The Coiled-Coil NLR *Rph1*, Confers Leaf Rust Resistance in Barley Cultivar Sudan[1][OPEN]

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Unraveling and exploiting mechanisms of disease resistance in cereal crops is currently limited by their large repeat-rich genomes and the lack of genetic recombination or cultivar (cv)-specific sequence information. We cloned the first leaf rust resistance gene *Rph1* (*Rph1.a*) from cultivated barley (*Hordeum vulgare*) using "MutChromSeq," a recently developed molecular genomics tool for the rapid cloning of genes in plants. Marker-trait association in the CI 9214/Stirling doubled haploid population mapped *Rph1* to the short arm of chromosome 2H in a physical region of 1.3 megabases relative to the barley cv Morex reference assembly. A sodium azide mutant population in cv Sudan was generated and 10 mutants were confirmed by progeny-testing. Flow-sorted 2H chromosomes from Sudan (wild type) and six of the mutants were sequenced and compared to identify candidate genes for the *Rph1* locus. MutChromSeq identified a single gene candidate encoding a coiled-coil nucleotide binding site Leucine-rich repeat (NLR) receptor protein that was altered in three different mutants. Further Sanger sequencing confirmed all three mutations and identified an additional two independent mutations within the same candidate gene. Phylogenetic analysis determined that *Rph1* clustered separately from all previously cloned NLRs from the Triticeae and displayed highest sequence similarity (89%) with a homolog of the Arabidopsis (*Arabidopsis thaliana*) disease resistance protein 1 protein in *Triticum urartu*. In this study we determined the molecular basis for *Rph1*-mediated resistance in cultivated barley enabling varietal improvement through diagnostic marker design, gene editing, and gene stacking technologies.

Barley (*Hordeum vulgare*) is the fourth most important cereal crop in the world and is mainly used for malt production, animal feed and, in some regions, human consumption. This agricultural practice of monoculture in addition to climate change favors the emergence of new pathogen variants that can significantly reduce yield, posing a serious threat to global food security. Since Biblical times, rust pathogens have plagued farmer's fields causing significant yield losses and, in severe cases, crop failure and famine (Kislev, 1982). In particular, plant pathogens of the *Puccinia* genus are some of the most feared and damaging diseases of cereal crops (Dean et al., 2012). Although leaf rust caused by *P. hordei* is the most widespread and serious foliar disease of barley, it can be controlled effectively by genetic resistance (Park et al., 2015). The leaf rust resistance (*R*) gene *Rph1* was first described in barley cultivars (cv) Oderbrucker, Speciale, and Sudan by Roane and Starling (1967) and was mapped to chromosome 2H using trisomic analysis (Tuleen and McDaniel, 1971). *Rph1*-mediated resistance was later

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designated *Rph1.a* to conform with recommended allele symbols for leaf rust resistance genes in barley (Franckowiak et al., 1992). Despite the existence of virulence for most *Rph* genes in barley by prevailing *P. hordei* variants, combining multiple genes conferring diverse resistance mechanisms, as demonstrated in wheat (*Triticum aestivum*; Park, 2003; Koller et al., 2018), could provide a sustained method for broad spectrum disease control (Brun et al., 2010).

The intense diversifying selection imposed on *R* genes has resulted in high accessional variation at *R* gene loci including sequence polymorphisms and copy number variation (Noël et al., 1999; Kuang et al., 2005; Chavan et al., 2015; Thind et al., 2018). In many cases, this has led to erosion of the orthogonal relationships between *R* gene analogs belonging to different accessions of the same species (Noël et al., 1999; Kuang et al., 2005; Chavan et al., 2015; Thind et al., 2018). Most map-based *R* gene cloning projects therefore include the generation of a high-quality physical sequence (e.g. a bacterial artifificial chromosome tiling path) spanning the flanking markers delimiting the *R* gene in the resistant accession. This is followed by identification of candidate *R* genes and experimental validation by transformation of the candidate gene(s) into a susceptible accession (Periyannan et al., 2013; Kawashima et al., 2016). Generating a physical tiling path is, however, expensive and time-consuming.

An alternative or complementary approach involves *R* gene identification by sequence-comparison of mutants. A line containing the desired *R* gene in a background that is susceptible to the pathogen isolate of interest is mutated and the progeny is screened for loss-of-resistance. If multiple, independently derived mutant alleles are obtained and found to have mutations in the same gene, this then provides very strong evidence for gene identification; the causal gene can be further substantiated by demonstrating genetic cosegregation between the candidate gene and resistance. The size of cereal genomes, such as 16.03 to 16.58 gigabases (Gb) for wheat (International Wheat Genome Sequencing Consortium, 2018) and 4.88 to 5.04 Gb for barley (Mascher et al., 2017) imposes a barrier, however, in terms of the cost of sequencing and computational analysis. This can be overcome by sequencing only a selected fraction of the genome, an approach known as "genome complexity reduction." Because most *R* genes encode nucleotide binding site Leu-rich repeats (NLR; Kourelis and van der Hoorn, 2018), NLR-exome capture and sequencing can be used to efficiently compare multiple mutant alleles. This strategy was successfully used to clone *Sr22*, *Sr45*, *Yr5*, *Yr7*, and *Pm21* from wheat or wheat-alien introgression lines (Steuernagel et al., 2016; Marchal et al., 2018; Xing et al., 2018). More recently, NLR exome capture on 151 genetically diverse individuals of the wild wheat D-genome progenitor *Aegilops tauschii* was coupled to association genetics to rapidly clone *Sr46* and *SrTA1662* (Arora et al., 2018).

The very strength of NLR exome capture in providing a stringent complexity reduction is also its weakness by excluding, perforce (1) NLRs with significant sequence divergence to the source sequences used in bait design, (2) NLRs with exotic integrated domains (Sarris et al., 2016), and (3) *R* genes not conforming to the canonical structure of an NLR (Fu et al., 2009; Krattinger et al., 2009; Moore et al., 2015). This bias can be overcome by chromosome flow sorting, where only a prior knowledge of the chromosome on which the gene resides is required (Giorgi et al., 2013; Steuernagel et al., 2017). Sánchez-Martín and colleagues flow-sorted and sequenced wheat chromosome 5D from six *Pm2* mutants and the parental wild type. Subsequent sequence-comparison was sufficient to identify a single candidate gene that could then be confirmed by sequencing additional mutants (Sánchez-Martín et al., 2016). A limitation of this approach is the high number of mutants (~5 in barley, ~6 in wheat) necessary to identify a single candidate gene (Sánchez-Martín et al., 2016). This requires the generation and screening of a large mutant population, typically numbering several thousand individuals (Mago et al., 2017), in particular in barley (a diploid) where the tolerated mutation density is approximately seven times less than that in hexaploid wheat (Uauy et al., 2017). The requirement for many mutants for unambiguous gene identification can be mitigated if combining chromosome flow sorting with positional mapping (Thind et al., 2017). A limitation of chromosome flow sorting is the generally low quality of assemblies ($N_{50} > 2$ kb; 75% genes assembled into contigs with ≥90% query coverage) obtained from flow-sorted and multiple displacement-amplified DNA (Sánchez-Martín et al., 2016). This can be vastly improved with Chicago long-range linked-read sequencing and assembly to obtain N_{50} scaffold sizes of 9.76 Mb to 22.39 Mb (Thind et al., 2017; Xing et al., 2018), although at high cost. In this study, we combined MutChromSeq with genetic mapping to rapidly clone *Rph1* in barley cv Sudan from a defined region on chromosome 2H. We also report on developing a cost-effective wild-type sequence assembly with high contiguity (contig $N_{50} > 20.1$ kb) and gene space representation (83% of genes with ≥90% query coverage).

RESULTS

The availability of an array of *P. hordei* pathotypes with contrasting virulence for *Rph1*-mediated resistance permitted a reliable phenotypic screen at the seedling stage of two barley populations segregating for the *Rph1* locus, with the aim of confirming the previous map location reported by Tuleen and McDaniel (1971). The barley cvs CI 9214, Sudan, and Berg were all postulated to carry *Rph1* based on greenhouse tests with *P. hordei* pathotypes with contrasting virulence/avirulence profiles (Table 1). Monogenic inheritance for *Rph1*-mediated resistance was confirmed by the observed segregation in both barley mapping populations (CI 9214/Baudin and CI 9214/Stirling) when

Table 1. *Infection response data of selected barley genotypes previously postulated to carry the* Rph1 *resistance gene tested with four* P. hordei *pathotypes and their* Rph1 *sequence haplotype*

Accession	220P+[a]	5457P+[b]	4610P+[c]	253P-[d]	*Rph1* Haplotype
CIho 11958	;1-CN	3+	;1-CN	;12C	Susceptible
ISR950.13	;1-CN	3+	;1-CN	3+	Susceptible
Seln 8861	0;N	3+	;N	3+	Susceptible
CI9214	;1-CN	3+	;1-CN	3+	Resistant
HOR 15560	0;N	3+	;N	3+	Resistant
Sudan	;1-CN	3+	;1-CN	3+	Resistant
Berg	0;N	3+	0;N	3+	Susceptible
Bowman+*Rph1*	;N	3+	;N	3+	Resistant
Bowman[5]	3+	3+	3+	3+	Susceptible
Stirling	3+	3+	3+	:1+C	Susceptible
Baudin	;12-C	3+	3+	;12-C	NA[e]
Gus[f]	3+	3+	3+	3+	NA[e]

[a]Pathotype 220P+ = avirulent on *Rph1* and *Rph12*. [b]Pathotype 5457P+ = virulent on *Rph1* and *Rph12*. [c]Pathotype 4610P+ = avirulent on *Rph1* and virulent on *Rph12*. [d]Pathotype 253P- = virulent on *Rph1* and avirulent on *Rph12*. [e]NA = not applicable. [f]Susceptible controls.

inoculated at the primary leaf stage in the greenhouse with *Rph1*-avirulent *P. hordei* pathotype 4610P+ (Table 2, Fig. 1).

A total of 61 representative genotypes of the CI 9214/Stirling doubled haploid (DH) population from both resistant and susceptible phenotypic classes were selected for genetic mapping of *Rph1*, and were subsequently genotyped using 10,258 Diversity Arrays Technology sequences (DArT-Seq) marker loci. Further genome-wide marker-trait association (MTA) analysis demonstrated that DArT-seq only on 2HS were significantly associated [−log10 (*P*-value) of 15] with *Rph1* phenotypic scores using Fischer's exact test, linkage disequilibrium-correlation coefficient, and χ^2 analysis (Fig. 2; Supplemental Fig. S1; Supplemental Table S1, A and B). Genetic mapping of *Rph1* in the CI 9214/Baudin population was also performed using a subset of 92 recombinant inbred lines (RILs) from the mapping population. A 1.3-Mb physical region in the Morex reference genome sequence known to harbor the *Rph1* gene flanked by two DArT-Seq markers (13,139,911 bp and 14,361,439 bp) from the CI 9214/Baudin mapping population was used to search for candidate genes using a modified version of MutantHunter (Sánchez-Martín et al., 2016). The flanking markers were identified based on the presence of two recombinants from the 92 RILs on either side of *Rph1*.

A conservative estimate of the genetic and physical interval known to harbor the *Rph1* gene enabled a second level of genome complexity reduction in addition to the chromosome sorting. A sodium azide mutant population was produced for the *Rph1* differential cv Sudan and 2,100 M_2 generation spikes were rust-tested with *P. hordei* pathotype 4610 P+ in the greenhouse for loss-of-function mutations in the *Rph1* gene. We identified 10 spikes that contained putative *rph1* knockouts. At least two susceptible seedlings per M_2 spike were advanced and their progeny were rust-tested in the M_3 generation; nine families were phenotyped as homozygous susceptible and one segregated. Of these, five homozygous susceptible families and one heterozygous mutant family were processed for chromosome sorting for 2H and Illumina sequencing (Fig. 3). Chromosomes were isolated according to Lysák et al. (1999), labeled in suspension with GAA microsatellite using fluorescence in situ hybridization (FISH) in suspension following the protocol of Giorgi et al. (2013), stained with diamidino-2-phenylindole dihydrochloride and analyzed by a FACSAria II SORP flow cytometer and sorter. The resulting bivariate flow karyotype with the 2H population highlighted is shown in Supplemental Fig. S2. In total, 80,000 copies of chromosome 2H were flow-sorted from Sudan wild-type and six Sudan-derived mutant lines. The purity of the sorted fractions determined by FISH was 86%. Multiple displacement amplification (MDA) was only performed to amplify chromosomal DNA from the six mutant lines; however, in contrast to Sánchez-Martín et al. (2016), importantly MDA was not performed on chromosomal DNA from the Sudan wild type. Illumina sequencing of amplified chromosomal DNA yielded 27.6–45.5 million of 250-bp paired-end (PE) reads for each of the six mutant chromosomes (representing 18–30× coverage). For the wild-type chromosome 2H of cv Sudan, 222 million of 250-bp PE reads were obtained (equivalent to 144× coverage) and assembled into 62,427 scaffolds with N_{50} = 8,032 and N_{50} length of 20.7 kb. The total assembly length of 587.7 Mb represents 75% of the estimated chromosome size.

Illumina sequence reads of six mutant lines (M422, M483, M544, M761, M763, and M767) were mapped to the wild-type Sudan chromosome 2H within the 1.3-kb region of interest to search for genes that contain multiple independent mutations. A single full-length gene encoding an NLR (corresponding to HORVU2Hr1G006480.6 in Morex) in scaffold 2895_0 from Sudan contained mutations in lines M422, M761, M763, and M767 (Fig. 3; Supplemental Fig. S3; Table 3). No mutations were identified in lines M483 and M544 using both Illumina or Sanger sequencing, suggesting

Table 2. *Segregation frequencies and genetic analysis of three mapping populations when inoculated with two barley leaf rust pathotypes at seedling stage*

Population	Generation	Pathotype	No. of Lines			Genetic Ratio	X2	*P*
			Res	Seg	Sus			
CI 9214/Stirling	DH	4610P+	122	0	138	1:1[a]	0.99	>0.32
CI 9214/Baudin	RIL	4610P+	168	0	194	1:1[a]	1.87	>0.17
Sudan/Berg	F_3	220P+	122	0	0	7:8:1[b]	366.0	<0.0001

[a]Res:Sus. [b]Res:Seg:Sus.

either a cis regulatory mutation or a second-site suppressor. Further examination of the Illumina reads for line M767 identified a heterozygous C/T mutation changing a Gly to Glu in the conserved GLPL motif. Rust testing in the M3 progeny of M767 determined that it segregated with *Rph1*-mediated resistance, whereas all other mutant lines were homozygous-susceptible. We PCR-amplified and Sanger-sequenced the *Rph1* gene for seven mutant lines (M199, M422, M430, M727, M761, M763, and M767). We confirmed the MutChromSeq results and identified two additional mutations in lines M199 and M430 in the NBS domain. Three mutant lines (M727, M761, and M763) shared the same C/T mutation in the Leucine-rich repeat (LRR) domain (Gly to Asp), despite originating from different M_2 spikes, therefore a total of five independent mutations were confirmed in *Rph1* (Table 3).

We also performed MutChromSeq on the entire chromosome 2H assembly to rule out the involvement of additional gene/s outside the defined *Rph1* region that show a concordance of multiple independent nonsynonymous mutations. As part of this analysis, we assessed: (1) whether reported single nucleotide polymorphisms were induced by sodium azide mutagenesis (i.e. G > A or C > T), (2) whether the contig reported contains predicted high confidence (HC) coding regions, and (3) whether the reported mutations are located in a coding region of aligned genes. We identified 45 candidate contigs across the entire Sudan chromosome 2H containing mutations. Further examination determined that 31 of the 45 contigs either carried no predicted coding genes or that the identified mutations were not located in predicted genes. In a further five contigs where mutations were identified in genes, manual checking determined they were not induced by sodium azide and were therefore considered false-positives. The remaining contigs carried only a single mutation within a candidate HC gene; however, these mutations were likely nonfunctional. Scaffold 2895_0 identified initially in the 1.3-kb region of interest remained the only plausible *Rph1* candidate based on the presence of multiple independent nonsynonymous mutations.

The *Rph1* resistance gene in Sudan encoded for an NLR receptor protein (981 amino acid residues) with a predicted Rx coiled-coil (CC) domain and carried a single intron in the NBS domain, which is commonly found in NLRs in the Triticeae subtribe (Steuernagel et al., 2018; Fig. 3). Phylogenetic analysis comparing the full-length Sudan Rph1 protein sequence with that of previously cloned Triticeae NLRs determined that Rph1 was more closely related to the Mla clade than other more divergent rust- and mildew-resistance NLRs, mainly from bread wheat (Fig. 4). The closest ortholog to Rph1 was an Arabidopsis (*Arabidopsis thaliana*) RPM1 NLR homolog from *Triticum urartu*, which shared 89% amino acid identity. Further sequence comparison of the Sudan resistance allele with three barley cvs all lacking *Rph1*-mediated resistance and with available genomic sequence (Morex, Bowman, and Barke) identified seven amino acid substitutions that were common among the susceptible accessions.

Rph1 barley differential lines Sudan and Berg are known to carry the same race-specificity in response to multiple Australian- and American-derived *P. hordei* pathotypes; however, the immune (Im) infection type displayed by Berg when challenged with *Rph1*-avirulent isolates was different from the hypersensitive response (HR) necrosis observed in Sudan. We therefore hypothesized that the *R* genes carried by Sudan and Berg might be allelic at *Rph1* or be different but carry closely linked genes. Rust tests using *P. hordei*

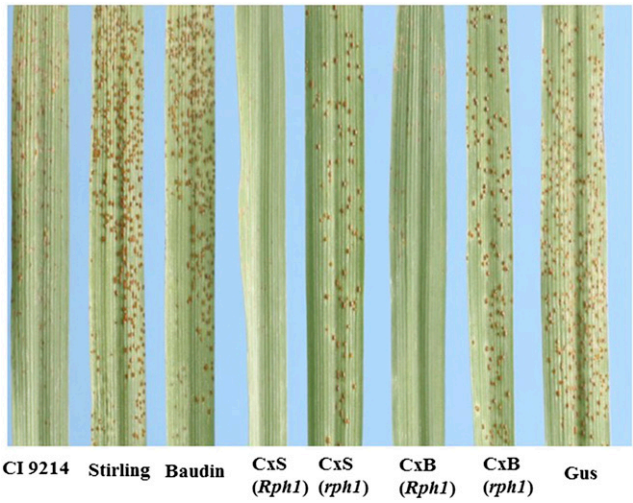

Figure 1. Monogenic inheritance for *Rph1*-mediated resistance in the CI 9214 x Stirling and CI 9214 x Baudin mapping populations. Seedling leaves of the infection types of (left to right): CI 9214 (C), Stirling (S), Baudin (B), C/S DH line (*Rph1*), C/S DH line (*rph1*), C/B RIL (*Rph1*), C/B RIL (*rph1*), and susceptible control Gus inoculated with *P. hordei* pathotype 4610P+ (virulent on *Rph12* in Baudin, *Rph9.am* in Stirling, and avirulent to *Rph1* in CI 9214).

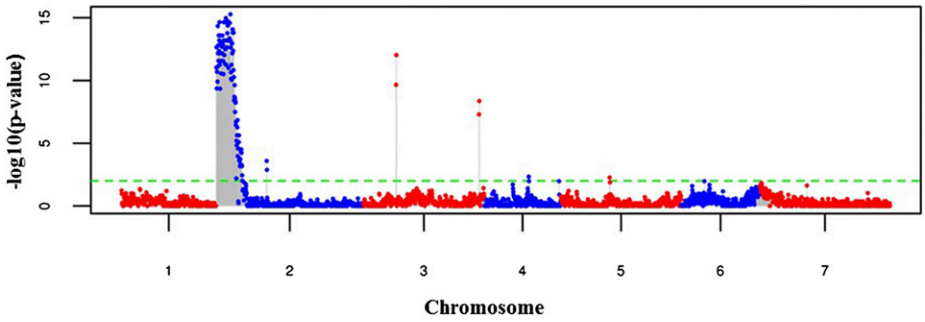

Figure 2. Marker trait association analysis in the CI 9214 x Stirling DH population maps *Rph1* to the short arm of chromosome 2H. Genome-wide Manhattan plot derived from MTA analysis using Fisher's exact test on 2 × 2 count table for seedling resistance to *P. hordei* pathotype 4610P+ (binary scoring data) in the CI9214/Stirling DH population using >10,000 DArT-Seq markers. The –log10 of *P*-values were plotted against the positions on the physical Bowman genome assembly. The peaks above minimum threshold of 2 (*P*-value = 0.03) can be considered as significantly associated. Vertical axis represents −log10 (*P*) values of the *P*-value of the MTA. The colors blue and red were used to differentiate between chromosomes (1H–7H, indicated by tick marks).

pathotype 220P+ in a Sudan/Berg F_3 population (n = 122) did not reveal any segregation (resistance vs susceptible); with all families showing a nonsegregating resistant pattern. The χ^2 value (χ^2 = 366) significantly ($P < 0.0001$) deviated from the segregation ratio expected for two independent genes (Table 2). No susceptible individual was recovered among the entire progeny (n = 1,860 seedlings), indicating that the loci conferring seedling resistance in both genotypes is most likely the same, that is, allelic, or two physically proximal genes linked in repulsion. The maximum recombination frequency between two alleles was estimated to be r = 1.2 at P = 0.05.

We assessed the response of eight different barley lines (accessions and cvs) postulated to carry *Rph1*-mediated resistance with four *P. hordei* pathotypes with contrasting virulence to determine if they had the Im or HR response characteristic of the resistance observed in Berg and Sudan, respectively (Table 1; Supplemental Fig. S4). Tests indicated that five lines had the HR response and two lines (UWA Seln 8861 and HOR15560) were Im as observed for Berg. We sequenced the *Rph1* gene in these three Im lines (Berg, UWA Seln 8861 and HOR15560) and three HR lines (CI 9214, ISR950.13 and CIho 119558) to confirm the haplotype of resistant parent CI 9214 and to determine whether there was any molecular correlation between the haplotypes of lines with the Berg (Im) versus Sudan (HR) phenotypes. The results revealed that both different phenotypes are not determined by their *Rph1.a* allele sequence. For example, the CI 9214 (HR) and HOR15560 (Im) alleles were identical to that in Sudan (HR); in contrast Berg (Im), CIho 119558 (HR), ISR950.13 (HR), and UWA seln 8861 (Im) all carried the susceptibility allele, suggesting their resistance is conferred by a different gene (Table 1).

DISCUSSION

Understanding and exploiting the mechanisms that underpin important agronomic traits such as resistance

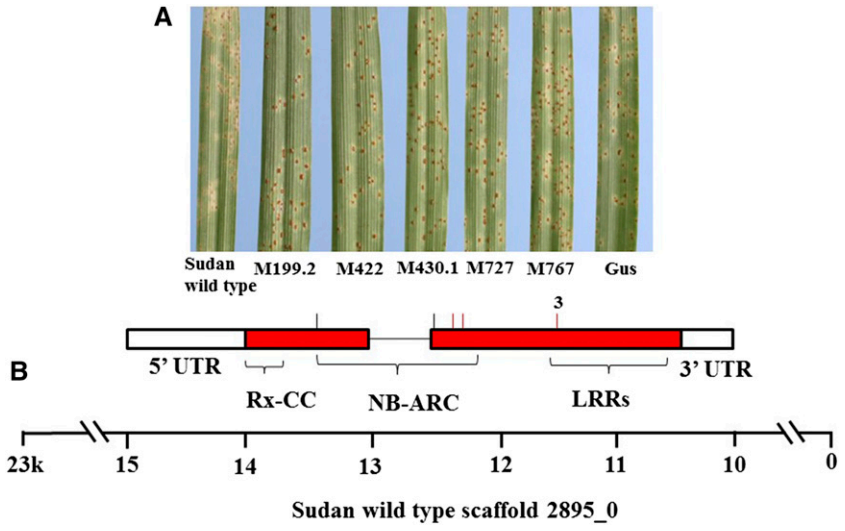

Figure 3. MutChromSeq and subsequent Sanger sequence confirmation of five susceptible *rph1* knockout mutants reveals that Rph1-mediated leaf rust resistance is conferred by a CC NLR gene on chromosome 2H. Cloning of the barley *Rph1* gene using MutChromSeq. A, Resistant *Rph1* barley wild-type donor cv Sudan, five sodium azide-derived susceptible mutants (M199.2, M422, M430.1, M727, and M767), and the susceptible control Gus. B, *Rph1* locus showing intron–exon boundaries, protein domains, and 5′ and 3′ untranslated regions. Mutations identified by both MutChromSeq and Sanger sequencing are indicated by red vertical lines, whereas mutations identified by Sanger sequencing of additional mutants are indicated by black vertical lines. A number above the line indicates identical mutations occurring in independent lines. UTR, untranslated region.

Table 3. *Sodium azide induced mutations in the* Rph1 *candidate scaffold confirmed by Sanger sequencing*

bp Position Scaffold 2895_0	Mutational Change	Affected Lines	Amino Acid Change
11,416	G > A	763.2, 761.2, 727	Gly > Asp
12,268	C > T	422	Gly > Asp
12,298	C > T	767	Gly > Glu
12,458	C > T	430.1	Ala > Pro
13,365	G > A	199.2	Gly > Glu

to biotic and abiotic stresses is only possible through identifying variants at the molecular level that define their genetic control. Chromosome sorting and sequencing of wild type and multiple independent mutants, referred to as MutChromSeq, provides a lossless complexity reduction method suitable for the cloning of any gene of interest (Sánchez-Martín et al., 2016; Steuernagel et al., 2017). In this study, we used this approach to clone the leaf rust resistance gene, *Rph1*, from cultivated barley. *Rph1* was an attractive target due to the ability to reliably phenotype mutants with ablated resistance. We determined that in both the CI 9214/Stirling and the CI 9214/Baudin mapping populations, *Rph1* was inherited as a monogenic trait. Although there was no requirement for recombination-based genetic mapping, we used MTA analysis (>10,000 DArT-Seq markers) and mapped *Rph1* to the short arm of chromosome 2H in the CI 9214/Stirling DH population, confirming previous trisomic analysis by Tuleen and McDaniel (1971). The coupling of MutChromSeq with genetic mapping in this study reduced the size of the genomic region interrogated and the number of mutants required. This is particularly important for diploid organisms, such as barley, where generating multiple independent knock-outs in the same gene requires more work (i.e. generating and screening of larger mutant populations) compared to that in a polyploid organism, such as bread wheat.

The MutantHunter pipeline reported in Sánchez-Martín et al. (2016) was modified to search for candidates within the 1.3-Mb region harboring *Rph1* on chromosome 2HS. We identified multiple independent nonsynonymous mutations within a gene on a single contig (Scaffold 2895_0). It was expected, however, that other sodium azide-induced mutations would also be identified outside the regions of interest. Therefore, we mapped the same mutant reads to the entire Sudan chromosome 2H assembly and identified the same *Rph1* candidate on Scaffold 2895_0, thus validating our targeted approach and ruling out the involvement of other genes outside the defined *Rph1* region. Nevertheless, numerous contigs (45) were identified outside the target region harboring mutations that, after further examination, were ruled out as candidates due to either

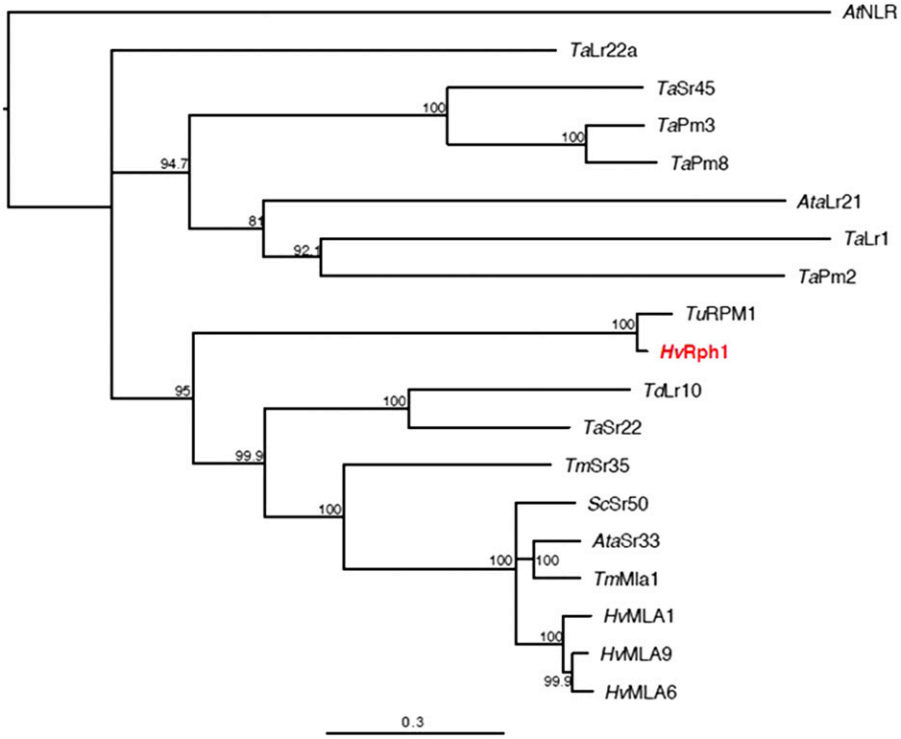

Figure 4. Phylogenetic analysis groups Rph1 in a separate cluster from other NLRs conferring rust resistance in cereal crops. Neighbor-joining tree analysis of Rph1 from barley cv Sudan. The *Rph1* amino acid sequence was then compared with related NLR sequences from the Triticeae including: *A. tauschii* (*Ata*; Lr21-AAP74647.1 and Sr33-AGQ17384.1). *S. cereale* (*Sc*; Sr50-ALO61074.1), *T. aestivum* (*Ta*; Lr22a-ARO38244.1, Sr22-CUM44212.1, Lr1-ABS29034.1, Sr45-CUM44213.1, Pm2-CZT14023.1, Pm3-ADH04488.1, and Pm8-AGY30894.1), *T. urartu* (*Tu*; RPM1-like EMS67965.1), *T. monococcum* subsp. *monococcum* (*Tm*; Sr35-AGP75918.1 and MLA1-ADX06722.1), *T. dicoccoides* (*Td*; Lr10-ADM65840.1), and *H. vulgare* (*Hv*; MLA1-AAG37354.1, MLA6-CAC29242.1, and MLA9-ACZ65487.1). Statistical support for individual nodes was estimates from 1,000 bootstrap replicates and values are represented as percentages on the nodes (values of >70% are shown). The scale bar represented the proportion of site changes along each branch.

(1) the absence of coding sequence, (2) mutations not in coding genes, (3) false-positives, or (4) single non-function mutations within genes. Due to the higher N_{50} of this study (20.1 kb), this number is higher than that reported in the study of Sánchez-Martín et al. (2016) where the N_{50} was 1.4 kb. Taken together, we identified the *Rph1* resistance gene candidate on the short arm of chromosome 2H based on the sequence comparison of three *rph1* mutants with wild-type Sudan. All three mutations were confirmed by Sanger sequencing in addition to a further two independent mutants derived from additional spikes tested at a later stage. Consequently, based on the confirmation of five non-synonymous mutations, we now refer to this gene as *Rph1*.

Rph1 encodes a predicted CC NLR receptor protein. Phylogenetic analysis in our study suggested that *Rph1* was located in a separate clade and is likely representative of a subfamily of NLRs that is distinct from other recently cloned rust- and mildew-resistance NLR proteins from the Triticeae. Recent cloning studies in wheat have determined that stem rust (*Sr33* and *Sr50*) and leaf rust (*Lr22a*) protein sequences show marked similarity to previously characterized NLR families such as Mla from barley and RPM1 from Arabidopsis, respectively (Periyannan et al., 2013; Mago et al., 2015; Thind et al., 2017). The closest known ortholog to Rph1 was an RPM1-like NLR protein from *T. urartu* (89% sequence identity), suggesting both shared a common ancestor; however, we show that Rph1 showed greater similarity to the Mla family relative to that for Lr22a. Although *Lr22a* was located in a syntenic region on chromosome 2DS to *Rph1*, bioinformatics analysis using the 2016 Morex genome browser suggests the true ortholog of *Lr22a* and RPM1 is located ~1 Mb downstream from the *Rph1* gene. Interestingly, the predicted protein sequences of Rph1 and TuRPM1 carried an Rx-like coil-coiled domain at the N-terminal that has been shown to be essential for resistance against virus X in potato (*Solanum tuberosum*; Bendahmane et al., 2002).

The nucleotide binding ATPase (NB-ARC) domain of NB-LRR proteins is known to act as a molecular switch that regulates their activity. Previous characterization of loss-of-function mutants suggests that the GLPL motif functions as a hinge, facilitating nucleotide-dependent movement of the flanking helices, and that the ARC1 subdomain transmits these conformational changes to the other parts of the protein (Leipe et al., 2004). Dodds et al. (2001) determined that a Gly to Glu substitution in the highly conserved GLPL motif of the P2 NLR protein from flax (*Linum sativum*) compromised resistance to flax rust (*Melampsora lini*). The HR induced by transient expression of the Rx NLR from potato was inhibited by the presence of two mutations targeting the Gly and Pro residues of the GLPL motif (Bendahmane et al., 2002). We identified the same mutational variant as Dodds et al. (2001) in our study for *Rph1* mutant M767. We also identified a second mutation six amino acids downstream from the GLPL motif, suggesting that this conserved region is critical for *Rph1*-mediated resistance. Interestingly, three mutant families derived from different M_2 spikes shared the same mutation in the LRR domain that caused a Gly to change to an Asp. It is likely these M_2 spikes were derived from the same meristem cell from a single M_1 plant, and hence carry the same mutation that are therefore not independent. Sanger sequencing of the full-length coding sequence in our study failed to detect mutations in M544 and M483, suggesting these families possibly either carry mutations within the regulatory sequences of *Rph1* or possibly in a secondary-site repressor. Previous studies in wheat (Feuillet et al., 2003; McGrann et al., 2014), barley (Torp and Jørgensen, 1986), and Arabidopsis (Tornero et al., 2002) indicated that second site or extragenic mutants during R gene loss-of-function screens was a common occurrence.

Genes that confer pathogen defense are often clustered in plant genomes, evolving via duplication, diversifying selection through mutation and transposon-insertion events. In barley, the best example of this is the *Mla* locus, which is organized into three gene-rich islands separated by retro-transposable elements (Wei et al., 2002). Such gene clusters in plants often comprise allelic series or represent closely related genes with distinct recognition specificities. *Rph1* was one of the four clustered NLRs in Morex; however, the corresponding homologous 22-kb scaffold 2895_0 from Sudan carried two full-length NLRs separated by only 2 kb relative to 80 kb in Morex (Supplemental Fig. S3). Taken together, *Rph1* appears to be part of a complex resistance locus. In wheat, three closely linked stripe rust resistance genes were recently cloned using MutRenSeq based on the presence of multiple independent mutants in each of the three genes (Marchal et al., 2018). *Yr5* and *Yr7* were originally hypothesized to be allelic and closely linked with *YrSP* (Zhang et al., 2009); however, molecular analysis performed on this complex locus on chromosome 2B determined that the *Yr5*- and *Yr7*-mediated resistance were each conferred by neighboring paralogous NLR genes (Marchal et al., 2018). In contrast to that for *Yr5* and *Yr7*, Sudan and Berg in our study share the same recognition specificity in response to all Australian and North American *P. hordei* isolates tested, but differ in the infection type, with Berg displaying immunity and Sudan being characterized by HR necrosis. An allelism test between Sudan and Berg determined phenotypically that no recombinants were found that gave rise to susceptible individuals, indicating that the two genes are either closely linked in repulsion or allelic. Direct comparison of the Rph1 protein sequence between Sudan and three sequenced barley genotypes lacking functional *Rph1* (Morex, Bowman, and Barke) identified seven amino acid substitutions that are different between the resistant and susceptible alleles. Sanger sequencing of the *Rph1* gene using six barley accessions, including CI 9214, Berg, HOR15560, UWA seln 8861, ISR950.13, and CIho 11958, revealed that both CI 9214 and HOR15560 *Rph1* alleles were identical to that in Sudan (*Rph1.a*); however, Berg and the remaining three

accessions carried the same susceptible haplotype as that in Morex, Bowman, and Barke with no correlation between *Rph1* haplotype and infection type. The observation that Berg has the *rph1* haplotype that is associated with multiple susceptible lines suggests there may be another gene conferring resistance to *P. hordei* in Berg that is epistatic to the susceptible *Rph1* haplotype. It is therefore more likely that the Berg resistance gene is not an allele of *Rph1* as hypothesized but likely, as in the case for *Yr5/Yr7*, a closely linked or neighboring NLR gene. As we did not generate mutants for Berg, we can only speculate that the resistance present in Berg forms part of the same NLR cluster as that for *Rph1*-mediated resistance. We further hypothesize that due to overlapping specificity, possibly the resistance genes present in Sudan and Berg recognize the same avirulence product in *P. hordei*.

The major bottleneck for gene cloning in cereal crops is the development of high-quality genomic sequence from the genotype carrying the trait. We significantly improved the wild-type cv Sudan assembly of chromosome 2H and increased the N_{50} value by 15-fold, from 1.4 kb (Sánchez-Martín et al., 2016) to 20.1 kb. We compared the quality of chromosome 2H assemblies for Sudan and Foma (Sánchez-Martín et al., 2016) relative to that of the 2D Dovetail assembly reported by Thind et al. (2017) by performing a Benchmarking Universal Single-Copy Orthologs analysis (http://busco.ezlab.org; Simão et al., 2015) and by mapping all predicted HC genes to the respective chromosome two assemblies (Supplemental Table S2, A and B). All forms of analysis indicated that not using MDA before Illumina sequencing and library construction improved the overall assembly quality in wild-type Sudan. Sánchez-Martín and colleagues were able to successfully clone a gene from both wheat and barley with an average N_{50} of 1.4 kb, meaning that 50% of the chromosome was assembled with contigs of 1.4 kb or longer. The full-length *Pm2* gene in wheat, however, is >4 kb, suggesting that the contig harboring *Pm2* was likely derived from a rather small frequency of large sequence contigs. Methods such as MutChromSeq and the recently developed targeted chromosome-based cloning via long-range assembly are unique as they generate cv-specific sequence information that allows the functional exploration of sequences not found in a reference genome (Steuernagel et al., 2016; Thind et al., 2017). Although our reported N_{50} is far from comparable to the recently developed Chicago long-range sequencing technology, it permits a low-cost effective haplotype analysis of nonreference genomes. Furthermore, our improved wild-type chromosome assembly provides increased confidence when cloning genes with large introns using the MutChromSeq approach.

In conclusion, recently developed genomic methodologies such as MutChromSeq, targeted chromosome-based cloning via long-range assembly, MutRenSeq, and AgRenSeq can now be used to mitigate the limitations of traditional map-based cloning approaches in crop plants. We used MutChromSeq to clone *Rph1*, which confers leaf rust resistance in barley. Seven amino acid changes were identified as diagnostic for resistance and susceptibility, enabling the prospect of enhanced marker-assisted selection. Although virulence for *Rph1*-mediated resistance is present at varying frequencies in global *P. hordei* pathogen populations, identifying the basis of resistance in a wide array of *R* genes from crops will allow effective pyramiding of multiple resistances into elite crop varieties. The rapid introduction of multiple favorable disease-resistance alleles in plants can also now be accelerated because of the emergence of new breeding technologies such as gene editing coupled with speed breeding (Ghosh et al., 2018; Watson et al., 2018). Future work will also involve determining the effectiveness of *Rph1*-mediated resistance in response to other diseases in crops such as wheat, maize (*Zea mays*), and rice (*Oryza sativa*) using a transgenic approach.

MATERIALS AND METHODS

Plant and Pathogen Materials

The barley (*Hordeum vulgare*) cv CI9214 was postulated to carry *Rph1*, whereas cvs Baudin (*Rph9.z*) and Stirling (*Rph9.am*) were known to lack *Rph1* based on their seedling response in multipathotype tests. Two barley mapping populations were produced to map the *Rph1* resistance gene, including a CI 9214/Stirling (258 lines) DH population and an F_9 generation RIL population derived from the cross CI 9214/Baudin (385 lines). Both populations were sourced from the Plant Breeding Institute–University of Sydney. For tests of allelism, barley leaf rust differential genotypes cvs Sudan and Berg were crossed to produce F_1 seed. Each F_1 plant was then selfed to produce F_2 seed, and 150 F_2 seeds derived from a single F_1 plant were randomly selected and space planted, then selfed in the field in 2017 and 130 F_3 families were harvested for rust testing.

The four pathotypes of *Puccinia hordei* used in the study, along with their virulence/avirulence profiles, are listed in Table 1. Both mapping populations and a collection of barley accessions postulated to carry *Rph1* were phenotypically assessed separately with the four *P. hordei* pathotypes, which have contrasting virulence. We used two *Rph1*-avirulent [4610P+ and 220P+] and two *Rph1*-virulent [5457P+ and 253P-] pathotypes, respectively, to rule out the involvement of known all-stage resistance genes in parental genotypes Stirling (*Rph9.am*) and Baudin (*Rph12*).

Statistical Analyses

The Chi squared (χ^2) test was used to determine the goodness-of-fit between the phenotypic data recorded from disease infection types versus expected genetic ratios.

Inoculation Procedure and Disease Assessment

Seedlings were inoculated as described by Dracatos et al. (2014) for both mapping and mutant populations. Infection types were recorded 10 d post-inoculation using the "0" to "4" scale (Park and Karakousis, 2002). Infection types of test lines of each population were compared with those displayed by the parents and barley differential lines to assure accurate classification into resistant and susceptible classes.

Mapping *Rph1* in the CI 9214/Stirling and CI 9214/Baudin Mapping Populations

Genomic DNA was extracted from the leaf tissues of a single plant from a subset of 61 CI 9214/Stirling DH lines and 92 CI 9214/Baudin RILs from both mapping populations, using the CTAB method as described by Fulton et al.

(1995). The DNA was prepared and shipped to DArT for genotypic analysis as detailed on their Web site: www.diversityarrays.com.au.

Marker-Trait Analysis of Closely Linked DArT Markers at the *Rph1* Locus

The phenotypic data using *P. hordei* pathotype 4610P+ was converted to binary data (susceptible 3+ = 0, resistant ;1-CN = 1). Marker-trait analysis of each DArT-Seq marker with the *Rph1* phenotype was conducted by computing Fisher's exact test on 2 × 2 count tables using R statistical software (www.r-project.org). The null hypothesis was that the DArT-Seq marker genotypes were not associated with resistance to *P. hordei*, hence a random distribution of genotypes in the resistant and susceptible phenotypic groups. The log10 of *P*-values were plotted against the positions on the physical Bowman genome assembly by means of chromosome-wise and genome-wide "Manhattan" plots. DArT-Seq markers associated with the *Rph1* resistance gene are detailed in Supplemental Table S2.

Generation of Mutant Population and Phenotypic Analysis

The mutagenesis procedure was performed according to that described in Chandler and Harding (2013) with some modifications. Approximately 2,500 seeds of barley cv Sudan were immersed in water at 4°C overnight. The imbibed seed were transferred to a 2-L measuring cylinder filled with water and aerated with pressurized air for 8 h, with one change of water after 4 h. The water was drained. Seeds were incubated in a shaker for 2 h in freshly prepared 1 mM sodium azide dissolved in 0.1 M sodium citrate buffer (pH 3.0), washed extensively in running water for at least 2 h, and placed in a fume hood to dry overnight. Seeds were sown in pots. After two weeks, pots were transferred to an outside standout area. After another two weeks, seedlings were space transplanted to the field. Approximately 9,400 spikes were harvested from ~1,700 M_1 plants, that is, on average 5–6 spikes/plant. Because each tiller is usually derived from independent (genetically distinct) meristem cells in the seed embryo (Stadler, 1928), the M_2 seeds were threshed from single spikes.

The Sudan M_2 spikes and M_2-derived M_3 families were phenotypically assessed using the *Rph1*-avirulent pathotype 4610P+. In all cases at least two susceptible plants were transplanted for each candidate M_2 family, segregating for *rph1* knockouts for progeny testing. All sequence-confirmed mutants were progeny-tested at both the M_3 and M_4 generations and found to be homozygous-susceptible. Sanger sequence confirmation of all mutants was performed using sequence-specific primers designed to capture both exon regions of the *Rph1* candidate gene.

Flow Sorting and Preparation of Chromosomal DNA

Suspensions of intact mitotic chromosomes were prepared as described by Lysák et al. (1999). Briefly, root tip meristem cells were synchronized using hydroxyurea, accumulated in metaphase using amiprophos-methyl, and mildly fixed by formaldehyde. Chromosome suspensions were prepared by mechanical homogenization of 50 root tips in 1 mL ice-cold LB01 buffer (Doležel et al., 1989). Chromosome analysis and sorting was done on a FACSAria II SORP flow cytometer and sorter (Becton Dickinson Immunocytometry Systems). Barley 2H chromosomes were sorted after bivariate analysis of diamidino-2-phenylindole dihydrochloride fluorescence and fluorescence of GAA microsatellites labeled with fluorescein isothiocyanate using FISH in suspension (Giorgi et al., 2013).

Illumina Library Construction and Sequencing

Flow-sorted chromosomes were treated with freshly prepared proteinase K and purified on Microcon YM-100 columns (Millipore). For 2H chromosomes from mutant lines, DNA was amplified by MDA using Illustra GenomiPhi V2 DNA Amplification Kit (GE Healthcare) as described by Šimková et al. (2008). Amplified DNA was then fragmented by a Bioruptor sonication device (Diagenode) and used to prepare sequencing libraries with a TruSeq DNA PCR-Free Library Prep kit (Illumina). For 2H chromosome of cv Sudan (wild type), nonamplified DNA (16 ng) was directly fragmented with a Bioruptor sonication device and subsequently used to prepare a sequencing library with the NEBNext UltraII DNA library Prep Kit for Illumina (New England Biolabs). Pooled libraries of wild-type and five mutant chromosomes were sequenced on an Illumina HiSeq in Rapid Run mode to gain 2 × 250-bp PE reads. Trimmomatic was used to remove sequencing adaptors and trim raw reads (LEADING: 20 TRAILING: 20 SLIDINGWINDOW: 4:15 MINLEN: 100). The assembly of the wild-type chromosome sequence was performed with Meraculous (Chapman et al., 2011) using *k*-mer size 111.

MutChromSeq

We extended the functionality of the MutChromSeq (Sánchez-Martín et al., 2016) pipeline by adding the possibility to filter for a set of reference scaffolds. The updated pipeline is available at GitHub (https://github.com/steuernb/MutChromSeq). In the specific case of *Rph1*, we used available mapping information. In relation to the barley reference genome sequence of cv Morex (Mascher et al., 2017), this was on chromosome 2H between nucleotide positions 13,139,911 bp and 14,361,439 bp. All genes that had been annotated within that interval were aligned to scaffolds from our Sudan assembly of 2H. The list of scaffolds with the best alignment for each gene were used as an additional filter for the updated MutChromSeq pipeline. The Sudan 2H assembly was masked for repeats using RepeatMasker (http://repeatmasker.org) with external repeat library TREP (http://wheat.pw.usda.gov/ITMI/Repeats; Wicker et al., 2002). Raw data from flow-sorted chromosomes of wild type and mutants were quality-trimmed using Trimmomatic (Bolger et al., 2014). Trimmed reads were aligned to repeat-masked reference using the software BWA (Li and Durbin, 2009), version 0.7.12. Mappings were further processed using SAMtools (Li et al., 2009), version 0.1.19, and the following subprograms; parameters diverging from default are mentioned below: samtools view -Shub -f 2 input.sam output.bam; samtools sort; samtools rmdup; samtools index; samtools mpileup -B -Q 0 -f Sudan_2H.masked.fasta. Pileup formatted files for wild type and mutants were converted to MutChromSeq input with Pileup2XML.jar -c 5 -a 0.7. Finally, MutChromSeq.jar was executed with -n 2 -c 5 -a 0.1 -z 1. The candidate contig was manually inspected using IGV (http://software.broadinstitute.org/software/igv/book/export/html/6).

Phylogenetic Analysis

The predicted Rph1 amino acid sequence was used as a query in GenBank using the program BlastP to identify closely related sequences. The Rph1 amino acid sequence was then compared with that of related NLR sequences from Triticeae including: *Aegilops tauschii* (*Ata*) Lr21-AAP74647.1 and Sr33-AGQ17384.1), *Secale cereale* (*Sc*; Sr50-ALO61074.1), *Triticum aestivum* (*Ta*; Lr22a-ARO38244.1, Sr22-CUM44212.1, Lr1-ABS29034.1, Sr45-CUM44213.1, Pm2-CZT14023.1, Pm3-ADH04488.1, and Pm8-AGY30894.1), *T. urartu* (*Tu*; RPM1-like EMS67965.1), *T. monococcum* subsp. *monococcum* (*Tm*; Sr35-AGP75918.1 and MLA1-ADX06722.1), *T. dicoccoides* (*Td*; Lr10-ADM65840.1), and *H. vulgare* (*Hv*; MLA1-AAG37354.1, MLA6-CAC29242.1, and MLA9-ACZ65487.1). An unrelated NLR from Arabidopsis (*Arabidopsis thaliana*), At5g45510-Q8VZC7.2, was included as an out-group. A multiple sequence alignment was performed using ClustalW (Larkin et al., 2007), in Geneious version 11.0.2 (https://www.geneious.com) with the BLOSUM scoring matrix, and settings of gap creation at −10 cost, and gap extension at −0.1 cost per element. After removing all ambiguously aligned regions using trimAl (Capella-Gutiérrez et al., 2009) the final sequence alignment of length 1,026 amino acids (n = 19) was determined. A phylogenetic tree based on this alignment was then inferred using the Neighbor-Joining method in the Geneious Tree Builder software, employing the Jukes-Cantor genetic distance model. Bootstrap support for individual nodes was generated using 1,000 bootstrap replicates.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL under accession number MK376319.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Genome-wide MTA analysis in the CI 9214/Stirling DH population.

Supplemental Figure S2. Biparametric flow karyotype of barley cv Sudan.

Supplemental Figure S3. Sequence comparison at the *Rph1* locus between the Morex reference and wild-type resistance donor cv Sudan.

Supplemental Figure S4. Infection response of seven barley accessions postulated to carry leaf rust resistance gene *Rph1*.

Supplemental **Table S1.** Summary table of most closely associated DArT-Seq markers at the *Rph1* locus on chromosome 2H.

Supplemental Table S2. Assessment of sequence assembly quality for chromosome 2H in barley cvs Foma (Sánchez-Martín et al., 2016) and Sudan and chromosome 2D in wheat cv Campala (Thind et al., 2017) using (A) Benchmarking Universal Single-Copy Orthologs and (B) mapping all predicted HC genes.

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