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ORIGINAL RESEARCH

Adult resistance genes to barley powdery mildew confer basal penetration resistance associated with broad-spectrum resistance

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Abstract

Powdery mildew isa major disease of barley (*Hordeum vulgare* L.) for which breeders have traditionally relied on dominant, pathogen race-specific resistance genes for genetic control. Directional selection pressures in extensive monocultures invariably result in such genes being overcome as the pathogen mutates to evade recognition. This has led to a widespread reliance on fungicides and a single broad-spectrum recessive resistance provided by the *mlo* gene. The range of resistance genes and alleles found in wild crop relatives and landraces has been reduced in agricultural cultivars through an erosion of genetic diversity during domestication and selective breeding. Three novel major-effect adult plant resistance (APR) genes from landraces, designated Resistance to *Blumeria graminis* f. sp. *hordei* (*Rbgh1* to *Rbgh3*), were identified in the terminal regions of barley chromosomes 5HL, 7HS, and 1HS, respectively. The phenotype of the new APR genes showed neither pronounced penetration resistance, nor the spontaneous necrosis and mesophyll cell death typical of *mlo* resistance, nor a whole epidermal cell hypersensitive response, typical of race-specific resistance. Instead, resistance was localized to the site of attempted penetration in an epidermal cell and was associated with cell wall appositions and cytosolic vesicle-like bodies, and lacked strong induction of reactive oxygen species. The APR genes exhibited differences in vesicle-like body sizes, their distribution, and the extent of localized 3,3-diaminobenzidine staining in individual doubled haploid lines. The results revealed a set of unique basal penetration resistance genes that offer opportunities for combining different resistance mechanisms in breeding programs for robust mildew resistance.

Abbreviations: APR, adult plant resistance; *Bgh*, *Blumeria graminis* f. sp. *hordei*; CWA, cell wall apposition; DAB, 3,3-diaminobenzidine; dai, days after inoculation; DH, doubled haploid; HR, hypersensitive response; IT, infection type; LOD, logarithm of odds; QTL, quantitative trait locus; *Rbgh*, Resistance to *Blumeria graminis* f. sp. *hordei*; *R*-gene, race-specific resistance gene; SSR, simple sequence repeat; VLB, vesicle-like body.

1 | INTRODUCTION

Core Ideas

- Atypical adult powdery mildew resistance was identified in barley landraces.
- Three major-effect adult resistance loci were mapped close to chromosomal ends.
- Cytological studies indicated shared basal penetration phenotypes.

Assimilation of new genes and alleles into breeding programs from exotic germplasm is essential for sustaining improvements in desirable traits such as yield, environmental adaption, and disease resistance. Elite barley (*Hordeum vulgare* L.) lines are less diverse than ancestral forms (Caldwell et al., 2006), with selection by breeders leading to elevated levels of linkage disequilibrium (nonrandom association of alleles) at distinct loci. Muñoz-Amatriaín et al. (2014) used genome-wide genotyping of a worldwide collection of barley landraces and cultivars to confirm that modern barley contains regions of high linkage disequilibrium associated with traits involved in domestication and breeding selection. Tondelli et al. (2013) compared recent and old European two-row spring barley germplasm and also suggested breeders' selection of elite lines has led to fixation of traits at the expense of genetic diversity, although some genomic regions display diversity, possibly through efforts to introduce disease resistance from wild relatives. Fortunately for cultivated barley, introducing desirable traits is aided by significant genetic repositories that include historic cultivars, landraces, and closely related wild species.

Barley powdery mildew [*Blumeria graminis* f. sp. *hordei* (*Bgh*)] exists throughout temperate regions where barley is grown. The disease is highly mobile, with airborne spores capable of spreading hundreds of kilometers (Brown & Hovmøller, 2002). *Blumeria graminis* f. sp. *hordei*, like all crop powdery mildews, is especially prone to developing fungicide resistance (Grimmer et al., 2015). *Blumeria graminis* f. sp. *hordei* also has a propensity to acquire virulence against race-specific resistance genes (*R*-genes) traditionally favored by breeders (Jørgensen, 1994), and their transience is illustrated by virulence to almost all recently deployed *R*-genes in Europe being present in just two isolates (Spies et al., 2012). Durable resistance genes to powdery mildew are therefore preferable, two forms of which are known in barley. The most common is based on the use of recessive alleles of the *Mlo* gene. Out of the more than 40 *mlo* alleles known to date (Reinstädler et al., 2010), only *mlo-11*, *mlo-11(cnv2)*, and a point mutation have been reported to occur naturally (Ge et al., 2016; Reinstädler et al., 2010). Two mutants, *mlo-11* and *mlo-9*, are widely used in European spring barley cultivars and were present in over 75% of cultivars registered in the Czech Republic from 2011 to 2015 (Dreiseitl, 2017; Jørgensen, 1992). Nevertheless, although *mlo* resistance has proven to be stable over several decades, over-reliance on a single resistance mechanism in broad-scale agriculture poses strong directional pressure on the pathogen to evolve virulence.

The second form of durable resistance involves genes other than major *R*-genes. These may be expressed at all growth stages or at different stages, and include partial or quantitative resistance and adult plant resistance (APR). Although *R*-gene resistance is race-specific and nondurable, quantitative resistance and APR may be considered non-race-specific and durable (Hwang & Heitefuss, 1982). Adult plant resistance to cereal fungal diseases provides protection in the host's later growth stages, normally between tillering and booting. In cereals, APR is effective against a range of pathogens, such as wheat powdery mildew (Vikas et al., 2020; Wang et al., 2005), wheat rust (Huerta-Espino et al., 2020), barley leaf rust (Rothwell et al., 2019), and spot and net forms of barley net blotch (reviewed in Clare et al., 2020). Adult plant resistance to barley powdery mildew has been documented previously (Heitefuss et al., 1997; Hwang & Heitefuss, 1982), with Gupta et al. (2018) being the first to report an APR quantitative trait locus (QTL) in the accessions 'CLE210' from Uruguay and 'Denar' from the Czech Republic. In this study, APR refers to resistance expressed in tissues other than seedlings, normally from the fifth leaf stage. APR resistance genes offer the opportunity of combining genes with different underlying mechanisms such as the wheat rust and powdery mildew resistance genes *Lr34* and *Lr67* (Moore et al., 2015; Ren et al., 2017), under the hypothesis that combinations of resistance mechanisms are more difficult for a pathogen to overcome, as well as potentially prolonging the effectiveness of any new major resistance genes.

The objectives of this study were to identify novel and potentially durable barley powdery mildew resistance genes and to determine their characteristics and genetic basis. To achieve this, barley landraces from centers of barley genetic diversity (Ethiopia, the Near East, and Asia) were previously screened with Western Australian *Bgh* pathotypes that are virulent against several major *R*-genes. Landraces were screened for seedling plant resistance and APR, and included the durably resistant barley accessions described by Spies et al. (2012) as candidates. Here, we report the underlying genetic basis for resistance found in a six-rowed landrace from Turkey ('HOR3270'), which was resistant at both the seedling and adult stages, and a six-rowed landrace from Azerbaijan ('Eth069'), which was resistant at the adult stage. Resistance genes were mapped in doubled haploid (DH) populations and evaluated phenotypically at the macroscopic and cytological levels.

2 | MATERIALS AND METHODS

2.1 | Plant and fungal materials

Barley accessions used in this study were obtained from the Australian Grains Genebank (Horsham, Vic.). The identifying codes are Eth069 (PI 68192) and HOR3270. 'Baudin', used as a susceptible parent, is an Australian malting cultivar. A Western Australian *Bgh* isolate, Chi-001, which is virulent to the *Mlo* resistance genes *a7*, *a8*, *a10*, *a12*, and *a13* in near-isogenic lines of 'Pallas' (Kølster et al., 1986), was used for inoculations. This isolate was selected as representing *Bgh* virulence genes that are common in Western Australia following pathotype screening.

2.2 | Detached leaf and whole plant disease assays

Blumeria graminis f. sp. *hordei* maintenance, plant growth, and *Bgh* inoculation conditions were as described previously (Ge et al., 2016). Disease symptoms were rated on an infection type (IT) scale from 0 to 4, where 0 = no visible mycelium; 1 = sparse mycelial development with no sporulation; 2 = mycelia present with very few spore chains; 3 = moderate mycelial development and discrete lesions with sporulation; 4 = amorphous mycelial development and abundant sporulation (Mains & Dietz, 1930). Doubled haploid, F_1, and F_2 seedlings (first leaf) were inoculated and phenotyped as detached leaves on benzimidazole agar plates (Tucker et al., 2013), with leaves from three replicate plants used for the DH lines. Adult DH population plants were grown in a polyethylene tunnel between June and September and inoculated at 7 wk, then phenotyped at 8 wk. In Australia, the main growing season for barley is over these winter months when the mean temperatures in Perth are <15 ˚C and the daylength is <11 h, which are conditions conducive to vegetative growth, although the high levels of humidity in a covered environment favor powdery mildew. For each population, three plants per DH line were planted per pot, with the DH lines randomized in a complete block with two experimental replications. Doubled haploid lines showing aberrant characteristics resulting from unfavorable gene combinations in wide crosses (chlorotic leaves and stressed or stunted growth) were excluded from phenotyping. Powdery mildew colonies of individual DH lines containing single APR genes only and parental lines were counted on detached leaves. Four replicate experiments were conducted with three detached leaves per growth stage. Each detached leaf was taken from a different plant, with seeds germinated to synchronize fully expanded leaves at each growth stage. Detached leaves were inoculated on benzimidazole agar plates (Tucker et al., 2013) and *Bgh* colonies were counted within a single 2.5- by 0.6-cm section per leaf. A ruler was applied across all leaves on a plate to establish a common 2.5-cm-long region and the width was set from the left-hand side of each leaf. All experiments, unless specified otherwise, were phenotyped at 7 d after inoculation (dai) and rescored at 10 dai.

2.3 | Cytology

Two staining protocols were used to examine disease progression and host responses following inoculation with *Bgh* conidia. Reactive oxygen species production was detected via the 3,3-diaminobenzidine (DAB) uptake protocol described by Thordal-Christensen et al. (1997). Triple staining with aniline blue, calcofluor white, and Evans blue was performed following the protocol Herburger and Holzinger (2016), with the exception of staining with 0.1% calcofluor white. Staining protocols were performed with 0.5-cm^2 leaf segments from 10 independent leaves per accession. Images were captured with an Olympus BX51 microscope (Olympus Corporation) or a Nikon A1 confocal microscope (Nikon Corporation) for triple-stained samples.

2.4 | Construction of DH populations

All plants used to produce F_1 crosses were derived from a single seed from each accession, with the cultivar Baudin used as the powdery-mildew-susceptible parent. To verify F_1 individuals, a set of 19 simple sequence repeats (SSRs) (Hearnden et al., 2007) were screened to detect polymorphisms between the parent lines. No heterozygous SSRs were observed within the parent lines. Doubled haploid populations were developed via androgenesis with anther culture (Broughton et al., 2014) at the Department of Primary Industries and Regional Development, Western Australia. Over 400 DH lines per population were produced. Progeny exhibiting chimeric or tetraploid traits were excluded from genetic mapping.

2.5 | DNA extraction and genotyping

Leaf tissues were sampled from seedlings of each parent or DH line, and genomic DNA was isolated using the cetyltrimethylammonium bromide method originally described by Doyle and Doyle (1987) with the modifications made by Michiels et al. (2003). DNA integrity was examined by agarose gel electrophoresis, and the quantity was measured with a Qubit 2.0 fluorometer (Invitrogen). Aliquots(2 μg each) of DNA dissolved in a 10-mM tris(hydroxymethyl)aminomethane–HCl buffer were sent

to Agribio (Agriculture Victoria Research) for targeted genotyping-by-sequencing.

Genotyping was performed with a targeted genotyping-by-sequencing panel comprising 7,571 probes selected for uniform genome coverage and designed to have minimal ascertainment bias (Matthew Hayden, Agriculture Victoria, personal communication, 24 Feb. 2021). Samples were analyzed via a custom bioinformatics pipeline that processes the sampled targeted genotyping-by-sequencing reads into genotype calls by building an allele-specific reference. The allele-specific reference ensures that sample genotype calls are provided for loci that are assayed but not represented in the Morex Version 1.0 assembly (http://plants.ensembl.org/, hereafter referred to as the barley reference genome), either because of their biological absence or nonassembly in the reference. Codominant genotype calls were based on the haplotype of the entire length of the gap-fill region rather than individual single nucleotide polymorphisms, enabling genotype calls for multiple alleles at a marker locus.

Additional genetic markers were developed for the HOR3270 × Baudin DH population to reduce the distance between the closest flanking markers and the 5HL resistance locus, which was approximately 2 Mbp. Twenty-nine SSR markers were designed on the basis of the barley reference genome, and polymorphic SSRs were genotyped following PCR amplification and gel electrophoresis. The SSR primer sequences for the flanking markers 5HL_SSR1 and 5HL_SSR2 are listed in Supplemental Table S1.

2.6 | Genetic map construction and genetic mapping of barley powdery mildew resistance loci

A preliminary genetic map was constructed by Agribio at Agriculture Victoria Research in ASMap (Taylor & Butler, 2016) with a logarithm of odds (LOD) value of 6 after removing samples with high heterozygosity (because the samples were derived from maternal F_1 tissue, had chromosome abnormalities, or resulted from DNA cross-contamination), unexpectedly high numbers of crossovers, and clonal samples. The data were further filtered to retain markers with an amplification rate of >80% and to exclude co-segregating markers. Markers showing segregation distortion were excluded in JoinMap Version 5 (Van Ooijen, 2018), unless clustered together with the correct Morex genome assembly order, under the assumption of biological significance which is common in wide crosses (Zamir & Tadmor, 1986), or that they are linked to traits affecting regeneration of plantlets following microspore-culture (Sayed et al., 2002). Markers were grouped into linkage groups by their independence LOD in JoinMap Version 5 and ordered within each linkage group by the maximum likelihood algorithm with the default parameters.

Quantitative trait locus mapping for powdery mildew resistance was performed in MapQTL Version 6 (Van Ooijen, 2009) with the genetic map data files exported from JoinMap Version 5. In the Baudin × HOR3270 population, resistant adult individuals (asymptomatic or typically had fewer than 20 pustules of with an IT = 3–4 at 7 dai) were scored as 1, individuals with seedling resistance were scored as 2, and susceptible individuals were scored as 4. For the Baudin × Eth069 population, asymptomatic resistant adult individuals (plants with no pustules or fewer than five delayed pustules; IT = 2–3 after 10 dai) were scored as 1. Adult individuals with sparse mildew colonies with a characteristic dark brown leaf epidermis, with an IT = 3 at 7 dai and fewer than 10 pustules were scored as 2. Susceptible individuals were scored as 4. Interval mapping was used to detect the QTLs, and the genome-wide LOD significance threshold (at a relative cumulative value of 0.95) was determined by a permutation test with 10,000 permutations. Residual variance was controlled for by selecting the closest markers associated with QTLs as cofactors in multiple-QTL model mapping. To test for minor linked QTLs, multiple-QTL models mapping was repeated with the automatic cofactor selection procedure. Epistatic-effect QTLs were analyzed via the inclusive composite interval mapping of epistatic QTLs function implemented in QTL IciMapping Version 4.1 with a probability value of 0.0001 at a threshold LOD of 5 (Li et al., 2008; Meng et al., 2015).

3 | RESULTS

In the landrace HOR3270, which showed both seedling and adult resistance following inoculation with the *Bgh* isolate Chi-001 (IT = 0), resistance was codominant in F_1 progeny seedlings from a cross with the *Bgh*-susceptible cultivar Baudin and was dominant in adult leaves. Eth069 showed IT 4 in seedlings, although these had low colony counts and full resistance (IT = 0) in adult leaves, with infrequent, small, and sparse pustules. Eth069 was difficult to cross with Baudin, with only two being successful F_1 progeny that were used for DH production. For that reason, the F_1 plants were not scored for their phenotype. These results suggest that HOR3270 and Eth069 potentially contained one or more adult powdery mildew resistance genes.

The number of resistance genes in HOR3270 and Eth069 was inferred from F_2 seedlings and DH adult plants, with phenotyping data subjected to χ^2 analysis to confirm the goodness of fit of the observed ratios against theoretical predictions (Table 1). HOR3270 × Baudin F_2 seedling, DH seedlings, and DH adult resistance segregation ratios confirmed the single codominant seedling gene suggested by the F_1 phenotype (IT = 2–3), with APR conferred by a single dominant gene.

TABLE 1 Modes of inheritance of powdery mildew disease resistance genes and infection type scoring categories. Data for observed phenotypes and expected segregation ratios are presented, together with χ^2 values for the null hypothesis of no difference between the observed and expected segregation ratios, with $P > .05$ being significant

Parental cross	Population	*n*	R[a]	I[b]	S[c]	Expected ratio	χ^2	*P*-value
Baudin × HOR3270	F_2 seedling	196	46	**102**	48	1:2:1	0.37	>.8
	DH seedling	305	170	–	135	1:0.95[e]	2.27	>.1
	DH adult	305	230	–	75	3:1	0.03	>.8
Baudin × Eth069	F_2 seedling	100	–	–	100	N/A	–	–
	DH adult	309	159	56	94	2:0.77:1.23[e]	0.38	>.8

[a]R indicates resistant phenotypes with an overall infection type (IT) scoring category = 0. Further information on the resistance scoring categories is provided in the Methods section.

[b]Intermediate infection types (I) have a codominant seedling phenotype (IT = 2–3, in bold). Resistant adult DH plants are characterized by sparse pustules, typically <10 per plant, but with an IT of 3 (underlined).

[c]S indicates a susceptible (IT = 4).

[d] DH, doubled haploid.

[e]Marker segregation distortion was observed at the 1H resistance locus in the Baudin × HOR3270 population and the 5H locus in the Baudin × Eth069 population.

Both genes contributed to the resistant to susceptible ratio of 3:1 in the adult DH population. Slight segregation distortion was present at the seedling resistance locus, coincident with segregation distortion of nearby markers explained in the next section.

Eth069 × Baudin F_2 seedling progeny were susceptible, consistent with the resistance gene(s) acting at a later stage. Resistance in the DH population did not conform to any conventional Mendelian segregation ratio. The QTLs and genetic mapping (see below) indicated that two genes conferred APR, with segregation distortion in a region linked to resistance on 1HS. The skewed segregation accounted for the observed segregation ratio for the phenotypes if one resistance gene masked or was epistatic over a second, giving a modified expected ratio of 2:0.77:1.23 from a hypothesized 2:1:1 (ratio of resistance at epistatic locus one to resistance at locus two to fully susceptible). Statistical support for epistasis is provided in the following section.

3.1 | Genetic mapping of powdery mildew resistance genes

In both mapping populations in this study, the markers were clustered into seven linkage groups by the independence LOD function in JoinMap Version 5 (Van Ooijen, 2018). The markers coalesced up to a LOD value of 5 and showed no further linkage group splitting until the most stringent linkage in the test was reached (LOD = 10). The average marker density across the populations ranged from 1.16 to 1.59 per cM. Summary statistics for each mapping population are provided in Table 2, and the genotyping data are given in Supplemental Table S2a, b.

Significant segregation distortion of clusters of the markers was detected in each population (Supplemental Figure S1). In total, 10 distinct regions were evident. Both populations showed distortion on a distal section of 2HL (χ^2 = ~28–31, $P < .0001$) and the central region of 3H (χ^2 = ~25–50, $P < .0001$), with modest distortion shared at the long end of 7H (χ^2 = ~10–15, $P < .005$). Regions unique to the populations were: 1HL and 5HS in Baudin × HOR3270; in Baudin × Eth069, distortion was present across most of 6H, on 1HS, and in the mid-region of 2H.

The distorted regions in common on barley chromosomes 2 and 3 overlapped with the regions associated with anther culture survival and regeneration found by Manninen (2000). However, in more recent barley studies (Bélanger et al., 2016; Li et al., 2010), a large number of regions have been identified that either overlap or are different between populations, indicating that inferring the same gene(s) that may be associated with a trait affecting distortion may be unreliable. Two regions showing segregation distortion affected the predicted Mendelian segregation ratios for powdery mildew resistance

TABLE 2 Characteristics of genetic maps for doubled haploid (DH) populations in this study

DH population	Progeny in genetic map construction[a]	Polymorphic markers	Filtered codominant markers	Length of genetic map	Average marker spacing
	n			cM	
Baudin × HOR3270	305	3,726	762	1,211	1.59
Baudin × Eth069	325	5,333	966	1,124	1.16

[a]Genetic map values are based on linkage groups identified using the independent logarithm of odds in JoinMap Version 5 (Van Ooijen, 2018), ordered within each linkage group by the maximum likelihood algorithm.

loci (Table 1). Modest distortion was observed in the Baudin x HOR3270 population at the 1HS locus for seedling resistance. The inferred segregation from the marker with the strongest trait association was used in Table 1; however, distortion was more pronounced among a group of markers to one side of that marker, which would provide a more significant segregation *P*-value. Opposing marker distortion was observed at the second resistance locus, appearing to explain the balanced segregation in adult DH plants. A high level of segregation distortion was present in the markers closest to the Baudin × Eth069 1HS locus (Supplemental Figure S1b). According to the closest markers, a ratio of 1.58 Baudin alleles to 1 Eth069 allele was used to modify the expected Mendelian segregation ratio.

Genetic mapping of powdery mildew resistance in the two DH populations identified four major loci in distal or subtelomeric chromosomal regions. The final genetic linkage data for each DH population are available in Supplemental Table S3a,b, together with genetic linkage map data files (.dat) in Supplemental File S1. Linkage mapping in the Baudin × HOR3270 population detected a major locus for adult leaf resistance on the chromosome 5HL. The locus cosegregated with a SSR marker and was placed between two adjacent markers, within 0.33 cM of the nearest marker, spanning a region from 663.08 to 63.73 Mbp relative to the barley reference genome. A locus for seedling resistance was detected on chromosome 1HS between flanking markers that corresponded to the Morex genomic region of 0.427 to 6.97 Mbp. To determine if minor or suggestive QTLs might also govern resistance in Baudin x HOR3270, QTL mapping was conducted. The results supported the genetic linkage mapping, with no additional loci found (Supplemental Figure S2a).

In Eth069, APR alone was observed, and the DH adult plant segregation ratios suggested the involvement of more than one gene. Two categories of resistance were observed in adult DH plants, largely asymptomatic resistant individuals and individuals with relatively sparse mildew colonies on lower leaves associated with delayed surface browning. Resistance was therefore initially mapped as a quantitative trait. Only two significant loci were found, both with a major effect, on 7HS and on 1HS (Supplemental Figure S2b). After we controlled for segregation distortion on 1HS, the QTL results informed the two resistance loci segregation ratio in Table 1, whereas the genotype and phenotype data suggested that the 7H locus masked or was epistatic to the 1H locus. Genome-wide inclusive composite interval mapping of epistatic QTLs with IciMapping Version 4.1 (Li et al., 2008; Meng et al., 2015) confirmed a major epistatic interaction between chromosome 7H and chromosome 1H (Supplemental Table S4). The percentage of phenotype variance explained was 92.06% with a LOD value of 108.72, and the marker interval positions corresponded to the APR loci *Resistance to*Blumeria graminis *f. sp.* hordei 2 (*Rbgh2*) and *Rbgh3*, respectively. A second, putative epistatic interaction was detected between chromosomes 2H and 5H with a phenotypic variation explained of 1.54%.

In contrast to the 7H QTL, the QTL on 1H spanned some 10 cM with a broad, flat peak of 3 cM, reflecting poor recombinant resolution in that region (Supplemental Figure S2b). These two loci were positioned at the start of 7HS, with the closest genotyping-by-sequencing GBS single nucleotide polymorphism marker at 0.7 cM or 2.5 Mbp in the Morex reference genome, and on 1H between the flanking markers at 7.65 and 13.20 Mbp, respectively. Genetic linkage mapping of resistance was made possible by separating asymptomatic adult individuals from those with the sparse colony phenotype, with the results agreeing with the QTL mapping. A summary of the map positions of the new resistance loci is given in Table 3, flanking marker sequences are provided in Supplemental Table S5, and summary QTL data are provided in Supplemental Table S6.

3.2 | Detached leaf assays and whole-plant phenotypes of the APR genes

The colony numbers of each APR gene were studied at the first and fifth leaf stages in detached leaves in selected DH lines containing the gene of interest (Figure 1). The parental line HOR3270 was highly resistant at both the first and fifth leaf stages, with visible hypersensitive response (HR) symptoms supporting seedling phenotype observations that HOR3270 appears to possess a major *R*-gene. Eth069 seedling colony IT scores ranged from 2 to 4; in the fifth leaves, very few colonies formed, with an IT of 2–3. The lower number of colonies in Eth069 seedlings than in Baudin may be attributed to

TABLE 3 Chromosomal locations of *Blumeria graminis* f. sp. *hordei* (*Bgh*) adult plant resistance loci based on genetic linkage mapping

Parental cross	Resistance locus name[a]	Chromosome	Flanking markers	Interval	Distance
				cM	
Baudin × HOR3270	*RBgh1*	5HL	SSR1_663,081,098–SSR2_**663,733,599**	0.33	0
	Ml-like	1HS	2_427,296–6,101_**6,977,461**	14.56	5.54
Baudin × Eth069	*RBgh2*	7HS	Telomere–5,261_**2,474,271**	0.69	0.69
	RBgh3	1HS	17_7,656,241–6,110_**10,967,260**[c]	3.92	1.47

[a]Resistance loci were determined using the maximum likelihood algorithm in JoinMap Version 5 (Van Ooijen, 2018).

[b] Marker bp positions in the barley cultivar Morex Version 1.0 reference genome are indicated following the underscore in each marker name.

[c]Distance is the genetic distance in cM to the closest or cosegregating marker (shown in bold).

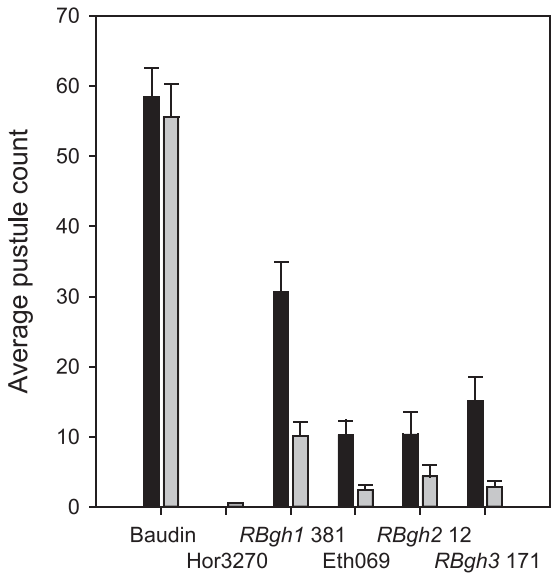

FIGURE 1 Powdery mildew colony counts for doubled haploid (DH) lines containing single powdery mildew adult plant resistance (APR) genes. Doubled haploid parental lines are shown in the graph with the DH lines named by their APR locus and line number. The DH line *RBgh1* 381 is from the Baudin × HOR3270 population; lines *RBgh2* 12 and *RBgh3* 171 are from Baudin × Eth069. The mean number of colonies for the first leaves (black bars) and fifth leaves (gray bars) are shown per 2.5- by 0.6-cm leaf section. Standard error bars and colony counts represent the average of four experiments, each consisting of three replicate leaves per line and growth stage, with all leaves inoculated together in a mildew settling tower

prehaustorial traits, which are common in susceptible barley lines (Aghnoum et al., 2010).

Doubled haploid APR lines all showed a reduction in total colony numbers between the first and fifth leaves, with the mean IT also reducing from scores of 3 to 4 to 2 to 3. The detached DH leaves showed more pustules than whole resistant adult plants inoculated at 7 wk in a hoop house, where all leaves showed resistance to powdery mildew, suggesting systemic resistance and that the resistance may be affected by the physiological condition of the detached leaves.

Inoculation of whole APR plants at the 5th leaf stage leads to small, sporadic pustules with an unhealthy appearance that fail to spread on the lower leaves of *RBgh1* and *RBgh3* genotypes by 7 dai and later on *RBgh2* by 14 dai (Supplemental Figure S3). The pustules were often surrounded by surface leaf pigmentation, particularly in *RBgh3* lines that have the darkest surface color. The pigmentation does not correspond to a HR early in infection and appears to be potentiated by prolonged infection, as the susceptible parent Baudin also shows surface browning beneath older mildew colonies.

3.3 | Atypical penetration resistance phenotypes characterize APR at the microscopic level

Cytological examination of the barley powdery mildew-resistant parents at 72 h after inoculation showed effective inhibition of powdery mildew growth. In HOR3270, resistance in younger leaves was accompanied by whole-cell auto-fluorescence in epidermal cells directly undergoing penetration by *Bgh* (Figure 2a, third leaf stage). The DAB staining often showed H_2O_2 accumulation throughout or in parts of the target cells, indicating a typical HR (Figure 2b,c). In Eth069 at the fifth leaf stage, strong cell wall appositions (CWAs) were apparent and tertiary appressorial penetration attempts were more common than in HOR3270 or Baudin (Figure 2d,e). These parental phenotypes contrasted with hyphal growth in a susceptible line (Figure 2l). The yellow staining in Figure 2k, which may have been caused by polyphenol accumulation (Hückelhoven et al., 1999), was not specific to the APR genes and was rarely observed.

Examination of the individual DH lines possessing a single APR gene suggested that all three APR genes displayed features consistent with penetration resistance. Cell wall appositions and DAB staining localized to penetration sites were prominent and there was a lack of spontaneous necrosis and mesophyll cell death, which is commonly seen with *mlo* alleles (Behn et al., 2005; Ge et al., 2016), and infrequent

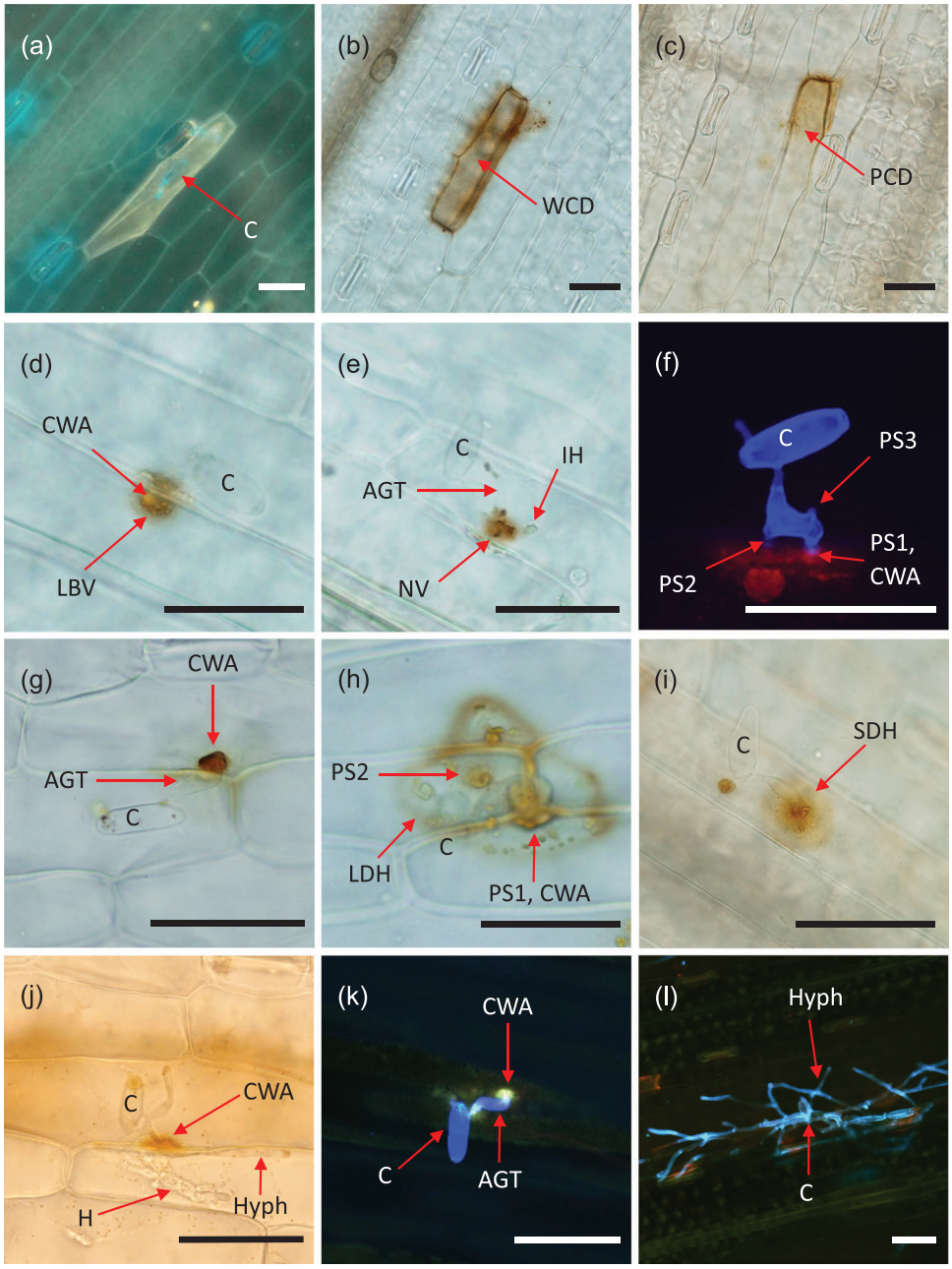

FIGURE 2 Typical responses to attempted penetration by *Blumeria graminis* f. sp. *hordei* observed in resistant parental accessions and in doubled haploid lines containing adult plant resistance (APR) genes. Images were taken at 72 h after inoculation (hai), except for Panel (a), which was taken at 48 hai. In (a), (f), (k), and (l), the images show samples were stained by aniline blue, calcofluor white, and Evans blue triple staining; the remaining images were stained with 3,3-diaminobenzidine (DAB). AGT, appressorial germ tube; C, conidia; CWA, cell wall apposition; Hyph, hyphae; H, haustorium; IH, initial haustorium; LBV, light brown vesicle-like bodies; LDH, large DAB halo; NV, no or indistinct vesicle-like bodies; PCD, partial epidermal cell DAB staining; PS, penetration site with site number; WCD, whole epidermal cell DAB staining. Scale bars represent 50 μm

epidermal cell HR. At the fifth leaf stage, *RBgh1* showed tight aggregation of cytosolic vesicle-like bodies (VLBs) at the sites of attempted penetration, visible upon close examination of Figure 2d, and fewer DAB staining halos around the penetration sites than *RBgh2* and *RBgh3*. Ineffective CWAs were occasionally observed, allowing an initial haustorium (Figure 2e). *RBgh2* (Figure 2h) was distinguished by large DAB halos around the penetration sites, with prominent staining at the halo edges. Vesicle-like bodies were diffused throughout the halos but also aggregated at penetration sites. In younger leaves before the onset of APR, successful penetration of CWA, haustorium formation and the development of weak whole-cell autofluorescence was observed (Figure 2j). Cytologically, *RBgh3* was similar to *RBgh2*, with fewer large DAB

halos and with the VLBs grouping closer to the penetration sites (Figure 2i).

The frequencies of different cytological features were quantified in adult DH lines containing individual APR genes, in their parents, and in the *mlo-11* cultivar 'Westminster'. Westminster showed complete resistance and had the highest proportion of CWAs, which Aghnoum et al. (2010) associated with *mlo* resistance. Westminster also showed the highest frequency of failed secondary and tertiary appressorial penetration attempts (Supplemental Table S7). However, penetration resistance associated with CWAs was not a specific indicator of resistance, being present in all lines with the susceptible cultivar Baudin showing similar frequency to HOR3270 and *RBgh1*. Cytological features involving the distribution of VLBs, the extent of DAB staining, successful penetration, and hyphal growth are shown in Figure 3. HOR3270 also exhibited complete resistance with no hyphal growth, and was characterized by the highest levels of whole or partial epidermal cell DAB staining, again indicating a typical HR, presumably resulting from the *Ml* locus on 1HS.

Among the APR lines, a high frequency of VLBs clustering at penetration sites was observed in *RBgh1*, *RBgh2*, and *RBgh3*, compared with the resistant parents. These were typified by CWAs with small DAB halos. In *RBgh2* in particular, CWAs were associated with large DAB halos with diffuse VLBs. All the APR lines showed low levels of penetration and initial hyphal growth (1.56–4.94%) compared with Baudin (21.40%). Cytological hyphal growth was consistent with macroscopic APR phenotypes across the mapping populations, with *RBgh2* genotypes showing the fewest sporadic pustules, followed by *RBgh3*, then *RBgh1* (see the Methods section). However, the majority of sites with initial hyphal growth failed to develop, as only low numbers or no pustules were seen in adult individuals.

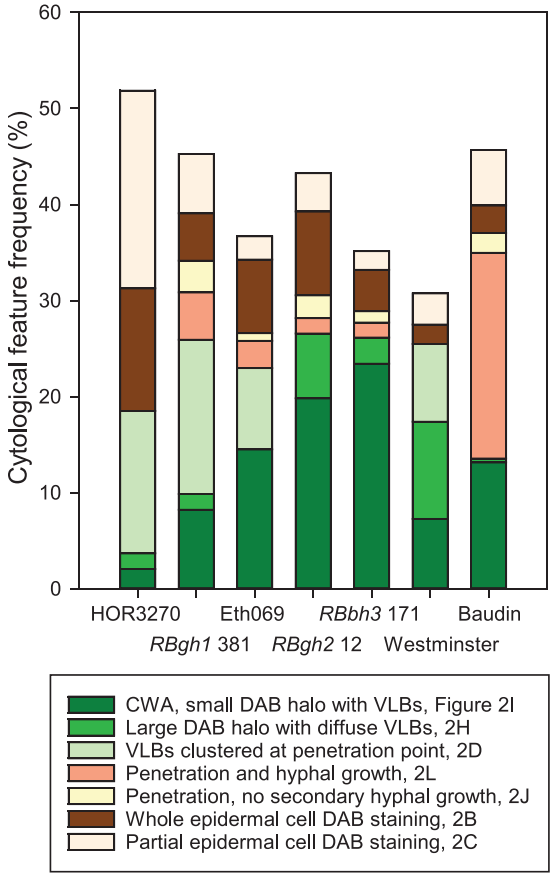

FIGURE 3 Frequencies of cytological features in different doubled haploid (DH) adult plant resistance (APR) lines in response to *Blumeria graminis* f. sp. *hordei* at 72 h after inoculation. The frequencies are based on observations of 3,3-diaminobenzidine (DAB)-stained fifth leaf samples from four replicate leaves per line with over 50 infection sites assessed per sample. The DH line *RBgh1* 381 is from the Baudin × HOR3270 population; lines *RBgh2* 12 and *RBgh3* 171 are from Baudin × Eth069. The cultivar Westminster contains the *mlo-11* powdery mildew resistance domain. The identity of the corresponding cytological features from Figure 2 are provided in the key. The figure excludes cell wall apposition counts, which are presented in Supplemental Table S7. CWA, cell wall apposition; VLB, vesicle-like bodies

4 | DISCUSSION

Barley landraces are postulated to contain genes and alleles that are not present in modern cultivars, which inspired this study to detect new, potentially durable sources of resistance to *Bgh*. Eth069 represents an accession that showed adult leaf resistance in preliminary phenotyping screens rather than major *R*-gene seedling resistance, which is generally effective by the fifth leaf stage (Heitefuss et al., 1997; Wright & Heale, 1984). We also included an accession with all-stage resistance, HOR3270, which was shown to be resistant when infected with two German *Bgh* isolates that was virulent to 44 accessions and four Israeli *Bgh* isolates that were virulent to 18 (Spies et al., 2012). The two accessions originate from very different locations: Eth069 from Azerbaijan and HOR3270 from Turkey. The APR in HOR3270 and the epistatic or main-effect locus in Eth069 were notable for being detected alongside a second resistance gene. In the Baudin x Eth069 DH population, atypical progeny segregation ratios initially indicated a QTL mapping approach as being appropriate. However, only two loci were detected, which had ratios altered by significant segregation distortion at the 1HS resistance locus.

The genes twinned with APR genes were mechanistically and phenotypically unrelated and located on 1HS. In HOR3270, resistance was codominant in F_2 seedlings and associated with a HR; in Eth069, resistance showed a small and sparse colony phenotype in adult leaves. The resistance of HOR3270 would therefore appear to be a major race-specific *R*-gene, whereas the resistance Eth069 is an APR.

Chromosome 1HS contains several *R*-genes ordered from the centromere: *Mlnn*, *Mlk*, *Mla6*, and *Mlra* (https://wheat.pw.usda.gov/GG3/BarleyBinMaps). *Mla6* is a representative of the race-specific *Mla R*-gene cluster (Halterman & Wise, 2004) that is positioned at ~30.29 Mbp in the barley reference genome. A BLASTN homolog of *Mla6* is present at 8.74 Mbp, which corresponds to the approximate position of *Mlra*, suggesting that the gene is likely to be present in HOR3270.

At the cytological level, the APR genes in this study showed two prominent features: cell wall apposition and cytosolic VLBs. Slight cytological differences occurred in the size and distribution of cytosolic VLBs, together with the extent of a DAB halo surrounding the penetration sites. They also lacked HR or mesophyll cell death, which are usually associated with race-specific *R*-genes or *mlo* resistance, respectively. Defense-related VLBs are known to be multifunctional and to accrue around CWAs at sites of attempted penetration by biotrophs. An et al. (2006) proposed three forms of *Bgh*: VLBs intensely colored by reducing dyes were found to be small papillae. Multi-vesicular bodies appeared to contain the precursors for CWAs and were suggested to also give rise to vesicles similar to the exosomes in animals that discharge antimicrobial compounds at the plasma membrane. In *Arabidopsis thaliana* (L.) Heynh., such exosomes were associated with the transfer of silencing sRNA to fungal pathogens, albeit against the necrotroph *Botrytis cinerea* (Cai et al., 2018).

Growth stage dependent resistance to barley powdery mildew has been reported previously as partial APR (Mastebroek & Balkema-Boomstra, 1991) and full APR (Hwang & Heitefuss, 1982). These are regarded as a form of basal penetration resistance (Aghnoum et al., 2010), as CWAs act before successful penetration and formation of haustoria. Basal resistance may also be considered as non-race -specific and durable, in common with both *mlo* and non-host resistance (Collins et al., 2003; Freialdenhoven et al., 1996). Basal and *mlo* resistance are mechanistically related through the involvement of cell wall appositions (Trujillo et al., 2004) and through a SNARE protein complex that appears to be necessary for the release of defense compounds from VLBs (Collins et al., 2003). The HR, typical of race-specific *R*-gene mediated resistance, is expressed as a second line of defense if penetration resistance fails (Trujillo et al., 2004).

Previous studies have reported *Bgh* resistance in broadly similar chromosomal regions to the APR loci. Gupta et al. (2018) used DH populations to determine a quantitative APR gene region on 5HL that was present in two barley lines, CLE210 and Denar. Significant overlapping QTLs, one from each parent, were found between 619.71 and 624.88 Mbp. These contrast with *RBgh1*, which manifested as a single locus rather than a quantitative trait and occupied a more distal position at approximately 663 Mbp.

Ames et al. (2015) conducted association mapping of 316 wild barley accessions and uncovered a QTL in a similar location to *RBgh1*. This locus was also effective in seedlings and appeared to be less distal. Aghnoum et al. (2010) used natural field infection to map QTLs for powdery mildew resistance in six biparental mapping populations. *Rbgq20* was found at the end of 7HS but was effective in seedling leaves only, coincident with the *R*-gene *Mlt*. Silvar et al. (2010) also found a QTL based on seedling phenotyping in the Spanish landrace 'SBCC097' towards the end of 7HS that explained up to 18.5% of the variance. The combination of resistance genes in SBCC097 was intriguingly effective against all pathotypes tested and acted after penetration with delayed HR (Silvar et al., 2013); however, compared with *RBgh2*, the QTL was several cM from the chromosome end. These QTLs appear to be similar to those found by Řepková et al. (2009). Perhaps a more relevant QTL may be the APR gene *Rbgq17*, which occupies a similar position to *RBgh1* on 5HL (Aghnoum et al., 2010), albeit with a modest effect (the proportion of phenotypic variance explained was 9.5%). The significance of the distal positions of the APR genes in this study lies in their high recombination rates and transposable element activity (Künzel et al., 2000; Wicker et al., 2016, 2017), which contribute to more rapid evolution.

In Australia, limited deployment of major *R*-genes (Dreiseitl & Platz, 2012) means that fewer virulence genes are present in the barley powdery mildew population than in Europe. Quarantine restrictions mean the range of virulence genes that may be assayed against new resistance genes is relatively narrow. The APR in HOR3270 provides the best evidence of broad resistance to a diverse collection of virulence genes, as Spies et al. (2012) included *Mlra* virulence, which is likely to be present on 1H, in their disease screens. The landrace was still resistant to 62 virulence genes. By association, the common mechanistic phenotypes of the other APR genes suggest they may share broad-spectrum resistance.

Although the APR genes in this study in themselves provide useful resistance, they may be an important means of potentiating major *R*-genes. Adult plant resistance can serve to back up such resistance if it fails, reducing the proliferation and spread of novel types of virulence. Combined with their basal mode of action, APR exerts less selective pressure on developing plants, reducing the likelihood of their breakdown. Indeed, a feature of natural plant populations is a lack of epidemics, which may be explained by combinations of resistance genes alongside phenotypic and genotypic spatial diversity (Ames et al., 2015; Bevan et al., 1993; Mastebroek et al., 1995).

In summary, this study has uncovered single major-effect APR genes in previously undetected regions near chromosomal termini. The shared basal resistance characteristics of the APR genes suggests that they are associated with a first line of defense against powdery mildew and may be useful alternative sources of resistance for barley breeders. Further

studies are needed to reveal shared or unique resistance signaling pathways compared with other resistance mechanisms.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Cynthia Ge: data curation, formal analysis, investigation, writing—original draft. Elzette Wentzel: data curation, investigation, methodology. Nola D'Souza: data curation, methodology. Kefei Chen: formal analysis. Richard Oliver: conceptualization, visualization. Simon R Ellwood: conceptualization, formal analysis, funding acquisition, investigation, writing—original draft, writing—review and editing

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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