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ORIGINAL PAPER

Association mapping of resistance to *Puccinia hordei* in Australian barley breeding germplasm

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Abstract

Key message **"To find stable resistance using association mapping tools, QTL with major and minor effects on leaf rust reactions were identified in barley breeding lines by assessing seedlings and adult plants."**

Abstract Three hundred and sixty (360) elite barley (*Hordeum vulgare* L.) breeding lines from the Northern Region Barley Breeding Program in Australia were genotyped with 3,244 polymorphic diversity arrays technology markers and the results used to map quantitative trait loci (QTL) conferring a reaction to leaf rust (*Puccinia hordei* Otth). The $F_{3:5}$ (Stage 2) lines were derived or sourced from different geographic origins or hubs of international barley breeding ventures representing two breeding cycles (2009 and 2011 trials) and were evaluated across eight environments for infection type at both seedling and adult plant stages. Association mapping was performed using mean scores for disease reaction, accounting for family effects using the eigenvalues from a matrix of genotype correlations. In this study, 15 QTL were detected; 5 QTL co-located with catalogued leaf rust resistance genes (*Rph1*, *Rph3/19*, *Rph8/14/15*, *Rph20*, *Rph21*), 6 QTL aligned with previously reported genomic regions and 4 QTL (3 on chromosome 1H and 1 on 7H) were novel. The adult plant resistance gene *Rph20* was identified across the majority of environments and pathotypes. The QTL detected in this study offer opportunities for breeding for more durable resistance to leaf rust through pyramiding multiple genomic regions via marker-assisted selection.

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Introduction

Puccinia hordei Otth is the causal agent of barley leaf rust, one of the most important diseases affecting this crop globally (Golegaonkar et al. 2009; Roane 1972). Leaf rust has been reported to cause yield losses up to 32 % in Australia and North America (Dill-Macky et al. 1989; Griffey et al. 1994). In Australia, resistance to this disease is a key trait for cultivar development, particularly in the northern grain growing region (i.e. northern New South Wales and southern Queensland), where severe epidemics may occur (Cotterill et al. 1992).

In cereals, resistance to rust is often distinguished as seedling resistance, partial resistance and adult plant resistance (APR) (Parlevliet and van Ommeren 1975). Seedling resistance, conferred commonly by one major effect gene, provides only one genetic barrier that may easily be eroded by mutations in the pathogen population and is therefore often pathotype specific (Parlevliet and van Ommeren 1975). In contrast, partial (quantitative) resistance is conferred by multiple minor effect genes that may influence factors such as infection frequency, pustule size and latent

period (Qi et al. 1998). These genes often do not provide adequate levels of resistance when deployed alone (Marcel et al. 2007; Qi et al. 1998; Hickey et al. 2011); however, the additive and/or epistatic effects of multiple partial resistance genes can provide effective adult plant resistance (APR) (Singh and Bowden 2011). APR is identified by seedling susceptibility and adult plant resistance and is largely non-pathotype specific (Marcel et al. 2007). Studies of rust resistance in wheat (*Triticum aestivum*) indicate that APR is a more durable form of resistance due to multiple genetic barriers (Hong and Singh 1996; Lagudah et al. 2006; McIntosh 1992; Pretorius et al. 2007).

Major effect barley leaf rust resistance genes have been described as *Rph* (reaction to *P. hordei*) genes. To date, 21 seedling resistance genes have been named: *Rph1–19*, *Rph21*, *Rph22* and one APR gene *Rph20*. Breeders in Australia have relied primarily on seedling resistances to leaf rust (i.e. *Rph1–12*, *Rph19* and *Rph21*), which lack durable effectiveness (Golegaonkar et al. 2009). Targeted APR is yet to be deployed intentionally in commercial barley cultivars. However, recent mapping and marker development for the first gene conferring APR to *P. hordei*; *Rph20* (Hickey et al. 2011) offers new opportunities for breeders to introgress this gene into breeding material using marker-assisted selection (MAS). This gene confers partial resistance to the pathogen that is expressed in adult plants. The remaining challenge is to identify additional genomic regions that may be combined with *Rph20* to provide higher levels of resistance and reduce the reliance upon a single resistance factor (Hickey et al. 2012).

Molecular markers can contribute to the dissection of genetic control of important traits (Lande and Thompson 1990). However, classical marker development methodologies for trait dissection can be inefficient and expensive as they typically involve the development of specifically structured, bi-parental mapping populations. These populations must segregate for the trait(s) of interest and require genotypic and repeated phenotypic analysis to validate marker/trait associations. Markers that show consistent and significant associations with traits, across environments and genetic backgrounds, can then be proposed as targets for MAS.

Association mapping (AM), also known as association genetics or linkage disequilibrium (LD) mapping, is an analytical approach for QTL detection that exploits LD between markers and closely linked QTL in lines with irregular population structures. In such populations, LD is generally lower than standard bi-parental populations and when used in combination with a high marker density can allow more accurate location of QTL compared with conventional mapping populations. When such an approach is used in breeding populations, it has the added advantage of identifying associations in the genetic context targeted by the breeder, thereby limiting the impact of context dependency on the value of marker/trait associations. The advent of cost-effective whole genome profiling utilising next-generation sequencing technologies (e.g. genotyping by sequencing; Elshire et al. 2011) has contributed to the increasing popularity of studies using large numbers of SNP markers for AM, in both animal and crop species. AM was initially adopted in model plants species such as *Arabidopsis* and maize (Wang et al. 2007). With the development of assay-based high-throughput marker systems, this approach for QTL discovery has gained popularity in many crop species. AM studies in barley using high-throughput DArT markers have examined genomic regions influencing yield and related traits under drought (Varshney et al. 2012), frost tolerance (Visioni et al. 2013) and salt tolerance (Long et al. 2013). The combined use of DArT and SNP markers has been used to identify QTL for resistance to spot blotch [*Cochliobolus*, *sativus* (Ito & Kurib.) Drechs. ex Dast.] (Roy et al. 2010; Zhou and Steffenson 2013a) and speckled leaf blotch (*Septoria passerinii* Sacc,) (Zhou and Steffenson 2013b).

This study aimed to identify genomic regions influencing resistance to *P. hordei* using breeding populations representative of the Northern Region Barley Breeding (NRBB) Program based in Queensland, Australia, using AM. The elite breeding lines were examined for reaction to *P. hordei* at both seedling and adult growth stages in Australian environments, and the results discussed in the context of previous QTL mapping studies for leaf rust.

Materials and methods

Germplasm selection

The barley breeding populations evaluated in this study consisted of germplasm sets, which included elite breeding lines, some parental lines and a small set of current Australian cultivars. These lines are representative of the NRBB Program based at the Hermitage Research Facility, Warwick, Queensland, Australia, and have been derived or sourced from different geographic origins or hubs of international barley breeding ventures. Entries in the populations were selected in part based on results from field screenings in inoculated nurseries for reactions to four foliar diseases: leaf rust, powdery mildew (*Blumeria graminis* (DC) E.O. Speer f. sp. *hordei* Ém. Marchal) and net and spot forms of net blotch (*Pyrenophora teres* Drechs. f. *teres* and *P. teres.* f. *maculata* Smedeg.) This material is being continuously developed and advanced, including many sources of resistance to biotic agents and agronomic traits.

The breeding populations fall into two distinct groups: breeding population 1 (BP1) consisted of 368 Stage 2 (equivalent F_3:F_5) lines selected from Stage 1 yield trials conducted in 2008, and breeding population 2 (BP2) consisted of 155 breeding lines selected from a mixture of Stage 1 and Stage 2 yield trials conducted in 2010. A small portion of elite lines overlap in both BP1 and BP2. The reduction in the number of lines in BP2 was caused in part by a leaf rust epiphytotic during the 2010 season that rendered previously resistant selections susceptible.

Pathogen materials

Breeding populations in this study were assessed for resistance to *P. hordei* using pathotypes 5453P+ (virulent for *Rph1*, *2*, *4*, *6*, *9*, *10*, *12* and *19*) and 5457P+ (virulent for *Rph1*, *2*, *3*, *4*, *6*, *9*, *10*, *12* and *19*) (Park 2009; Park and Williams 2011). Pathotypes present in field nurseries were confirmed using leaf samples taken from susceptible breeding lines.

Assessment of seedling resistance

BP1 was characterised for seedling resistance using *P. hordei* pathotype 5457P+ at the Cobbitty Research Station, The University of Sydney, New South Wales, Australia in 2009 (i.e. COB2009_Seedling). BP2 was assessed as seedlings in 2011 using pathotype 5457P+ at both Cobbitty (i.e. COB2011_Seedling) and at the Hermitage Research Facility, Warwick, Queensland, Australia (i.e. HRF2011_Seedling).

Seedlings were grown in pots in the glasshouse and inoculated with *P. hordei*, as per Park and Karakousis (2002). Post-inoculation, pots were returned to the glasshouse maintained at 20–25 °C, where disease developed and plants were assessed for resistance to *P. hordei* 10–11 days later. Disease infection type (IT) was recorded using the 0–4 scale (McIntosh 1992; Park and Karakousis 2002), where 0 is immune and >3 (3+ and above) is considered susceptible.

Assessment of resistance in the field

BP1 was assessed at the adult plant stage (post spike emergence) for resistance to *P. hordei* at two field sites in Australia in 2009: the Leslie Research Facility, Toowoomba, Queensland (i.e. LRF2009_Adult) at which two readings were taken, and the Sydney University Plant Breeding Institute, Cobbitty, New South Wales (i.e. COB2009_Adult). BP2 was assessed for adult resistance to *P. hordei* at the same field sites in 2011 (i.e. LRF2011_Adult and COB2011_Adult).

All entries were sown as hill plots of 15–20 seeds in rows 0.75 m apart with 0.50 m within-row spacing. Each pair of datum rows was separated from the succeeding pair of datum rows by a row of very susceptible spreader, the American cultivar ‘Gus’ (PI494521). Treatments were randomised and replicated twice. The nurseries were artificially inoculated by injecting an aqueous suspension of *P. hordei* urediniospores into one tiller/m of spreader row. Epidemics were promoted with sprinkler irrigation applied in the late evening when temperatures were favourable for infection and high humidities and low winds at night were expected. When epidemics were sufficiently developed to allow clear differentiation among entries, disease was assessed on a whole plot basis using a 0–9 scale (McNeal et al. 1971), where 0 is immune and 9 is very susceptible. The scale provides a single-digit summary of the amount of disease and reaction type and provided good differentiation among genotypes. The disease data collected were summarised as treatment means.

Data curation

Data for lines displaying a heterozygous disease response were assigned missing values. The seedling data collected at Cobbitty (COB2009_Seedling and COB2011_Seedling) was converted from the 0–4 scale to a 0–9 scale to standardise data. The mean disease response per line for each environment was then used for association mapping. A summary of the number of genotypes, number of

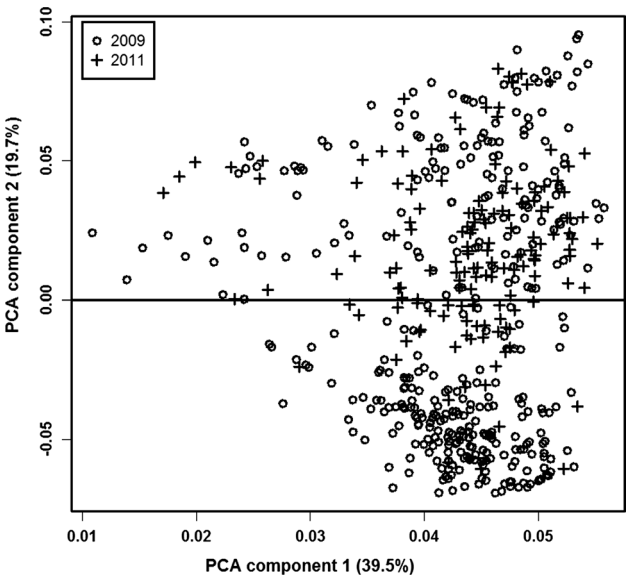

Fig. 1 PCA summary of breeding populations used in this study. Breeding population 1 (2009) across three environments, consisting of a total of 368 genotypes. Breeding population 2 (2011) across four environments and consisting of a total of 155 genotypes

polymorphic markers and environments used in this study is provided in Fig. 1 and Table S1.

Genotyping and construction of an integrated consensus map

Genomic DNA was extracted from bulked young leaf tissue sourced from 30 seeds representing each Stage 2 entry using the protocol recommended by diversity arrays technology (www.diversityarrays.com). A total of 523 lines (368 from BP1 and 155 from BP2) were genotyped with DArT markers using the Barley PstI (BstNI) v 1.7 array, which returned 1,411 polymorphic markers for BP1 and 1,159 for BP2, providing a total of 1,611 unique DArT markers across the two populations.

An integrated consensus map was constructed to maximise the number of DArT markers located on a single reference map, by manually projecting additional DArT markers onto the consensus map using bridge markers following the projection strategy detailed in Mace et al. (2009) (Table S2). The new DArT marker locations were based on genetic linkage maps for two doubled haploid (DH) populations: an ND19119-5/PI 642914 DH population that was developed for mapping Russian wheat aphid (RWA) resistance (W. Lawson, unpublished) and the ND24260/Flagship DH population (Hickey et al. 2011).

Phenotypic analysis

The mean disease response for each line obtained in each environment was used to generate frequency distribution figures for BP1 and BP2.

GxE between the trials used in this study was assessed using principal component biplots (Figs. 2, 3). Each year is presented in a separate figure due to the lack of genotype agreement between the years. In these figures the correlation between the trials can be found by assessing the direction and angle between the arrow vectors. Close arrows in the same direction indicate high correlation between trials.

Association mapping statistical analysis

A linear mixed model is used which simultaneously includes all the marker effects allowing for their correlations. Such methods have been proposed by Verbyla et al. (2007) and Smith (2011).

The model for data vector $\mathbf{y}^{n\times 1} = \mathbf{Y^n}$ can be written as

$$\mathbf{Y} = \mathbf{X}\tau + \mathbf{Z}_\mathrm{g}\left(\mathbf{M}\mathbf{u}_m + \mathbf{u}_\mathrm{g}\right) + \mathbf{Z}_\mathrm{o}\mathbf{u}_\mathrm{o} + \mathbf{e}$$

where the vector $\boldsymbol{\tau}$ is a vector of fixed effects, $\mathbf{u}_m$ is a $n_m \times 1$ vector of marker effects where n_m is the number of markers, $\mathbf{u}_\mathrm{g}$ is the $n_\mathrm{g} \times 1$ vector of residual genetic effects (not explained by the markers) where n_g is the number of genotypes and $\mathbf{u}_\mathrm{o}$ is the vector of random non-genetic (or peripheral, i.e. design and additional) effects. The matrix $\mathbf{X}$ is the design matrix for the fixed effects, the matrix $\mathbf{Z}_\mathrm{g}$ is the design matrix for the genotype effects and the matrix $\mathbf{Z}_\mathrm{o}$ is the design matrix for the non-genetic effects.

All random effects are assumed to follow a Gaussian distribution, with mean zero and each of the random effect vectors is assumed to be pairwise independent. Variances for the random and residual effects are $\sigma_m^2 I_{n_m}$, $\sigma_\mathrm{g}^2 I_{n_\mathrm{g}}$, $\sigma_o^2 I_o$ and $\sigma^2 I_n$ for the marker effects $\mathbf{u}_m$, the residual genetic effects $\mathbf{u}_\mathrm{g}$, the non-genetic random effects $\mathbf{u}_\mathrm{o}$ and the residual **e,** respectively, where we use I_n to denote an identity matrix of order n. σ_m^2 is a the genetic variance of the markers, σ_g^2 is a the residual genetic variance of the genotypes, σ_o^2 is the variance of the replicates and σ^2 is the residual variance.

The matrix M is the $n_\mathrm{g} \times n_m$ matrix of n_g genotypes by n_m markers with values of 1 and 0 representing the two alleles and missing values are imputed using the R package impute (Hastie et al. 2013).

The allele effects are the random BLUP effects $\mathbf{u}_m$ and are predicted by fitting this linear mixed model using the R package ASReml-R (Butler et al. 2009). The effects represent a linear regression of the trait of interest against the values representing the markers. In this instance, the effects are equivalent to the difference between the effect of allele "1" and allele "0".

Collation of previous QTL studies

Data on leaf rust resistance QTL from eight discovery publications were collated (Castro et al. 2012; Gonzalez et al. 2012; Hickey et al. 2011; Kicherer et al. 2000; Liu et al. 2011; Marcel et al. 2007; Qi et al. 1998; von Korff et al. 2005). From each study, data on the pedigree of the mapping population used, population type, population size, number of markers, number of linkage groups, map length, marker density and analysis method were collated (Table S3). In total, 11 populations were analysed across the eight publications.

The integrated map (Online Resource 1) was used as the reference map for QTL projection. From the eight studies, 60 individual QTL for resistance to *P. hordei* were included in the analysis (Fig. 4). Details were collected for each QTL or marker/trait association, including original marker interval, R^2 (% phenotypic variance explained), LOD value, direction of effect and published QTL symbol (where provided).

The locations of individual QTL were projected onto the integrated map based on flanking marker information in common between the individual study and the integrated map. For cases where flanking markers were not present

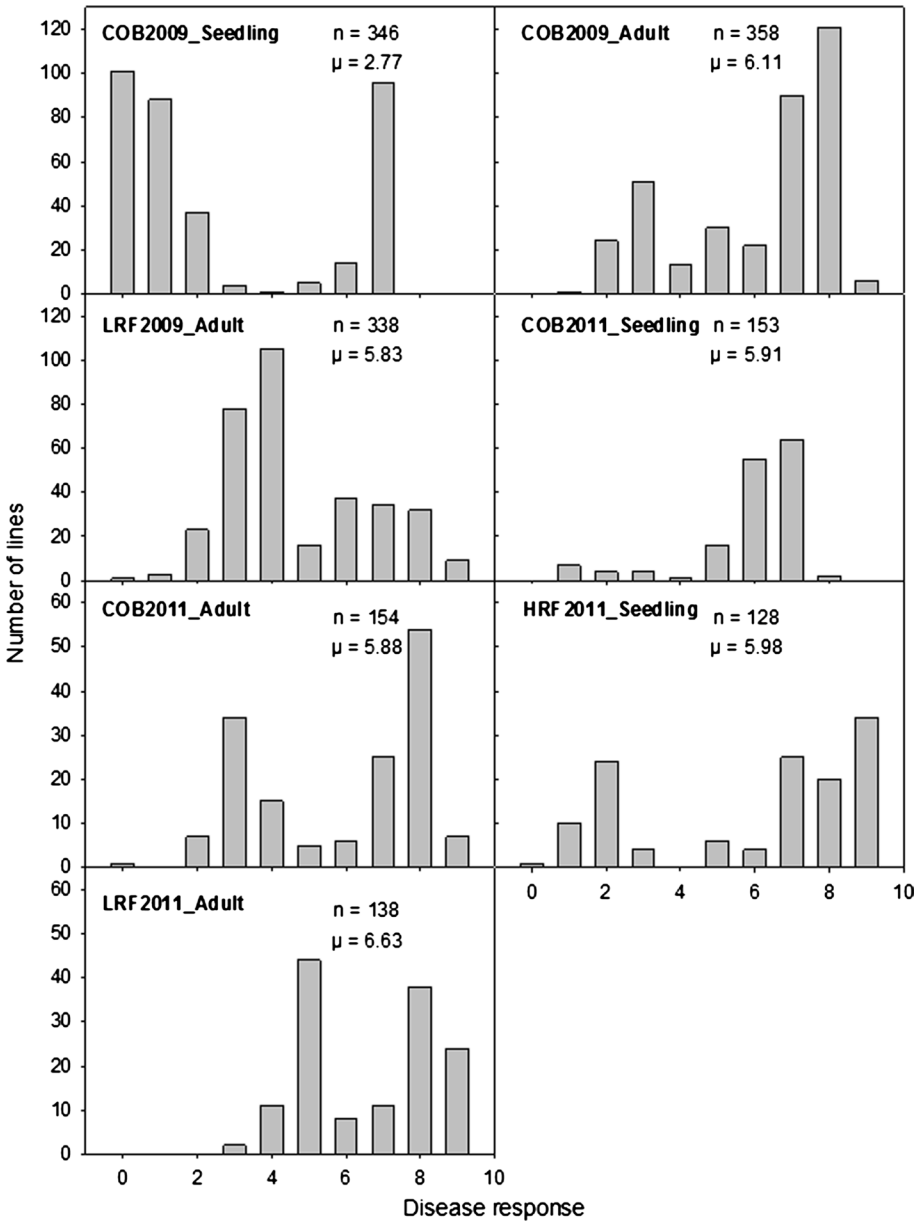

Fig. 2 Distribution of disease response to *P. hordei* in BP1 and BP2 assessed for seedling and adult plant resistance in 2009 and 2011, respectively

in the integrated map, their location was projected based on common markers (Cone et al. 2002; Mace and Jordan 2011). An individual QTL was not projected if the order of the flanking markers was inconsistent with the order of markers on the integrated map.

Confidence intervals for the projected QTL were calculated by adopting the formulae described by (Darvasi and Soller 1997; Guo et al. 2006) for F2/DH populations and recombinant inbred populations, respectively. These equations use population size and R^2 values (proportion of phenotypic variation explained) to estimate the confidence interval. For cases where R^2 values were not provided in a study, the projected QTL was assigned a confidence interval of 4 cM for graphical display purposes.

Diversity analysis of the *Rph20* region on chromosome 5HS

A diversity analysis of the *Rph20* region was performed using DARwin Version 5.0.158 (Perrier and Jacquemoud-Collet 2006). The analysis was performed using 10 DArT markers spanning 11.8 cM (21.2–33.0 cM) in the short arm of barley chromosome 4 (5HS) using the Sokal–Michener distance measure and a hierarchical neighbour-joining tree was generated. Analysis of the *Rph20* region was performed using lines from BP2 only due the high frequency of the *Rph3* gene in the breeding populations and the use of a pathotype virulent for *Rph3* for phenotyping in 2011. To investigate associations between marker haplotypes

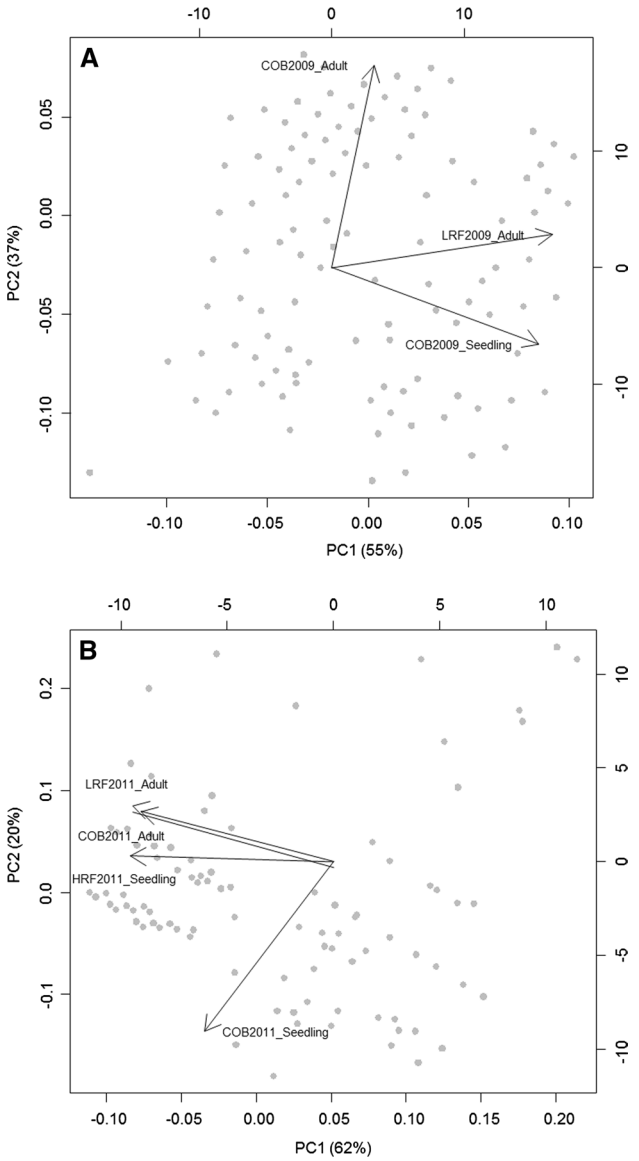

Fig. 3 **a** Biplots from principal component analysis using phenotype data for reaction to *P. hordei* for all lines in breeding population 1 (BP1), assessed for adult plant response in the field at Cobbitty 2009 (COB2009_Adult) and Toowoomba 2009 (LRF2009_Adult), and seedling response at Cobbitty 2009 (COB2009_Seedling). **b** Biplots from principal component analysis using phenotype data for reaction to *P. hordei* for all lines in breeding population 2 (BP2), assessed for adult plant response in the field at Cobbitty 2011 (COB2011_Adult) and Toowoomba 2011 (LRF2011_Adult), and seedling response at Cobbitty 2011 (COB2011_Seedling) and Warwick 2011 (HRF2011_Seedling)

and phenotype, lines were classed according to four phenotypic categories: (1) "seedling resistance" i.e. average seedling scores ≤2 at COB2011_Seedling (lines in this category were deemed to carry specific resistances as lines that showed APR in the field had scores of 2 or below in the Cobbitty seedling assessment), (2) "moderate-high levels of APR" i.e. average field scores ranging from 3 to 6 inclusive at LRF2011_Adult (lines with these scores were limiting rust development where pustules were constrained by chlorosis/necrosis and/or had markedly reduced sporulation), (3) "weak APR" i.e. average field scores from 7 to 7.5 at LRF2011_Adult (lines had 60–70 % infection, moderate sporulation, with no necrosis and little or no chlorosis), and (4) "susceptible" i.e. average field scores ≥8 at LRF2011_Adult (lines had >75 % infection, heavy sporulation and no tissue response).

Results

Phenotypic data summary

The disease response for entries in BP1 ranged from 1 to 9 at COB2009_Adult, 0 to 9 at LRF2009_Adult and 0 to 7 for the seedling assay performed at Cobbitty (COB2009_Seedling). The entries in BP2 displayed a similar range of resistance levels: 1 to 8 (COB2011_Seedling), 0 to 9 (COB2011_Adult), 0 to 9 (HRF2011_Seedling) and 3 to 9 (LRF2011_Adult). The mean disease response for BP1 assessed as seedlings at Cobbitty (COB2009_Seedling) was 2.77, and as adult plants assessed in the field the population mean at Cobbitty (COB2009_Adult) and Toowoomba (LRF2009_Adult) were 6.11 and 5.83, respectively (Fig. 2). The mean disease responses for BP2 assessed as seedlings at Cobbitty (COB2011_Seedling) and Warwick (HRF2011_Seedling) were 5.91 and 5.98, respectively (Fig. 2). When BP2 was assessed in the field at Cobbitty (COB2011_Adult) and Toowoomba (LRF2011_Adult), the mean disease responses were 5.88 and 6.63, respectively (Fig. 2).

In 2009, when the two different pathotypes were used for assessment of BP1 in field screening nurseries (i.e. COB2009_Adult and LRF2009_Adult; 5453P+ and 5457P+, respectively) there was only a weak correlation between responses to *P. hordei* observed in both environments (Fig. 3a). In comparison, reaction to *P. hordei* displayed in the seedling assay performed at Cobbitty (COB2009_Seedling) was strongly correlated with results from field screening conducted at Toowoomba (LRF2009_Adult) (Fig. 3a), in which the same pathotype was used.

Results from field screening nurseries conducted in 2011 for BP2 (i.e. LRF2011_Adult and COB2011_Adult) were highly correlated (Fig. 3b). Assessment of BP2 at the seedling stage performed at Warwick was also well correlated with field results in 2011. Despite the fact that the same pathotype was used for all experiments in 2011 (i.e. 5457P+), the results from seedling assessment conducted at Cobbitty had weaker correlations with the three other environments (Fig. 3b).

The quality of phenotypic data was very good; reflected by high estimates of heritability and strong correlations

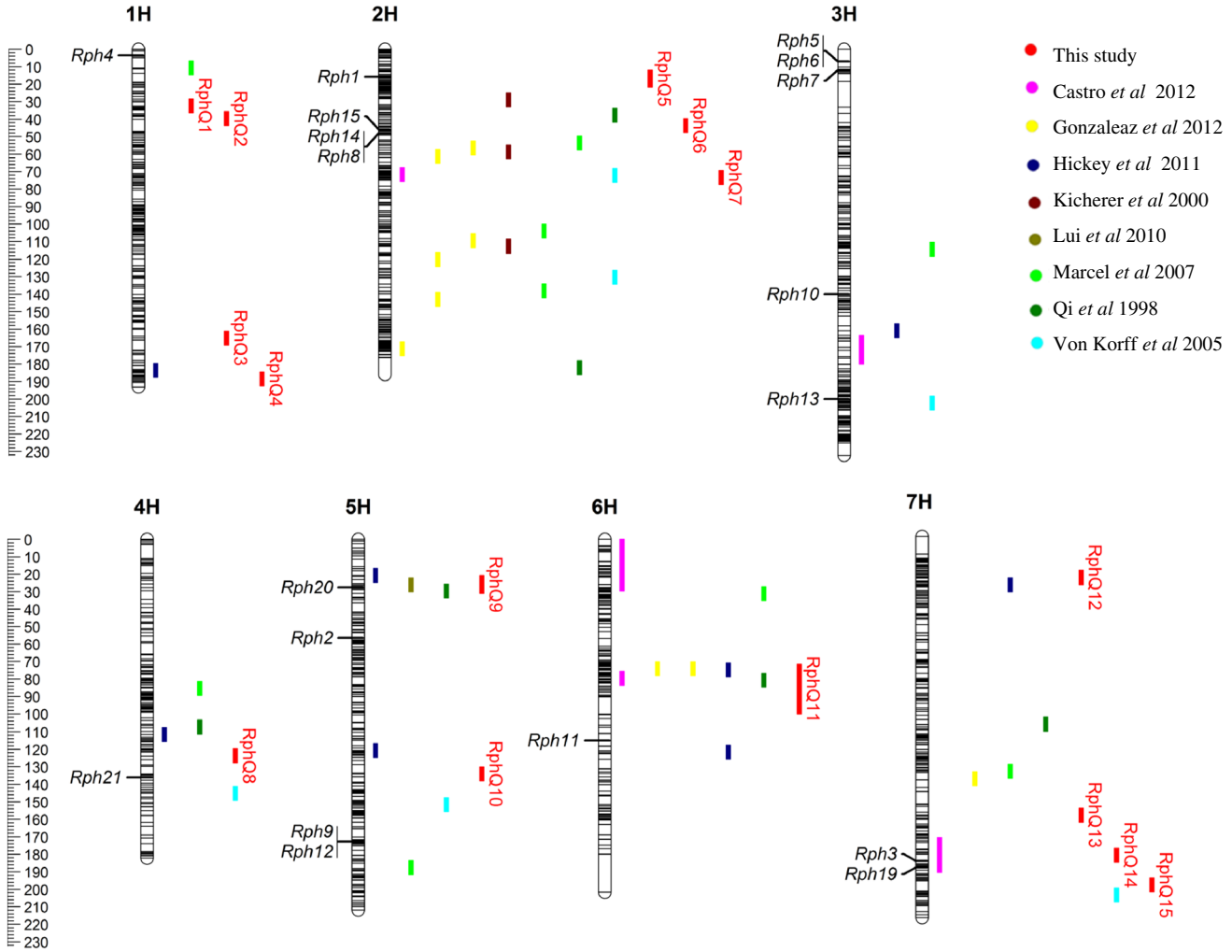

Fig. 4 Genomic regions for reaction to leaf rust identified from association mapping using elite breeding lines from the Northern Region Barley Breeding (NRBB) Program in Australia and projection of QTL reported in previous mapping studies. The key depicts the nine QTL studies displayed on the consensus map; *colours* indicate the different discovery studies as well as the association mapping performed in this study. For graphical display purposes, if the QTL confidence interval (CI) was <4 cM or the QTL was based on a single marker only, a CI of 4 cM was used. For further details about the eight QTL publications refer to Table S3

across environments, particularly when the same scoring system was adopted using the same pathotype. Heritabilities for disease response were calculated for two of the four environments in 2009 (BP1) and three of the four environments in 2011 (BP2). Heritability estimates were very high, ranging from 0.78 to 0.92. Heritabilities could not be estimated for environments where unreplicated experiments were conducted (i.e. COB2011_Seedling, COB2009_Seedling and COB2009_Adult); however, the disease responses of replicated check cultivars indicated a high degree of repeatability for these environments as well. The correlation between disease response for BP1 assessed using two different readings at LRF2009_Adult was very high (0.89). When the same pathotype of *P. hordei* was used for assessment of BP2 as adult plants in two different environments in 2011 (i.e. LRF2011_Adult and COB2011_Adult), the correlation between disease response data from each site was also very high (0.74).

Consensus map expansion

The barley DArT consensus genetic linkage map provided 2,957 locations for marker positioning (Wenzl et al. 2006). Integration of the RWA (ND19119-5/PI 642914) and ND24260/Flagship genetic linkage maps provided an additional 519 markers. The accumulation of these markers increased the total marker positions to 3,476 (Table S2). Final map length across the seven chromosomes was 1,417 cM with an average marker density of 2.8 (markers/cM) across the genome (Online Resource 1).

Of the 1,611 unique DArT markers initially obtained across BP1 and BP2, only approximately half (i.e. 845) could be positioned using the Wenzl et al. (2006) consensus map alone. The integrated map allowed positioning of an additional 393 markers. For BP1, 1,088 DArT markers could be positioned and 918 markers could be positioned for BP2. Of these markers, 768 were common across both populations, resulting in a total of 1,238 unique polymorphic markers with map locations.

Association mapping for rust resistance

Association mapping for resistance to *P. hordei* in BP1 (368 lines) and BP2 (155 lines) detected a total of 15 QTL across the seven environments, which were labelled as *RphQ1–15* (Table 1). Leaf rust QTL were detected on all chromosomes, except chromosome 3H (Table 1). Five QTL co-located with the genomic positions of catalogued leaf rust resistance genes *Rph1*, *3/19*, *8/14/15*, *20* and *21,* located on chromosomes 2H (*RphQ5 and RphQ6*), 4H (*RphQ8*), 5H (*RphQ9*) and 7H (*RphQ14*) (Fig. 4). Three QTL identified on chromosome 1H (i.e. *RphQ1–3*) and one QTL on chromosome 7H (*RphQ13*) did not align with any previously reported genomic regions influencing resistance to *P. hordei* and thus appear to be novel (Fig. 4). The remaining six QTL regions, *RphQ4*, *RphQ7*, *RphQ10–12* and *RphQ15*, corresponded with regions reported in previous QTL mapping studies (Fig. 4).

Of the 15 QTL identified in this study, 13 were detected in BP1 across the three environments (COB2009_Seedling, LRF2009_Adult and COB2009_Adult), represented by 25 markers with significant effects (Table 1). Eleven of these QTL were specific to this breeding population. Eleven of the QTL identified in BP1 were detected in field environments (i.e. *RphQ1, 3, 5, 8–15*), eight of which were not detected in the 2009 seedling assay (Table 1). Two of the QTL detected in BP1 were detected at the seedling stage only (i.e. *RphQ2* and *RphQ4*) and three at both developmental stages (i.e. *RphQ5*, *RphQ14* and *RphQ15*) (Table 1).

Despite the close proximity of *RphQ1* (32.4 cM) and *RphQ2* (39.7 cM) on chromosome 1H, the regions were identified as two separate QTL because *RphQ1* was identified in BP1 at the adult plant stage at Toowoomba, whereas *RphQ2* was identified at the seedling stage (Table 1).

Association mapping conducted for BP1 assessed at reading 1 (LRF2009_Adult) detected two QTL (*RphQ5* and *RphQ13*), which were not detected using the reading 2 datasets collected 11 days later (Table 1). Additionally, analysis of reading 2 data detected *RphQ3, 8, 10, 11* and *15*, which were not detected using the data from reading 1. The QTL region with the largest effect detected in BP1 was *RphQ14* on 7HL (178.9–185.4 cM), which corresponds to catalogued seedling resistance gene *Rph3*. *RphQ14* was detected in all environments where the pathotype avirulent for *Rph3* (i.e. 5453P+) was used for inoculation. In this region, DArT marker bPb-3145 had the most significant association, where the presence of marker allele "1" was associated with increasing susceptibility (marker effect +7.18; Table 1). Based on the DArT marker haplotype, the resistance allele for *Rph3* (*RphQ14*) was present in 182 of 368 lines in BP1 (i.e. 49 %). However, only 43 of 155 lines (i.e. 28 %) in BP2 carried the *Rph3* resistance allele.

In BP2, four QTL were detected; *RphQ6* (2H), *RphQ7* (2H), *RphQ8* (4H) and *RphQ9* (5H). *RphQ6* and *RphQ7* were specific to BP2. All four QTL were detected at the adult plant stage in the field environments; however, *RphQ7* and *RphQ9* were detected also at the seedling stage (Table 1).

Only *RphQ8* and *RphQ9* were detected in both breeding populations (Table 1). While *RphQ8* was detected in both BP1 and BP2, the QTL was detected for field screening nurseries conducted at Toowoomba only (i.e. LRF2009_Adult and LRF2011_Adult). In contrast, *RphQ9*, corresponding with the catalogued APR gene *Rph20* in chromosome 5HS, was detected in all field environments of this study (Table 1). DArT marker bPb-0837 (26.7 cM) was the most consistently associated with resistance in the region and its marker effect ranged from −1.87 to −3.10 across the four field environments (Table 1). Another marker, bPb-0292 (26.4 cM), was also strongly associated with the *Rph20* resistance (marker effect −3.38 in LRF2011_Adult), but data for this marker were only available for BP2. Based on the DArT marker bPb-0837, 89 lines in BP1 (i.e. 23 %) carried the resistance allele for *Rph20*; however, adjacent markers suggested that a number of them were heterogeneous. A higher frequency of the resistance allele for *Rph20* was observed for BP2 (i.e. 38 %).

DArT marker haplotypes in the Rph20 region

The critical marker haplotype associated with expression of APR gene *Rph20* was the presence of two DArT markers: bPb-0837 (26.7 cM) and bPb-0292 (26.4 cM). The majority of lines in BP2 with marker allele "1" for these two markers displayed moderate to high levels of APR (Fig. 5). Lines with this marker haplotype were clustered with standards previously reported to carry *Rph20*, such as elite breeding line NRB06059 (Mackay*2/WI2314) and Australian cultivar Mackay (Cameo/Koru) (Fig. 5). Breeding lines lacking these two critical DArT markers (marker allele "0") clustered together and the majority of these lines displayed either a susceptible or only weak APR phenotype. These lines clustered together with standards (e.g. Fitzroy, Grout and Gairdner) that are known to lack *Rph20* and are susceptible to *P. hordei* pathotype 5457P+ (Fig. 5). Lines that were postulated to carry seedling resistance (based on data

Table 1 Summary of the 15 leaf rust reaction QTL detected in in BP1 (2009) and BP2 (2011) at two developmental stages: APR (Adult) and seedling stage

QTL name	Chr.	Marker name	Co-located *Rph* gene	Position DArT map (cM)[a]	COB2009 seedling[b]	LRF2009 adult[b, c] Reading 1	LRF2009 adult[b, c] Reading 2	COB2009 adult[b, c]	HRF2011 seedling[c]	COB2011 seedling[c]	LRF2011 adult[c]	COB2011 adult[c]
RphQ1	1H	bPb-3117	[d]	32.4	NS[1]	2.17*	1.83*	NS	NA[2]	NA	NA	NA
RphQ2	1H	bPb-4793	[d]	39.7	**2.98***	**NS**	NS	NS	NS	**NS**	NS	**NS**
RphQ3	1H	bPb-2565	[d]	165.2	**NS**	**NS**	**1.90***	NS	NA	NA	NA	NA
RphQ4	1H	bPb-8308	[e]	188.5	3.08*	NS	NS	**NS**	NS	NS	NS	NS
RphQ5	2H	bPb-2279	*Rph1*	12	**NS**	NS	NS	1.68*	NS	**NS**	NS	NS
		bPb-7445		21.7	**2.49***	**1.81***	**NS**	**NS**	NS	NS	**NS**	**NS**
RphQ6	2H	bPb-6755	*Rph8/14/15*	42.8	**NS**	NS	NS	NS	NS	NS	2.56**	2.07*
		bPb-9682		43.9	**NS**	**NS**	NS	**NS**	NS	NS	2.33*	NS
		bPb-4261		43.9	**NS**	**NS**	**NS**	NS	NS	NS	2.33*	NS
RphQ7	2H	bPb-9925	[e]	73.4	**NS**	**NS**	**NS**	**NS**	**2.65***	**NS**	**2***	**NS**
RphQ8	4H	bPb-3809	*Rph21*	120.8	**NS**	**NS**	**NS**	NS	**1.56**	**NS**	**1.93***	**NS**
		bPb-9440		128.9	NS	NS	1.62*	NS	NA	NA	NA	NA
RphQ9	5H	bPb-8580	*Rph20*	20.7	NS	NS	NS	NS	NS	**NS**	1.85*	NS
		bPb-1084		25.3	**NS**	NS	NS	NS	NS	**NS**	3.21***	2.11*
		bPb-33276		25.4	NS	NS	NS	NS	NS	**NS**	2.41**	2.77**
		bPb-47406		25.4	**NS**	NS	NS	1.68*	NS	**NS**	2.77**	NS
		bPb-4814		25.5	**NS**	NS	NS	1.87*	NA	NA	NA	NA
		bPb-0292		26.4	NA	NA	NA	NA	**NS**	**NS**	**3.38*****	**NS**
		bPb0837		26.7	**NS**	**1.87***	**2.65*****	**2.61****	**3.10***	**NS**	**3.1*****	**NS**
		bPb-8572		28.2	**NS**	NS	NS	1.68*	NS	NS	2.56**	NS
		bPb-2460		31	**NS**	NS	NS	NS	NS	NS	2.31*	NS
		bPb-8072		31	**NS**	NS	NS	NS	NS	NS	2.41*	NS
RphQ10	5H	bPb-6126	[e]	134.1	**NS**	NS	1.78*	NS	NS	**NS**	**NS**	**NS**
RphQ11	6H	bPb-3722	[e]	71.6	NS	NS	1.68*	NS	**NS**	**NS**	NS	NS
		bPb-3744		81.5	**NS**	**NS**	**1.71***	**NS**	NS	NS	**NS**	**NS**
		bPb-0432		100.1	NS	NS	1.53*	NS	**NS**	NS	NS	**NS**
RphQ12	7H	bPb-0398	[e]	19.3	**NS**	NS	1.58*	**NS**	**NS**	**NS**	**NS**	**NS**
		bPb-9202		27.7	**NS**	2.18**	NS	NS	NS	**NS**	**NS**	**NS**
RphQ13	7H	bPb-5260	[d]	159.3	**NS**	**1.96***	**NS**	**NS**	NA	NA	NA	NA
RphQ14	7H	bPb-3484	*Rph3/19*	178.9	NS	2.17**	NS	NS	NS	NS	**NS**	NS
		bPb-9104		180.3	**4.95*****	**1.77***	**1.71***	**NS**	NS	NS	NS	NS
		bPb-0364		185.4	7.04***	6.87***	5.99***	**NS**	**NS**	**NS**	**NS**	**NS**
		bPb-1767		185.4	6.93***	7.05***	5.68***	**NS**	**NS**	**NS**	NS	**NS**
		bPb-3145		185.4	7.18***	6.46***	5.39***	**NS**	**NS**	**NS**	NS	**NS**
		bPb-3875		185.4	6.68***	6.39***	5.65***	**NS**	NS	**NS**	NS	**NS**
RphQ15	7H	bPb-1232	*Rph3/19*	199.2	**2.76***	**NS**	**1.82***	NS	NS	NS	NS	NS

Catalogued *Rph* genes that aligned with QTL detected are presented here. List of all markers significantly associated with reaction to *P. hordei* in at least one environment. Marker effects are calculated as the difference between the allelic effect of the "1" allele minus the effect of the "0" allele

Bold indicates a negative association between leaf rust reaction and the positive DArT allele at each locus, and non-bold indicates a positive association between leaf rust reaction and the positive DArT allele at each locus

NS[1] no significant data at this location, *NA*[2] no data because marker data was missing in this population

* Significant at the 5 % level

** Significant at 1 % level

*** Significant at 0.1 % level—*P* values were derived from the fixed linear model

[a] Positioning of marker on the integrated consensus map (Online resource 1)

[b] Pathotype 5453 P+

[c] Pathotype 5457 P+

[d] QTL novel to this study

[e] Alignment with QTL presented in previous mapping study

from COB2011_Seedling) were scattered throughout the hierarchical tree, regardless of their marker haplotype in the *Rph20* region (Fig. 5). Interestingly, eight lines in BP2 lacked the critical marker haplotype for *Rph20*, but displayed moderate to high levels of APR and clustered with the lines displaying a susceptible phenotype (Fig. 5).

Discussion

This study reports on the use of two interrelated breeding populations to identify QTL for leaf rust resistance in multiple environments. Previous mapping studies detected four QTL for seedling resistance that co-located with previously catalogued major effect genes *Rph4*(1) and *Rph8/14/15*(3). Six QTL for APR co-located with catalogued genes *Rph13*(1), *Rph20*(3), *Rph11*(1) and *Rph3/19*(1). In the present study, we identified 15 QTL in total: 5 co-located with previously identified major effect resistance genes, 6 co-located with previously identified QTL for resistance and 4 were novel. Of the 15 QTL detected in this study, 13 were present in BP1; however, only 4 were identified within BP2. This is largely due to the reduction in population size from 368 in 2009 to 155 in 2011 as lines with susceptible reactions would have been removed from the breeding program. Another contributing factor would have been the increased virulence of the *P. hordei* isolate that was used to screen BP2 as well as the conditions under which the *Rph20* gene is expressed (Singh et al. 2013).

Association mapping detects pathogen evolution and effects of selection

During the course of this study a major change in the pathogenicity of *P. hordei* was observed (Park 2009). The pathotypes used for leaf rust screening during 2009 changed from avirulent to virulent on lines with the *Rph3* gene for resistance. Results from the 2009 seedling assay (COB2009_Seedling) and the two readings from the Toowoomba field nursery (LRF2009_Adult) showed that *RphQ14* on 7HL conferred a large portion of the resistance observed. However, this QTL was not observed in other leaf rust screenings (Table 1). Since *Rph3* (*RphQ14*) was used widely as an effective resistance source until 2009, 182 of 368 (49 %) entries in BP1 had the molecular markers associated with *Rph3*. In BP2, 43 of 155 (28 %) entries had the *Rph3* markers. The change in frequencies was most likely caused by the severe leaf rust epiphytotic in 2010 which shifted the BP2 population toward resistance genes other than *Rph3*. These observations highlight the effectiveness of association mapping to detect changes in pathogenicity (as a result of selection) and the utility of using specific markers to observe changes in gene frequencies.

One leaf rust resistance gene with a major effect (i.e. *Rph15*) was introduced purposely to the BP2 population. The *Rph15* gene is present in a few lines, which is reflected in the detection of QTL *RphQ6* (near DArT marker bPb-6755); however, the LD in this region is not adequate to predict *Rph15* reaction type based on molecular markers alone. A molecular marker platform with increased density (e.g. the DArTseq platform or a 10K SNP array) might enable detection of *Rph15* based on markers alone.

The only known major effect gene detected across all field environments in this study is *Rph20*, which confers moderate resistance to leaf rust as an adult plant and in some environments exhibits reduced pustule development on seedlings (Golegaonkar et al. 2010; Hickey et al. 2011). The QTL *RphQ9* is near the region of chromosome 5H where *Rph20* is located and molecular marker bPb-0837 is diagnostic for the presence of *Rph20* (Hickey et al. 2012). Eighty-nine lines in BP1 (i.e. 23 %) had the bPb-0837 marker, but adjacent markers suggest that a number of them were heterogeneous. However, 53 lines (38 %) in BP2 had the bPb-0837 marker. This indicates an increase in the frequency of the *Rph20* gene and a decrease for *Rph3* dictated by the pathotype change and a severe epiphytotic during 2010.

Alignment of QTL with those reported in previous studies

All other QTL for leaf rust resistance identified in this study appear to be environment specific. Although *RphQ8* was detected in both BP1 and BP2, it was detected in field nurseries conducted in Toowoomba only. The pattern of finding significant, but minor effect QTL for leaf rust reaction, which are environment specific, has been reported in other studies (Castro et al. 2012; Hickey et al. 2011; Qi et al. 1998; von Korff et al. 2005). Other than *Rph20*, none of the QTL detected in this study seemed to confer a major change in the level of leaf rust resistance that was consistent across the two breeding populations. For instance, *RphQ5* on chromosome 2H was consistently detected across three environments (seedling and adult stage) in 2009, but was not detected for BP2 in 2011. Another example is *RphQ6*, a QTL positioned on chromosome 2H, which was detected in two environments in BP2, but was not detected for BP1. As the marker density for whole genome profiles is increased and larger numbers of lines are scored, the ability to follow the minor QTL in breeding material will improve. As with major effect QTL, changes in gene frequencies in breeding populations could be determined over time.

The detection of QTL that appear to be population specific creates challenges for utilising these resistance factors in breeding programmes. A large number of QTL mapping studies Qi et al. (1998), Hickey et al. (2011), Liu et al. (2011), Castro et al. (2012) and Gonzalez et al. (2012) have

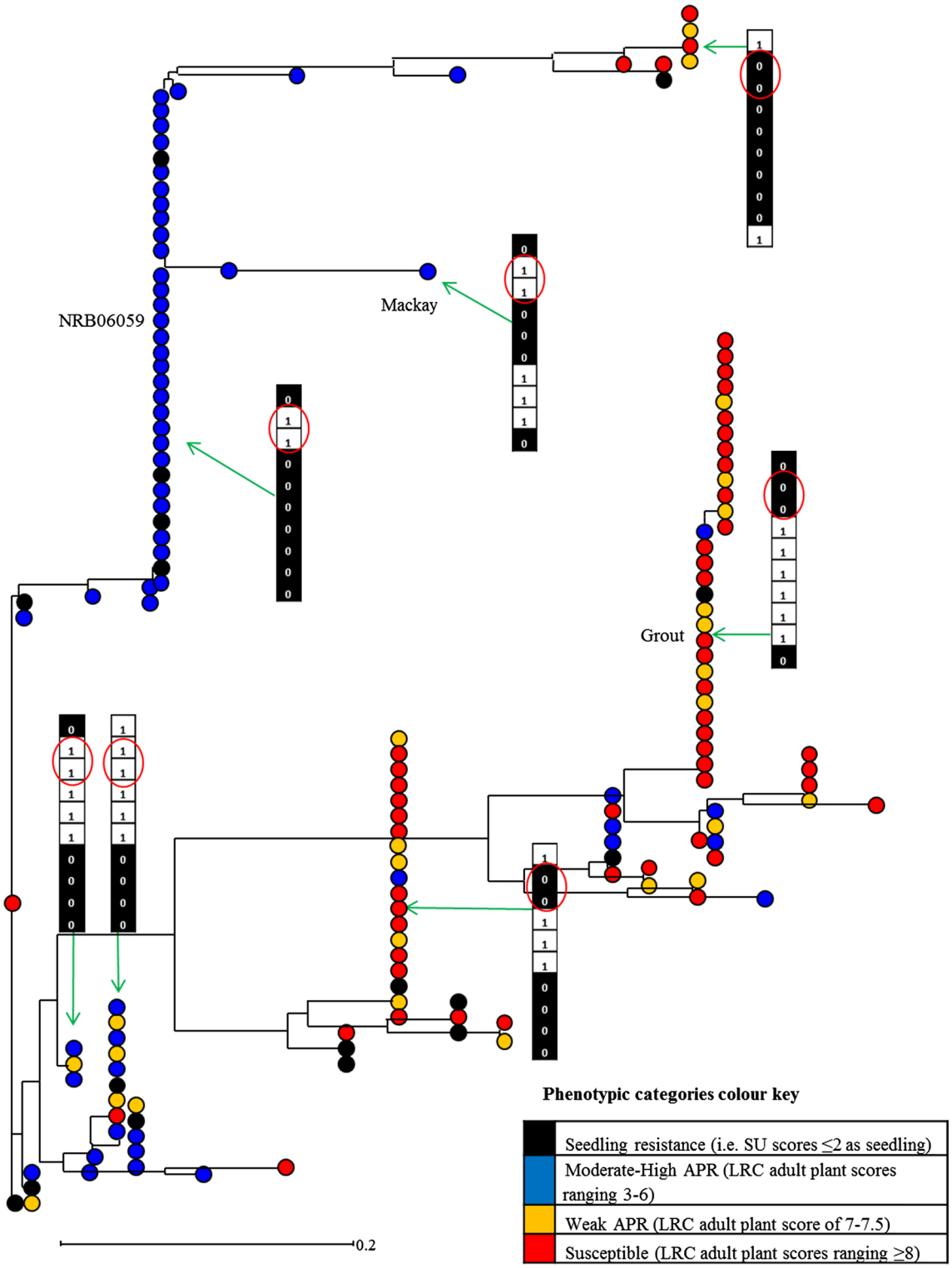

Fig. 5 Hierarchical neighbour-joining tree displaying results from diversity analysis of the *Rph20* region in breeding population 2 (BP2) using ten DArT markers spanning the 11.8 cM region (i.e. 21.2–33.0 cM) on chromosome 5HS. DArT marker haplotypes are displayed for key standards and clusters of lines. The two DArT markers critical for expression of *Rph20*; bPb-0837 (26.29 cM) and bPb0292 (26.36 cM) are *circled red*. *Lines* indicated by nodes, were classed according to four phenotypic categories, including *1* seedling resistance, *2* moderate-high levels of APR, *3* weak APR and *4* susceptible

investigated the genetic control of resistance to *P. hordei* using sources of resistance that trace back to the parent of Vada; *H. laevigatum* (Hickey et al. 2012). Despite a common source of resistance across mapping populations (Table S3), the QTL identified (and their effects) are highly variable. Different aspects of each genetic study are a probable cause, including genetic background, pathotypes, growth stage, methods used to quantify levels of resistance, environmental differences, DNA marker systems, mapping method, etc. Further, in the bi-parental populations, investigations are limited to only two segregating alleles at each locus. This has implications when comparing studies because populations subject to genetic analysis could be derived from parents that carry the same allele at a locus influencing reaction to *P. hordei*, in which case the resistance allele will not be detected and the QTL region will not be identified. Association mapping reduces this problem as more genetic diversity can be analysed and there is potential for multiple alleles segregating at any one locus. Also, as pointed out by Varshney et al. (2012), phenotypic detection of minor effect QTL may occur only when several are combined.

Despite such major differences between mapping studies, it is important to identify consistencies where possible. Thus, we have highlighted the regions that appear to align with QTL identified in this study by projecting onto our consensus map, the position of QTL reported in previous mapping studies (Table S3; Fig. 4).

Four QTL co-located with the genomic positions of catalogued seedling resistance genes *Rph1*, *3/19*, *8/14/15*, and *21,* located in chromosomes 2H, 4H and 7H (Fig. 4). It is reassuring to observe this alignment of QTL with well-characterised resistance genes because it confirms the accuracy of the association mapping approach. Perhaps, one of the most promising regions contributing APR is the 6HL QTL (*RphQ11*), which has been reported previously in numerous mapping studies (Castro et al. 2012; Gonzalez et al. 2012; Hickey et al. 2011; Qi et al. 1998) and the QTL intervals appear to align quite well on the consensus map (Fig. 4). In the study by Hickey et al. (2011), a QTL conferring resistance in the ND24260/Flagship DH population was donated by ND24260. The QTL conferred only a low level of resistance alone, but when combined with APR gene *Rph20*, it provided high levels of APR across environments.

Other sources of APR to *Puccinia hordei*

DArT marker bPb-0837 is associated strongly to the resistance allele for *Rph20* (Hickey et al. 2011). Linkage between the marker and the APR phenotype has remained despite considerable crossing and recombination in the region for more than 60 years (Hickey et al. 2012), and remains the only reported simply inherited gene conferring moderate levels of APR to *P. hordei*. We currently face overexploiting *Rph20* via selection and deployment of a single resistance factor. This places higher selection pressure on mutations in the pathogen population. Prior to identification of the DArT marker associated with *Rph20*, breeders around the world were selecting for resistance to leaf rust based on phenotype. This presumably resulted in selection of genotypes carrying *Rph20* in combination with other minor APR genes. Although *Rph20* confers partial resistance, which is regarded as pathotype non-specific, pathotype specificity has been reported in numerous APR pathosystems (Gonzalez et al. 2012). For example, a number of major genes for APR to leaf rust in wheat (*Lr12*, *Lr13*, *Lr22a* and *Lr37*) are race specific.

The current study identified a small set of eight lines in BP2 that may carry alternative sources of major APR gene(s) or multiple minor APR genes. These lines lack the *Rph20* resistance allele, based on DArT markers, but display moderate to high levels of APR. Allelism testing is required to confirm that these resistances are in fact different; however, allelism testing for APR genes in not straight forward because APR genes are known to interact with genetic backgrounds and expression is influenced by environmental conditions.

The three QTL identified in chromosome 1H (i.e. *RphQ1–3*) and the QTL in chromosome 7H (*RphQ13*) do not align with any previously reported regions influencing reaction to *P. hordei*, thus they appear to be novel. Of these, *RphQ2*, *RphQ3* and *RphQ13* were detected only at the adult plant stage in the field; however, they were not detected in BP2 in 2011 (Table 1). *RphQ8* positioned in chromosome 4H was detected only at the adult plant stage and in both BP1 and BP2, but not in all field environments. *RphQ6* in chromosome 6H was detected only in adult plant tests in BP2, but this region likely corresponds with seedling resistance gene *Rph15* (Fig. 4). It is possible that breeding lines carrying effective seedling resistance genes (e.g. *Rph7*, *Rph15*) may also carry genes for APR, but their effects would be masked by the seedling resistance gene. As mentioned above, the region of *RphQ11* in chromosome 6HL (*qRphND*) has been identified previously in several mapping studies and its effect when coupled with *Rph20* has been validated (Hickey et al. 2011). A number of entries in BP2 lack *Rph20*, but carry weak levels of APR, which were identified via haplotype analysis of the *Rph20* region (Fig. 5). These lines could be useful sources of minor APR genes, which when combined with *Rph20* may provide high levels of resistance across environments.

Future direction

The QTL detected in this study are relevant to the NRBB germplasm, thus MAS can be implemented immediately to

pyramid these genomic regions for durable control of leaf rust. Lines carrying desirable gene combinations could be used in backcrossing to other germplasm. Unfortunately, the value of one new APR gene is unlikely to justify its transfer into elite breeding material (typically a 10- to 20-year process). Transferring several APR genes at the same time into locally adapted germplasm may reduce the cost and time associated with their deployment.

Considering APR gene *Rph20* confers only a weak level of partial resistance when deployed alone, in theory elite breeding lines that carry the *Rph20* resistance allele and express high levels of resistance are likely to have other minor APR genes in addition to *Rph20*. Based on knowledge of their genome positions, these minor effect genes could be manipulated via MAS. We propose utilisation of elite breeding populations for rapid discovery and validation of minor APR genes. This approach is less expensive (phenotypic data can be collected as part of the breeding process), faster (eliminates need for population development), permits high-resolution mapping (populations with low LD) and encompasses more genetic diversity than a traditional bi-parental population. Elite breeding lines could be selected from crosses fixed for *Rph20*, but diverse for complementary minor APR genes, and used for QTL discovery. A targeted approach such as this would deliver relevant information to breeders who can pass the benefits to barley growers via new cultivars possessing high levels of APR to *P. hordei*.

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Conflict of interest The authors declare that they have no conflict of interest.

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