated (p-rep) experimental design (Cullis et al. 2006). Two ranges of hill plots were sown between rows of the susceptible cultivar Gus (Toowoomba 2009, 2010 and Cobbitty 2010) or a mixture of susceptible cultivars Morex, Bowman and INIA Ceibo (one nursery at La Estanzuela 2010), to ensure even coverage of the disease throughout the trials. Replication of DH lines ranged between 70 and 90%. Each design provided two blocks for replicates; for instance at Toowoomba in 2009 the replicates were split by range with the first replicate sown in ranges 1–2 and the second replicate in ranges 3–4. The p-rep design included an underlying component for autocorrelation in the range and plot direction and was Latinised along ranges. An exception to these experimental designs was the experiment conducted at Cobbitty, where the DH population was sown as unreplicated hill plots (10–15 seeds) in genotype order (i.e. DH number). This number is random and not associated with population structure. Parental lines (ND24260 and Flagship) were replicated three times each.

Inoculation of spreader rows was performed using the injection method, where emerging leaves were injected with a water suspension of urediniospores (Toowoomba 2009 and 2010) or sprayed with a mineral oil suspension (one nursery at La Estanzuela 2010 and Cobbity 2010). Initial assessment of APR to *P. hordei* was performed at anthesis using a 0–9 scale (McNeal et al. 1971), where 0 = immune and 9 = very susceptible. Multiple assessments were made at Toowoomba in 2009, to investigate gene effects as the disease incidence progressed during the season, i.e. under low, moderate and high disease pressure. Similarly, different levels of disease pressure were present in three separate field screening nurseries at La Estanzuela in 2010 (Table 1). These three nurseries were assessed over a 2-day period.

Phenotyping adult plant resistance under controlled environment

Before screening the DH population for APR to *P. hordei* under CEC, preliminary characterisation experiments were conducted using a set of barley genotypes known to possess high levels of APR: Vada, Pompadour, Gilbert, Mackay, Baronesse and Flagship (Golegaonkar et al. 2009). The experiments combined factors including growth stage, temperature and photoperiod, similar to those conducted in

Table 1 Results and statistics for disease incidence readings from spatial analysis of experiments characterising the ND24260 × Flagship doubled haploid population for adult plant resistance to *P. hordei*, assessed across five environments: Toowoomba 2009 (TWB09) and 2010 (TWB10), Cobbitty 2010 (COB10), La Estanzuela 2010 (URU10) and assessment under controlled environmental conditions in 2010 (CEC10)

Environment	Parental means[a]		Population mean (Av. BLUE)	Range	Av. LSD
	ND24260	Flagship			
TWB2009 (read 5/10/09)	na[b]	na	5.94	2.88–8.56	1.50
TWB2009 (read 16/10/09)[c]	8.5	5.0	6.98	3.04–9.19	1.26
TWB2009 (read 23/10/09)	na	na	7.93	4.48–9.26	1.32
TWB2010	8.48	5.12	7.05	3.81–9.35	1.18
URU2010 (nursery 1)	5.77	4.74	5.30	2.41–9.11	1.40
URU2010 (nursery 2)[c]	6.86	5.98	5.96	1.85–9.25	1.27
URU2010 (nursery 3)	7.52	5.95	6.47	2.82–9.31	1.21
CEC2010	6.48	6.20	6.79	3.73–9.08	1.15
COB2010[d]	6.5	4.0	5.55	2.0–9.0	na

[a] The disease reading scale used was 0 = immune and 9 = very susceptible

[b] na = no data collected or calculated

[c] Site selected for QTL analysis

[d] An unreplicated experiment was conducted with only parental cultivars replicated

Hickey et al. (2011), which were used to develop a rapid phenotyping method for APR to stripe rust (*Puccinia striiformis* f. sp. *tritici*) in wheat. Results from these preliminary experiments were used to develop the protocol described below.

Approximately, 5–10 grains per DH line and disease standards were imbibed with distilled water in Petri dishes lined with a filter paper (Whatman® 90 mm) for 24 h and then placed in a refrigerator (4°C) for 48 h to promote synchronous germination. Germinated grains were transplanted into 12 × 100-cell seedling trays (10 × 10 cells, with dimensions of 30 cm × 35 cm) arranged in two rows of six trays on a bench in a controlled temperature glasshouse maintained at 22 ± 1°C fitted with low-pressure sodium lamps to supplement natural light levels and extend the photoperiod to 24 h (i.e. constant light). An experimental design was generated with 20 rows × 60 columns, containing four replicate blocks. The design included an underlying component for autocorrelation in the row and column direction and was Latinised along rows. Each cell within a seedling tray contained a single plant. A total of 338 genotypes were assessed. Of these, 10 disease standards and 176 DH lines were replicated four times (i.e. four individual plants) and the remaining 152 DH lines were replicated three times. Plants were grown for 23 days and then inoculated with *P. hordei* using a suspension of urediniospores in light mineral oil at a rate of 0.01 g/mL and applied evenly to plants with an airbrush. Trays were incubated at 20°C overnight in a dew chamber (100% relative humidity maintained with a fogger). Trays were moved back into the glasshouse, the temperature lowered by 2° (20 ± 1°C constant) and the lights were set to a regular (12 h) diurnal photoperiod. Half of the plants (reps 1–2) were scored 8 days post-inoculation, whereas the remaining half (reps 3–4) were scored 11 days post-inoculation. Plants were assessed for disease response using the 0–9 scale, paying particular attention to the response on upper leaves (i.e. flag-1 and flag-2). Assessment of the barley lines for APR was completed within 5 weeks after sowing.

Genotyping and construction of genetic linkage map

Genomic DNA was extracted from young leaf tissue from a single plant of each DH line using the protocol recommended by Diversity Arrays Technology Pty Ltd (http://www.diversityarrays.com). A total of 334 DH lines were genotyped with DArT markers using the Barley PstI (BstNI) v 1.7 array, which comprises approximately 1,500 markers known to be polymorphic in a wide range of barley cultivars.

Prior to linkage mapping and QTL analyses, the resultant DArT data were curated to remove low quality markers ($P < 70$), markers with >60% missing data, monomorphic markers and markers with >75% segregation distortion in the DH population. The genetic linkage map for the ND24260 × Flagship DH population was constructed using Map Manager QTX (Manly et al. 2001). A total of 634 polymorphic DArT markers were used and the final marker order was confirmed rigorously using RECORD (Van Os et al. 2005) and compared with the DArT consensus map (Wenzl et al. 2006).

Spatial analysis of phenotypic data

A linear mixed model was fitted to the raw data, where genotype was considered a fixed factor and replicate blocking considered a random factor. An underlying autoregressive process was fitted to model autocorrelation between neighbouring plots in field experiments and cells within seedling trays for CEC. For each experiment, best linear unbiased estimates (BLUEs) were calculated using ASReml-R (Butler et al. 2007). The residual maximum likelihood (REML; Patterson and Thompson 1971) algorithm provided BLUEs as the predicted values for the DH lines. A second stage analysis (across environments) was not conducted due to differences in experimental design and pathotypes, etc. Data collected from multiple readings at the same location (Toowoomba 2009) and across different disease nurseries (La Estanzuela 2010) were analysed separately to derive BLUEs for APR to *P. hordei* for each DH line under the varying levels of disease pressure.

To evaluate patterns in APR expressed across the five environments, ordination analysis based on singular value decomposition was applied to the matrix of line by environment estimators (BLUEs). The data were column standardised and the first three principal components were displayed in biplots (Gabriel 1971). To demonstrate gene effects, DH lines were assigned to groups according to the APR genes detected through association analysis and composite interval mapping.

Association analysis and QTL mapping

BLUEs for APR to *P. hordei* in each environment were used for single-marker association analysis performed according to Arief et al. (2010). QTL were reported as regions containing DArT markers significantly influencing the trait. LOG scores ($-\log_{10}P$, where P is the probability of the mean between lines with and without the marker to be different) greater than 3.0 ($P < 0.001$) were considered to be significant. Positive and negative values assigned to LOG scores indicate the relationship between the marker state (present or absent) and disease response, i.e. a positive value indicates that the presence of the marker is associated with increased susceptibility (associated with higher phenotypic group mean), whereas a negative value indicates that

the presence of the marker is associated with increased resistance (associated with lower phenotypic group mean).

QTL identified in association analysis were confirmed through composite interval mapping (CIM) using WinQTLCartographer v2.0 (Wang et al. 2007). Graphical representation of the map was produced using MapChart 2.1 (Voorrips 2002). QTL were considered significant at an LOD threshold of 3.0 or higher based on genome-wide permutations to determine the experiment's wide significance for the test statistic threshold (1,000 permutations reading at 2 cM).

A PCR-based marker for qRphFlag

To permit screening of *qRphFlag* in elite breeding lines and selected cultivars, the DArT marker bPb-0837 was converted to a simple PCR-based marker system. Sequence information for the bPb-0837 DArT marker was provided by Diversity Arrays Technology (http://www.diversityarrays.com) and specific primers designed using Primer3 v0.4.0.

A 245-bp fragment for the *bPb-0837-PCR* marker was amplified in a 20-μL reaction mixture containing 0.2 mM dNTP, 1× PCR buffer (Qiagen), 1.5 mM $MgCl_2$, 0.5 μM each primer (bPb-0837F 5′GACACTTCGTGCCAGTTTG A3′; bPb-0837R 5′CCTCCCTCCCTCTTCTCAAC3′), 1U *Taq* DNA polymerase (Qiagen) and approximately 20 ng genomic DNA. Following an initial denaturation step of 2 min at 94°C, PCR was performed for a total of 35 cycles with the profile: 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C. The resultant fragment was analysed on a 2% agarose gel against a relevant molecular weight marker. The cultivar Flagship was used as the positive control.

Results

Expression of APR across diverse environments

The genes conferring resistance to *P. hordei* segregating in the ND24260 × Flagship DH population were associated with pustule size distribution depending upon the level of resistance (Fig. 1). The disease response for adult plants assessed across the five environments ranged between 2 and 9 (using the 0–9 scale; Fig. 2). In this study, this corresponded with a range of approximately 20 R-MR to 90 S of the disease severity (Peterson et al. 1948) and response (Stakman et al. 1962). There was obvious transgressive segregation for both resistance and susceptibility in all environments (Table 1). The population distribution for APR varied across the environments and so did the levels of APR expressed by both parents, although Flagship consistently showed higher levels of APR than ND24260 (Fig. 2). As the disease progressed in field experiments, the low levels of APR expressed by ND24260 became virtually ineffective under high disease pressure.

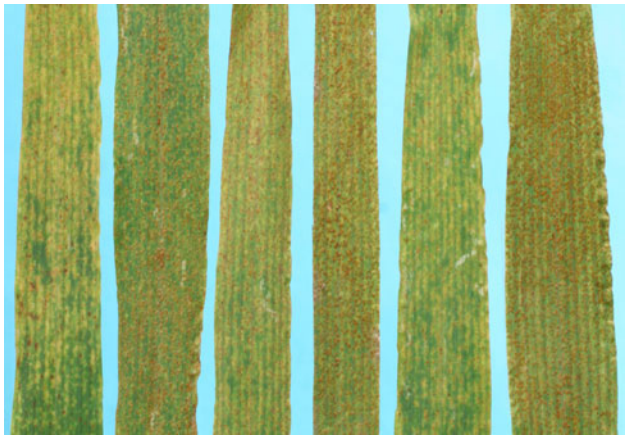

Fig. 1 Leaves from adult plants infected with *P. hordei*. Single leaves, *left* to *right*: Flagship, ND24260, DH046 (*qRphFlag* alone), DH285 (*qRphND* alone), DH137 (*qRphFlag* + *qRphND*) and DH033 (no genes)

Genetic linkage map for ND24260 × Flagship DH population

The genetic linkage map constructed for the DH population was based on 310 lines after 24 were eliminated as duplicate lines or as having off-type DArT marker patterns. The map consisted of ten linkage groups for the seven chromosomes, including two linkage groups per chromosomes 1H, 5H and 6H (Online Resource 1). The total map length was 2,038 cM, with an average distance of 3.21 cM between each DArT marker. Some gaps in the linkage map were due to uneven distribution of DArT markers and in some cases this was due to regions of markers that were not polymorphic between parents.

Mapping genes for APR

Association analysis for data sets from the five field environments consistently detected two QTL conferring APR to *P. hordei* in this ND24260 × Flagship DH population; one being *qRphFlag* that mapped to chromosome 5HS and the other mapped to 6HL (Fig. 3a). Results from CIM also detected these two QTL across all environments, with the exception of Cobbitty 2010, where the 6HL QTL was non-significant (Fig. 3b).

The QTL that mapped to chromosome 5HS (*qRphFlag*; donated by Flagship) had a large effect in all environments. This QTL explained 64–85% of the phenotypic variation for APR to *P. hordei* when assessed across the four field environments (Table 2). Markers in the region of this QTL had very high LOG scores (over 100 in some environments), which aligned with highly significant LOD scores

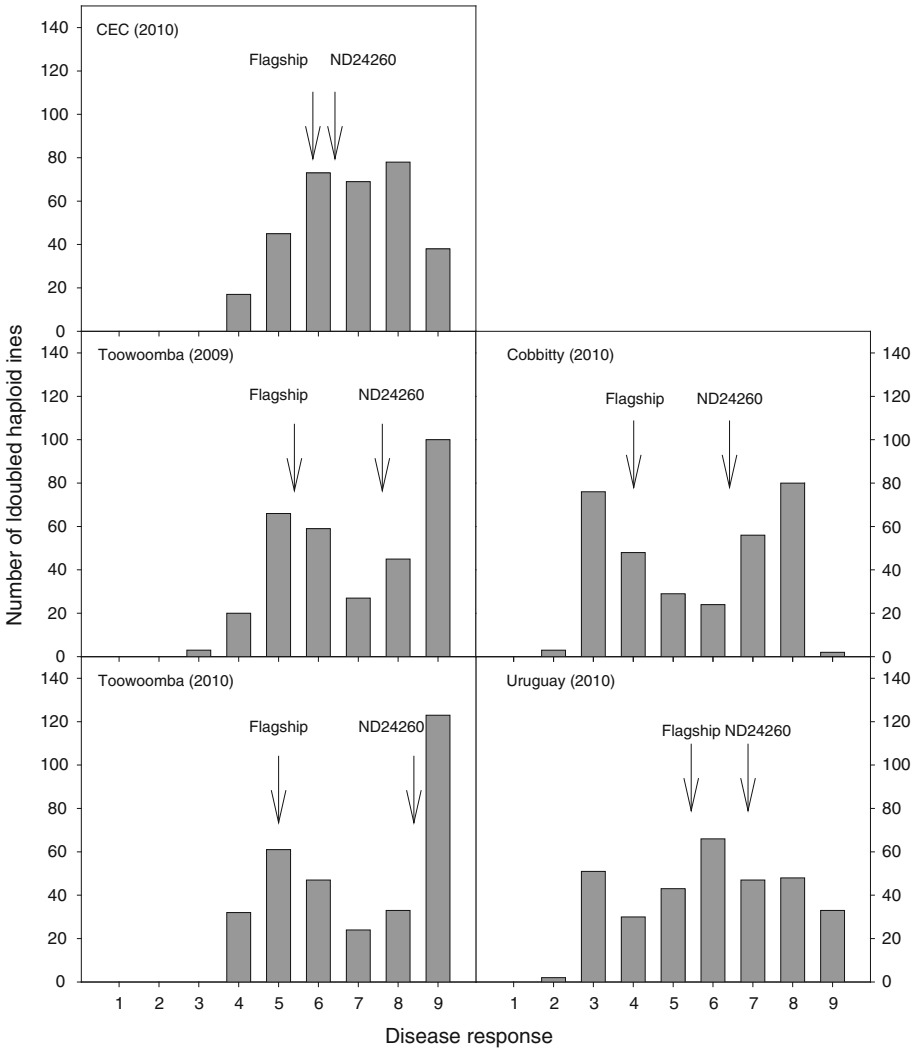

Fig. 2 Distribution of disease response to *P. hordei* in the ND24260 × Flagship doubled haploid population assessed for adult plant resistance in four field environments (Toowoomba 2009, 2010, Cobbitty 2010 and Uruguay 2010) and under controlled environment (CEC) in 2010. Mean disease response values for parents are indicated by *arrows*

from CIM (over 130 in some environments; Table 2). From the association analysis, the DArT marker that appeared to be most closely linked to *qRphFlag* was bPb-0837, positioned at 21.2 cM (5HS) on the DArT linkage map (Fig. 3b). However, from CIM the most significant marker varied between environments, but these markers were all located within 7 cM of each other (Table 2).

The presence of a second APR gene, *qRphND*, donated by ND24260 and corresponding with a QTL on chromosome 6HL, was detected in all environments except Cobbitty in 2010 (Table 2, Fig. 3b). This QTL displayed a smaller effect than the 5HS QTL (only explaining 2–5% phenotypic variation), except for La Estanzuela 2010 where it explained up to 25% variation (Table 2). From association analysis, the DArT marker most strongly associated with this QTL across all environments was bPb-9922 positioned at 107.7 cM on the linkage map (Fig. 3b). In this population, the presence of this DArT marker was associated with susceptibility to *P. hordei*, i.e. individuals carrying the 'Flagship' allele at this genomic location have a higher phenotypic group mean. However, the QTL peak varied across environments in CIM (Fig. 3b). The most significant markers detected in each environment were located within 28 cM of each other, but within only 7 cM for experiments conducted in 2010 (Table 2).

Some minor QTL were detected on chromosomes 1H, 3H, 4H, 6H and 7H (Table 2). Although the LOD/LOG scores were significant, the effects were only small (explained 1–4% variation) and were not consistently detected across environments.

When the population was assessed for APR under CEC, both the 5HS QTL (*qRphFlag*) and the 6HL QTL explained less phenotypic variation (Table 2). Two additional QTL with minor effects were detected on chromosomes 1H and 4H (Table 2). The largest LOG scores from association analyses for these QTL were 3.56 and 4.52, respectively. They were also detected in CIM. In addition, CIM detected

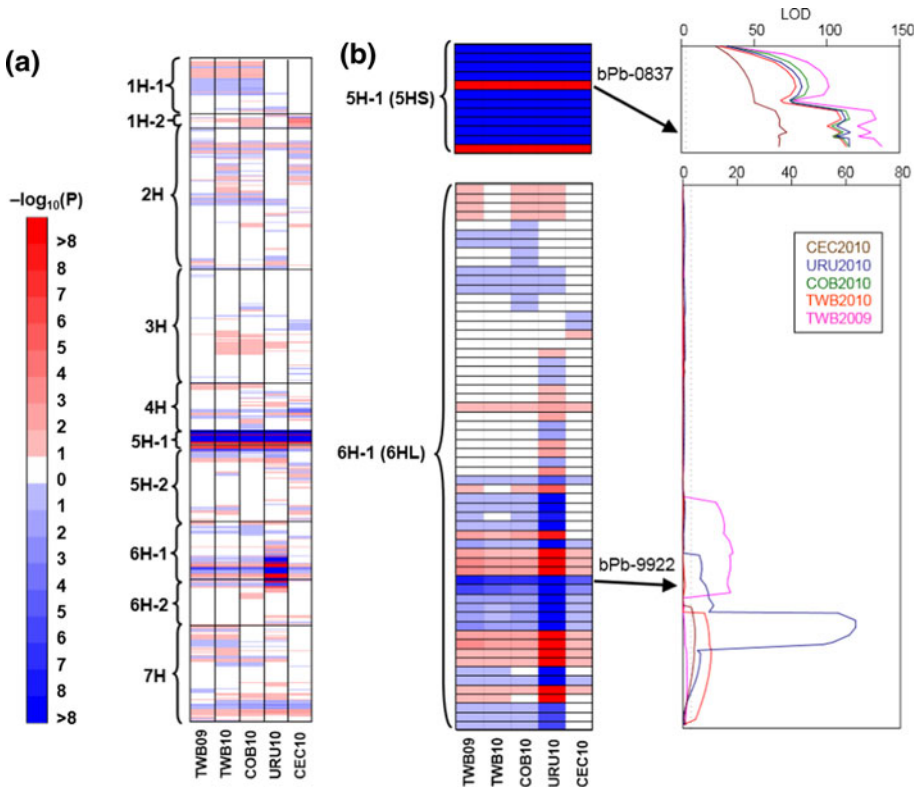

Fig. 3 Heat map displaying association results for **a** the whole genome (10 linkage groups), and **b** QTL regions for adult plant resistance to *P. hordei* on linkage groups 5H-1 (5HS) and 6H-1 (6HL) across five environments: Toowoomba 2009 (TWB09) and 2010 (TWB10), Cobbitty 2010 (COB10), La Estanzuela 2010 (URU10) and assessment under controlled environmental conditions in 2010 (CEC10). Associations with negative LOG scores (correlating with lower phenotypic group mean values) are indicated in *red* (i.e. the presence of the marker contributes to resistance) and associations with positive LOG scores (correlating with higher phenotypic group mean values) are indicated in *blue* (i.e. the presence of the marker contributes to susceptibility). Increasing colour intensity indicates a stronger association based on single-marker analysis. A graphical representation of results from composite interval mapping also displayed for *qRphFlag* on 5HS and *qRphND* on 6HL. The positions of closely linked DArT markers bPb-0837 (for *qRphFlag*) and bPb-9922 (for *qRphND*) are indicated by *arrows*

a minor QTL on chromosome 3H, which was also detected in the field at La Estanzuela 2010 (Table 2). Some DH lines, which were recorded as susceptible in field experiments, displayed low levels of resistance when assessed under CEC.

Association analysis found that DArT markers bPb-0837 and bPb-9922 were most strongly associated with the two APR genes on chromosomes 5HS and 6HL, respectively (across all environments). Therefore, DH lines were assigned gene combinations based on the presence of DArT marker bPb-0837 for *qRphFlag* and lack of the DArT marker bPb-9922 for *qRphND* (Fig. 4). Whilst CIM provided in general QTL in agreement with association analysis results, the estimated position of the peak corresponding to the QTL varied across the five environments (Fig. 3b). Phenotypic effects for the two APR genes correlated across the five environments (Fig. 4). DH lines that displayed the highest level of APR across all environments carried both genes, i.e. the marker haplotype associated with the favourable alleles for both loci.

The multiple assessments of leaf rust reactions conducted at Toowoomba 2009 and La Estanzuela 2010 showed that superior levels of resistance were expressed by DH lines that combined the two APR genes under low, moderate and high disease pressure and across diverse environments (Fig. 5). In most cases, *qRphFlag* conferred good levels of APR, even under high disease pressure. However, some DH lines with the DArT marker bPb-0837 displayed only a low level of APR. In the absence of *qRphFlag*, the second gene *qRphND* conferred weaker resistance, except under low disease pressure at La Estanzuela, where both genes conferred a similar level of resistance when deployed alone (Fig. 5). Under high disease pressure at Toowoomba in 2009, the effect of *qRphND* alone was difficult to detect (Fig. 5).

Detection of qRphFlag in a range of germplasm

The DArT marker bPb-0837 corresponded with APR in a selection of elite breeding lines and cultivars that lacked seedling resistance to *P. hordei*, as assessed at Cobbitty in

Table 2 Quantitative trait loci (QTL) for adult plant resistance to *P. hordei* mapped across five environments: Toowoomba 2009 (TWB2009) and 2010 (TWB2010), La Estanzuela 2010 (URU2010), Cobbitty 2010 (COB2010) and controlled environmental conditions in 2010 (CEC2010). DArT markers are listed for each QTL region that were a) most strongly associated in association analysis, and b) positioned closest to the QTL peak from composite interval mapping (CIM)

Chromo. bin[a]	Environment	Marker	Position (cM)[b]	LOG[c]	LOD[d]	R^2 (%)[e]	Source[f]
1H_bin14	CEC2010	bPb-8112	36.8	−3.8			ND24260
		bPb-9410	36.9		6.9	3.2	
3H_bin11	URU2010	bPb-4209	178.8		4.2	1.1	Flagship
	CEC2010		182.8		4.4	2.7	
4H_bin09	CEC2010	bPb-8701	108.6	4.5			Flagship
					5.7	2.7	
5H_bin02	TWB2009	bPb-0837	21.2	−107.7			Flagship
		bPb-8572	24.7		137.8	84.5	
	TWB2010	bPb-0837	21.2	−93.1			
		bPb-8572	24.7		113.5	82.3	
	URU2010	bPb-0837	21.2	−64.8			
					116.1	65.5	
	COB2010	bPb-0837	21.2	−97.8			
		bPb-8580	17.9		115.6	80.1	
	CEC2010	bPb-0837	21.2	−62.8			
					72.4	56.6	
6H_bin07	COB2010	bPb-9045	88.4		11.9	3.6	Flagship
6H_bin07	TWB2009	bPb-9922	107.7	8.5			ND24260
		bPb-5822	100.6		18.3	4.5	
	TWB2010	bPb-9922	107.7	6.7			
		bPb-0151	128.1		10.5	3.3	
	URU2010	bPb-9922	107.7	20.23			
		bPb-0696	121.0		63.5	24.8	
	COB2010	bPb-9922	107.7	6.9			
	CEC2010	bPb-9922	107.7	5.2			
		bPb-0696	121.0		4.5	2.3	
7HL_bin09	TWB2009	bPb-9662	148.3		4.7	1.1	ND24260
	URU2010	bPb-9202	172.4	3.4			Flagship

[a] Chromosomal location of the DArT marker and estimated position based on the barley bin maps

[b] Position on genetic linkage map in the ND24260 × Flagship doubled haploid population, i.e. position of the significant DArT marker from association analysis and QTL peak position derived from CIM

[c] LOG from association analysis, where negative values are associated with a low phenotypic trait mean value which correlates with resistance

[d] LOD score for QTL peak position derived from CIM

[e] Percentage of phenotypic variation explained by the QTL

[f] The parent donating resistance for the QTL (derived from CIM in WinQTLCartographer)

2009 (Table 3). The *bPb-0837-PCR* marker for *qRphFlag* aligned with DArT marker bPb-0837 (i.e. >95%) in the ND24260 × Flagship DH population. The 245-bp fragment for *bPb-0837-PCR* amplified in barley cultivars known to possess good levels of APR to *P. hordei*, such as Flagship, Vada, Baronesse, Pompadour, Mackay, WI3407 and Patty (Fig. 6). However, the fragment was not detected in the cultivar Gilbert. The PCR marker (*bPb-0837-PCR*) aligned in almost all cases with the DArT marker in a larger set of germplasm including elite breeding lines (data not presented).

Discussion

Expression of APR across diverse environments

The APR to *P. hordei* segregating in the ND24260 × Flagship DH population is effective across field environments in Australia and Uruguay, and assessment under CEC. A similar range in disease responses and high degrees of phenotypic correlation for APR across these environments suggested that one or more genetic factors were consistently expressed across diverse environments to pathotypes

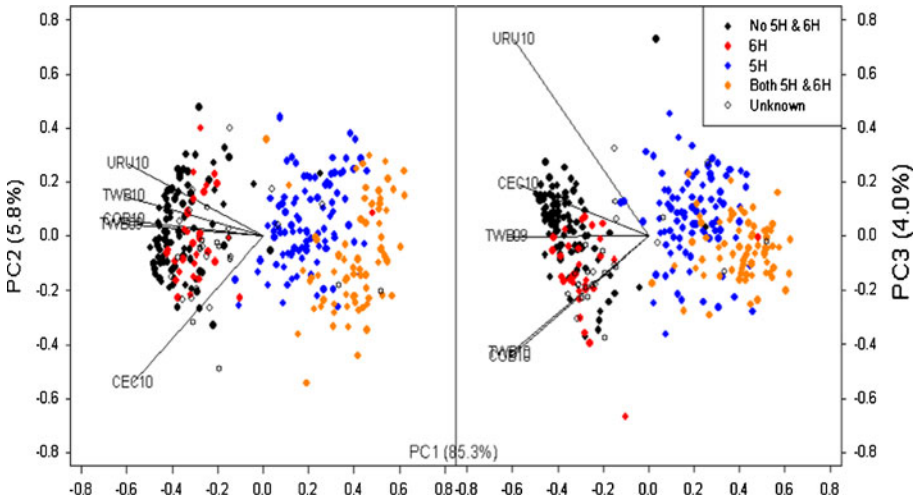

Fig. 4 Biplots from principal component analysis using best linear unbiased estimates (BLUEs) for adult plant resistance to *P. hordei* for all lines in the ND24260 × Flagship doubled haploid population, assessed across five environments: Toowoomba 2009 (TWB09) and 2010 (TWB10), Cobbitty 2010 (COB10), La Estanzuela 2010 (URU10) and assessment under controlled environmental conditions in 2010 (CEC10). DH lines assigned to groups according to the two APR genes, *qRphFlag* on 5HS and *qRphND* on 6HL, using DArT markers bPb-0837 and bPb-9922 respectively

of *P. hordei*. The transgressive segregation for APR to *P. hordei* observed in the current study is not entirely unexpected. Even susceptible parents have been reported to contribute genes (with additive effect) that confer transgressive levels of resistance, for instance stripe rust in wheat (Sharp and Fuchs 1982). In the ND24260 × Flagship DH population, it appears that lines displaying the highest levels of APR (superior to that of Flagship) combine resistance genes and/or modifiers (i.e. independent genomic regions influencing expression of the gene) donated by both parents.

Mapping genes for APR

Through this study we have validated the position of a gene conferring APR to *P. hordei* on chromosome 5HS, which was originally proposed by Golegaonkar et al. (2010) and in agreement with Liu et al. (2010). The gene is common in barley cultivars derived from Vada (Golegaonkar et al. 2009). The APR gene that we refer to as *qRphFlag* is likely the same gene underlying *Rphq4*; a QTL for partial resistance to *P. hordei* reported in the study by Qi et al. (1998). *Rphq4* is best expressed at the adult plant stage and is considered a key component of partial resistance in Vada, explaining 44.7% of the phenotypic variation (Qi et al. 1998). Both *Rphq4* and *qRphFlag* appear to have large effects. By comparing the mapped positions on chromosome 5HS across the two studies, Liu et al. (2010) suggested that the APR gene closely linked with the DArT marker bPb-0837 (donated by Pompadour) in the Pompadour × Stirling DH population may be different from the gene underlying *Rphq4*. The variation in position of these QTL, however, may be related to inconsistencies such as the level of recombination in the mapping population, the population size and the density of molecular markers. In fact, our results over trials show some variation in the location of the QTL peak. We also demonstrate that Pompadour and Vada carry the bPb-0837 DArT marker closely linked with *qRphFlag*. The additional QTL with minor effect reported in the current study and those reported by Qi et al. (1998) could be either related to other mechanisms of resistance or act as modifiers (i.e. independent genomic regions), which influence expression of the APR gene *qRphFlag*.

The APR gene *qRphND*, donated by ND24260 in the ND24260 × Flagship DH population, was mapped to chromosome 6HL and explained considerably less phenotypic variation across the five environments. In the absence of *qRphFlag*, the effect of this gene was difficult to detect, particularly under high disease pressures. Multiple assessments conducted for Toowoomba 2009 and the use of multiple disease nurseries at La Estanzuela showed that the gene conferred levels of resistance comparable to *qRphFlag* under low disease pressure. However, as the disease progressed, this gene and *qRphFlag* were less effective.

Under lower disease pressures, the two genes *qRphFlag* and *qRphND* displayed additive effects as suggested by Singh et al. (2000). However, when disease pressure was high, the enhanced level of resistance conferred by the presence of the two genes resulted in apparent epistasis. When the two APR genes were combined, we found increased expression, superior to the resistance expressed by Flagship. Therefore, these genes can be combined to confer more effective levels of APR. Combining these genes in cultivars could make APR to *P. hordei* a more effective control mechanism and may contribute to its durability.

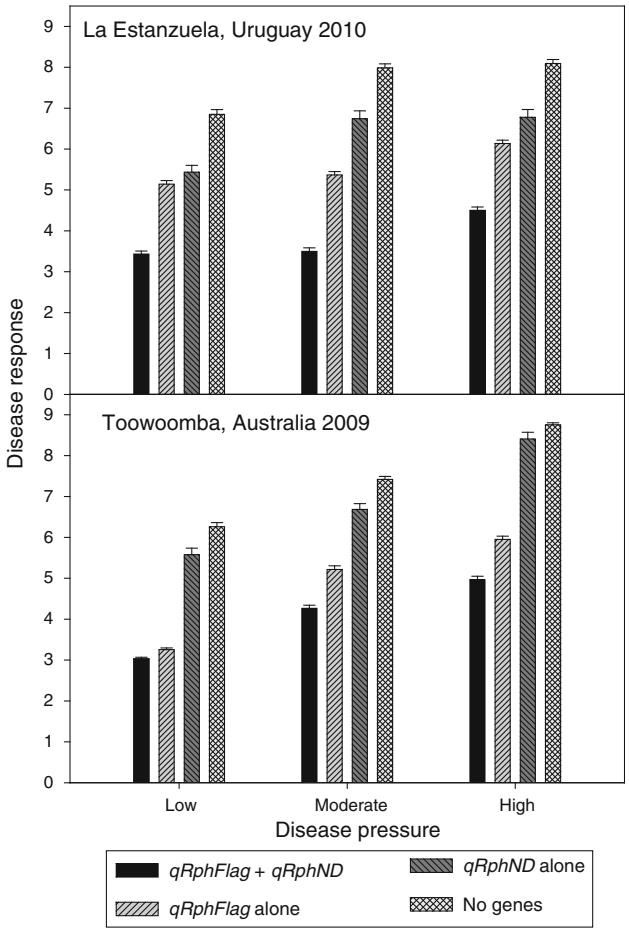

Fig. 5 Mean disease response and standard errors for doubled haploid (DH) lines assigned gene combinations: "*qRphFlag* + *qRphND*", "*qRphFlag* only", "*qRphND* only" and "no genes", for adult plant resistance (APR) to *P. hordei* assessed under varying levels of disease pressure (i.e. low, moderate and high) in the field at La Estanzuela (Uruguay) in 2010 and Toowoomba (Australia) in 2009. DH lines were assigned APR genes using the two DArT markers: bPb-0837 (*qRphFlag*) and bPb-9922 (*qRphND*). Data presented for La Estanzuela were attained from three experiments in separate disease nurseries, which had varying levels of disease pressure at the time of scoring, whereas data presented for Toowoomba were attained from multiple assessments as the disease progressed in the same experiment

The assessment of APR to *P. hordei* under CEC involved only a single inoculation to infect plants (all at a similar growth stage), whereas in the field, plants are exposed to repeated cycles of infection. Thus, the QTL that were detected only under CEC on chromosomes 1H and 4H could be associated with infection frequency or latent period. These QTL identified under CEC may contribute towards resistance by delaying disease progress, but their effects may be difficult to detect in the field where expression of APR is influenced by other factors including weather patterns and maturity. Expression of these two QTL could also be moderated by temperature, as a constant temperature was maintained in the CEC. Under CEC most photoperiod responses were eliminated, whilst in the field

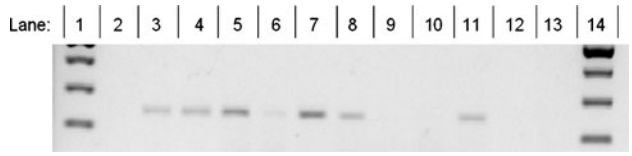

Fig. 6 Polymorphism of the *bPb-0837-PCR* DArT-PCR marker in key barley genotypes (*lanes 2–12*): ND24260, Flagship, Vada, Pompadour, Mackay, WI3407, Baronesse, Hindmarsh, Stirling, Patty and Gilbert. *Lanes 1* and *14* are 100 bp molecular weight marker controls and *lane 13* is a no-template control. The fragment for *qRphFlag* amplifies at 245 bp

at Hermitage Research Station (Warwick, QLD, Australia) in 2009, the same material showed a heading date range of 19 days. Although minor QTL detected only in field experiments (such as 6HS and 7HL) could be associated with maturity, they are not located where maturity genes such as *Ppd-H2* (1HL), *Eam1* (*Ppd-H1*, 2HS), *Eam6* (*eps2S*, 2HS) and *Eam5* (5HL) segregated in the ND24260 × Flagship DH population. The ability to identify APR to *P. hordei* under CEC (conferred by both major and minor QTL) within only 5 weeks provides breeding programs with the opportunity to conduct high-throughput disease screening all year round and validates a plant growth control technique that can be a useful tool for future mapping studies.

A molecular marker for qRphFlag

In the ND24260 × Flagship DH population, the DArT marker most closely linked to *qRphFlag* was bPb-0837. This was consistent across all environments in the current study and in agreement with Liu et al. (2010), where they mapped seven molecular markers within 5 cM of the APR gene on chromosome 5HS. Liu et al. (2010) estimated that DArT marker bPb-0837 was 0.7 cM away from the APR gene in a Pompadour × Stirling DH mapping population. In the current study, we derived the *bPb-0837-PCR* marker for *qRphFlag* and confirmed that Pompadour, Vada, Mackay, Baronesse, Flagship, WI3407 and Patty all contain the marker associated with the resistant allele in the 5HS region. Thus, the APR in these cultivars is likely of the same origin, as they are all related to either Vada or Emir (or their derivatives). Golegaonkar et al. (2009) first suggested that Vada (derived from a cross between *H. laevigatum* and Gull; Dros 1957) was the likely source of APR in a number of related barley cultivars. It was suggested that the resistance likely originated from *H. laevigatum* as opposed to Gull (Swedish for Gold), which is susceptible to *P. hordei* (Golegaonkar et al. 2009). However, both Vada and Emir have Gull in their parentage. Since the landrace Gull was widely utilised in the development of two-rowed barley cultivars in northern Europe, it is possible that the *qRphFlag* gene originated from Gull.

Table 3 Status of DArT marker bPb-0837 (linked to *qRphFlag*) in a selection of barley cultivars and elite breeding lines from the Barley Breeding Australia, Northern Region Program, Warwick, Australia

Genotype	Pedigree	Seedling[a]	Adult[b]	bPb-0837[c]
2ND25313	ND22182/ND19119-5	3+	8	0
2ND25479	ND20798//ND22201/ND19119-2	3	7	0
2ND25524	ND22007//ND22182/ND19119-5	3+	8	0
2ND25630	ND22201/2*ND19119-1-2	3+	7	0
C2-05-337-5	ND19119-5/200A12//Bowman*8/Mult. Dom.	3+	8	0
Fleet	Mundah/Keel//Barque	3+	3	1
Grout	Cameo/Arupo	3+	8	0
Hindmarsh	Dash/VB9409	33+	5	0
Kaputar	5604/1025//etc.	3+	8	0
Mackay	Cameo/Koru	3+	3	1
ND11993	ND8968/ND9163 = Norteña Daymán	3+	7	0
ND13111	ND9034/AC Oxbow = Norteña Carumbé	3+	7	0
ND14760	ND586/CIho2376//ND4880/3/4*Bowman/4/Eldo"S"/5/ND10278	3+	8	0
ND15562	ND13890//ND12567/CMB643-2A	3+	8	0
ND17293-1	ND14651/ND15062	33+	8	0
ND21089-1	ND18172/ND18076	3+	8	0
ND21867-1	ND18172/ND19130	3+	7	0
ND22185	ND18427/3/Mokkei 93-78/ND15462//ND16723/4/1ND19088	3+	8	0
ND24379	ND20824//ND20028/ND19119-1	3+	8	0
ND24393	ND17274/ND19119//ND19854	3+	8	0
NRB06059	Mackay*2/WI3214	3+	4	1
NRB07064	Mackay/Skiff	3+	3	1
NRB07067	Mackay/NRB01020	33+	3	1
NRB07162	Grout/NRB01628	3+	8	0
NRB07326	NRB01109/Mackay	33+	3	1
NRB07370	Cowabbie/NRB03471	3+	3	1
NRB07390	NRB01140/NRB04535	3+	4	1
NRB07437	Skiff/NRB04535	3+	3	1
NRB08072	Grout/Barke	3+	7	0
NRB08083	Grout/Barke	3+	6	1
NRB08087	Grout/Barke	3+	3	1
NRB08177	Valier/NRB01140	3+	8	0
NRB08434	Gairdner Plus/NRB03465	33+	2	1
NRB08446	Gairdner Plus/NRB03465	3+	3	1
NRB08449	Gairdner Plus/NRB03465	3+	2	1
NRB08466	Gairdner Plus/NRB03465	3+	3	1
NRB08531	NRB01145/NRB03467	23−	3	1
NRB08541	NRB01145/NRB03467	33+	4	1
NRB08543	NRB01145/NRB03467	33+	5	1
NRB08545	NRB01145/NRB03467	33−	3	1
NRB08847	Atahualpa/NRB03467//ND21089-1	3+	6	1
Roe	Doolup//Windich/Morex = WABAR2310	3+	7	0

Data presented for seedling and adult plant response to *P. hordei* assessed at Cobbitty in 2009. Lines that were postulated to not carry seedling resistance are displayed. The pathotype 5457P+ (virulent for *Rph* 1,2,3,4,6,9,10,12,19) was used for both adult plant and seedling tests

[a] Seedling test used a modified 0–4 scale, as described by Park and Karakousis (2002)

[b] Adult plant rating is based on 0–9 scale, where 9 = susceptible, 0 = immune

[b] 0 = absent, 1 = present

In a recent study by Golegaonkar et al. (2010), it was reported that the APR in Vada is conferred by a single, simply inherited gene. This finding contrasts with results from a previous study by Neervoort and Parlevliet (1978), who reported Vada to carry partial resistance to *P. hordei*, which they proposed was controlled by five to six genes. Other studies reported up to 10 QTL controlling the latent period in Vada (Qi et al. 1998, 1999). Results from our study demonstrate that the APR gene in Vada is also present in a number of other cultivars that show equivalent levels of APR to *P. hordei* (Golegaonkar et al. 2009). Golegaonkar et al. (2009) proposed that Pompadour and Patty may carry a different source of APR derived from the Dutch cultivar Emir. Our results suggest that cultivars derived from both Vada and Emir have the same APR gene on chromosome 5HS. Based on map position and donor source, this APR is likely the same one reported by Liu et al. (2010).

We detected *qRphFlag* in elite breeding lines and cultivars with high levels of APR (Table 3), which demonstrates that this gene is a common source of APR in a broad range of germplasm. Despite countless crosses and recombination events in breeding programs, the DArT marker bPb-0837 found in Vada is also linked with *qRphFlag* in modern breeding lines suggesting that the marker and QTL are strongly linked. However, some other accessions with APR trace to similar parentage, but lack the bPb-0837 marker.

The *bPb-0837-PCR* marker did not detect the presence of *qRphFlag* in Gilbert (Table 3). Based on the allelism tests conducted by Golegaonkar et al. (2010), Vada and Gilbert share the same gene for APR to *P. hordei*. It can be noted that Gilbert displayed a lower level of APR (30–40 MS) across the four environments reported in Golegaonkar et al. (2009). It is possible that *bPb-0837-PCR* has lost linkage with *qRphFlag* in the cultivar Gilbert and the lower level of APR is due to genetic background and/or lack of modifiers. The importance of modifiers in understanding gene interactions associated with APR to leaf rust is supported by previous studies indicating complex inheritance patterns in material having the *qRphFlag* gene (Neervoort and Parlevliet 1978; Qi et al. 1998, 1999). Liu et al. (2010) and our studies suggest simpler inheritance patterns, but in recent studies the probability of favourable modifiers being fixed in the bi-parental crosses is higher. Further studies are being done to verify the presence of *qRphFlag* in Gilbert and to understand the array of minor factors affecting the expression of APR.

Phenotypic expression of qRphFlag

In barley, there have been reports of partial resistance or slow rusting to *P. hordei* (Parlevliet 1979, 1983). This kind of resistance is different to that of either seedling or adult plant major gene resistance. Partial resistance is defined as a reduction in disease development despite susceptible infection types, which is typically not associated with a specific growth stage (Parlevliet and van Ommeren 1975). However, some components of partial resistance, such as increased latent period, are best expressed at the adult plant stage (Parlevliet 1975). It is known that some barley cultivars with APR to *P. hordei* (e.g. Patty, Pompadour and Vada) can also express some resistance at seedling growth stages (Golegaonkar et al. 2006). The variability in seedling response to *P. hordei* might be due to environmental factors. Other APR genes can be detected at seedling growth stages, for instance *Lr34* in wheat can be expressed in seedlings given specific environmental conditions (Drijepondt et al. 1991). Therefore, we consider partial resistance, as described by Parlevliet and van Ommeren (1975), the same type of resistance as APR reported in the current study and recent studies by Golegaonkar et al. (2009, 2010).

The resistance conferred by *qRphFlag* is not associated with a hypersensitive reaction in seedlings and adult plants. Histological studies have demonstrated that partial resistances in wheat and barley are based on restricted haustorium development, which is not associated with a hypersensitive response (Niks 1986; Jacobs 1989; Rubiales and Niks 1995). The enhanced expression of resistance conferred by *qRphFlag* in adult plants, rather than seedlings, is consistent with the typical APR expression pattern. APR is well documented across numerous host/pathogen systems such as leaf rust in wheat (Kaur et al. 2000), downy mildew in cauliflower (Jensen et al. 1999) and stripe rust in wheat (Singh and Rajaram 1994). APR to rust pathogens is often identified by slow rusting that is best expressed at adult plant growth stages, which affects pathogen growth via mechanisms such as low receptivity and production of fewer and smaller uredia. This is a common feature of the well-characterised APR genes in wheat such as *Lr34* (Drijepondt and Pretorius 1989) and *Lr46* (Martínez et al. 2001). We propose that *qRphFlag* is similar to these genes, which have provided durable APR to rust pathogens despite having a monogenic inheritance pattern (McIntosh et al. 1995). *Lr34* has contributed resistance to leaf rust in wheat cultivars for over 50 years (Krattinger et al. 2009). Tracing back *qRphFlag* to the Dutch barley cultivar Vada, or possibly Gull, suggests that this gene has contributed APR to *P. hordei* for more than 60 years and is now common in barley germplasm around the world where epiphytotics of leaf rust occur frequently.

The expression of *qRphFlag* can vary depending upon the genetic background and environmental conditions. When Vada was crossed to Gus for genetic studies by Golegaonkar (2007), resistant BC_1F_2 progeny deemed to carry the APR gene showed disease responses ranging between TR and 20MR. Also, the various barley cultivars found to carry *qRphFlag* display varying levels of APR to *P. hordei*.

In the current study, where *qRphND* was absent, DH lines carrying *qRphFlag* displayed variable levels of APR (ranging from resistant to moderately susceptible) under conditions with moderate disease pressure. The variation in expression of APR may in part be due to modifiers or suppressors. Perhaps, some of the QTL influencing components of partial resistance reported by Qi et al. (1998) are modifiers for the gene *qRphFlag* in Vada. However, the modifiers in Vada may not be the same as those segregating in the ND24260 × Flagship DH population.

Designation of Rph20 and searching for new sources of APR

In view of the results presented here, pedigree information and the allelism tests performed by Golegaonkar et al. (2009), *qRphFlag* is assigned the locus designation *Rph20* with the resistant allele designated as *Rph20.ai*. This gene seems a primary component of APR to *P. hordei* in barley cultivars related to Vada and Emir. The apparent lack of diversity for APR genes across the wide range of germplasm that has been assessed in the current and previous studies raises some concern, but also supports the critical importance of *Rph20* and its modifiers in offering a more effective protection against changes in the virulence of *P. hordei*. Current research is focussed on screening for alternate sources of APR and mapping of genes with minor effects to combine with *Rph20*, such as *qRphND* from ND24260. Although there is a need to conduct multi-pathotype testing to characterise seedling resistance genes, the *bPb-0837-PCR* marker might be effective to test for distinct sources of APR in barley genotypes from around the world. This marker should aid in efficient screening of barley breeding lines and avoid time-consuming allelism testing amongst potential parental lines.

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