Discovery and characterisation of a new leaf rust resistance gene introgressed in wheat from wild wheat *Aegilops peregrina*

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Wild wheat species *Aegilops peregrina* (*UpUpSpSp*), harbours resistance to various diseases including leaf rust and stripe rust. Inheritance studies in a recombinant inbred line population of wheat-*Ae. peregrina* introgression line IL pau16061 revealed the transfer of a single major dominant gene conditioning all stage resistance, herein temporarily designated as *LrAp*. Genomic in situ hybridisation of IL pau16061, resistant and susceptible RILs with U- and S-genome DNA probes confirmed that the introgression with leaf rust resistance is from the *Up* genome of *Ae. peregrina*. Fluorescence in situ hybridisation using chromosome specific probes identified *Up* genome introgression to be on the long arm of wheat chromosome 6B. To genetically map *LrAp*, bulked segregant analysis was combined with resistance gene enrichment sequencing (MapRenSeq). Five nucleotide binding leucine-rich repeat contigs distinguished resistant and susceptible bulks and single nucleotide polymorphism (SNP) markers from these contigs co-segregated with *LrAp*. All five RenSeq NB_ARC contigs showed identity with the long arm of wheat chromosome 6B confirming the introgression on 6BL which we propose is a compensating translocation from *Ae. peregrina* chromosome 6U due to homoeology between the alien and wheat chromosomes. The SNP markers developed in this study will aid in cloning and marker assisted gene pyramiding of *LrAp*.

Wheat is the prime food commodity of major part of the world’s population with an annual harvest of 733 million tons in 2015. It is a leading source of calories as well as protein both for humans and livestock. The limited genetic diversity at the farmers’ field increases genetic vulnerability to various biotic and abiotic stresses. Almost wherever wheat is grown, production is significantly constrained by one or more of the three rust diseases. These include leaf rust (*Puccinia triticina* Eriks); stem rust (*P. graminis* Pers. f. sp. *tritici*); and stripe rust (*P. striiformis* Westend f. sp. *tritici*). Among the three wheat rust pathogens, leaf rust cause less damage as compared to stripe and stem rust, however, due to its recurrent global occurrence, it would be anticipated that total annual losses incurred would be greater than any other rusts. In India, stem rust and stripe rust are geographically constrained, while leaf rust is endemic across all production areas. The disease not only reduce the kernel weight but also lessen number of kernels per head. The frequent emergence of novel pathotypes of *P. triticina* (Pt) and switching of virulence patterns constitute the main hurdles for its management.

Wild species of wheat, especially members of the tertiary gene pool, carry an immense diversity of disease resistance (*R*) genes that could enable more sustainable disease control. Till date, 79 genes for leaf rust resistance have been designated and ~44% are from wild progenitor and non-progenitor species. These include *Lr21, Lr22a, Lr32, Lr39/Lr41, Lr40, Lr42, Lr43 (Ae. tauschii); Lr28, Lr35, Lr36, Lr47, Lr51, Lr56 (Ae. speltoides); Lr63 (T. monococcum); Lr53, Lr64 (T. dicoccoides); Lr18, Lr50 (Triticum timopheevi); Lr37 (Ae. ventricosa); Lr9, Lr76 (Aegilops umbellulata); Lr19, Lr24, Lr29 (Thinopyrum ponticum); Lr25, Lr26 (Secale cereale); Lr38 (Th.
Ae. triuncialis seedling stage while WL711 was highly susceptible. The IL pau16061/WL711 F5:6 RIL population along with Ae. peregrina wild species (UpSpSp) with the wheat line WL711 (vulnerable to rust) 21. In the present study, the genes. R markers makes the RenSeq approach even more robust for mapping and tracking candidate genes. 

pau16061, i.e. whether from the Up or Sp genome, GISH was performed with genomic DNA from the U- and S-genome diploid species Ae. umbellulata and Ae. speltoides, respectively, as probes. Utilising S-genome DNA as probe, a strong signal was detected e distally in one pair of sub-metacentric chromosomes (Fig. 2a). GISH using S-genome DNA also detected a weak signal on the distal region of the long arm of a pair of chromosomes with satellites (Fig. 2b) indicating that IL pau16061 carries introgression from both the Up and Sp genomes of cytogenetic techniques 10,13,14 and limited transferability of microsatellite markers to the tertiary gene pool 15. These factors limited the utilisation of these attractive resources for wheat improvement. However, the recent developments in genomic approaches and the accessibility of various genome sequences has allowed the fast access to genes in wild species.

A leaf rust resistant introgression line (IL) pau16061, was developed previously from the hybridisation of the parental lines was tested at the seedling stage against parental pathotype 77-5. The F1 plants obtained from this cross

IL pau16061 was characterised cytogenetically to identify the donor genome of the introgression in IL Ae. peregrina. IL pau16061 was resistant to the majority of the leaf rust pathotypes prevalent in the Indian subcontinent Pt11, Pt12-5, Pt16-1, Pt77-5, Pt77-8, Pt77-10, Pt104-2, Pt104B, Pt106 and Pt162-2 at the seedling stage while WL711 was highly susceptible. The IL pau16061/WL711 F5:6 RIL population along with parental lines was tested at the seedling stage against Pt pathotype 77-5. The F1 plants obtained from this cross were resistant with near-immune infection type, indicating the dominant behavior of the leaf rust resistance gene LrAp. Twelve seedlings from each of 160 F5:6 families were screened. All the seedlings in 69 families showed resistant infection type (1) and these RILs were classified as homozygous resistant, while all the seedlings in 81 families showed infection type 3-3 and these RILs were classified as homozygous susceptible (Fig. 1) 22. Ten families were found to to be segregating for the leaf rust resistance gene. Seedlings were transplanted in the field and tested for terminal disease severity. At adult plant stage, WL711 showed terminal disease severity of 80-80, whereas Ae. peregrina and IL pau16061 were highly resistant (0-TR). Out of the 160 RILs, 69 were totally free from leaf rust (0) whereas 81 showed leaf rust severity of 60-80S, and 10 were found segregating. The segregation pattern confirmed a monogenic inheritance of leaf rust resistance (X2 = 1.875, p = 0.342) and the resistance locus was tentatively designated as LrAp.

To identify the genomic origin of the Ae. peregrina introgression in IL pau16061, i.e. whether from the Up or Sp genome, GISH was performed with genomic DNA from the U- and S-genome diploid species Ae. umbellulata and Ae. speltoides, respectively, as probes. Utilising S-genome DNA as probe, a strong signal was detected e distally in one pair of sub-metacentric chromosomes (Fig. 2a). GISH using U-genome DNA also detected a weak signal on the distal region of the long arm of a pair of chromosomes with satellites (Fig. 2b) indicating that IL pau16061 carries introgression from both the Up and Sp genomes of

Results

Inheritance studies. IL pau16061 was resistant to the majority of the leaf rust pathotypes prevalent in the Indian subcontinent Pt11, Pt12-5, Pt16-1, Pt77-5, Pt77-8, Pt77-10, Pt104-2, Pt104B, Pt106 and Pt162-2 at the seedling stage while WL711 was highly susceptible. The IL pau16061/WL711 F5:6 RIL population along with parental lines was tested at the seedling stage against Pt pathotype 77-5. The F1 plants obtained from this cross were resistant with near-immune infection type, indicating the dominant behavior of the leaf rust resistance gene LrAp. Twelve seedlings from each of 160 F5:6 families were screened. All the seedlings in 69 families showed resistant infection type (1) and these RILs were classified as homozygous resistant, while all the seedlings in 81 families showed infection type 3-3 and these RILs were classified as homozygous susceptible (Fig. 1) 22. Ten families were found to to be segregating for the leaf rust resistance gene. Seedlings were transplanted in the field and tested for terminal disease severity. At adult plant stage, WL711 showed terminal disease severity of 80-80, whereas Ae. peregrina and IL pau16061 were highly resistant (0-TR). Out of the 160 RILs, 69 were totally free from leaf rust (0) whereas 81 showed leaf rust severity of 60-80S, and 10 were found segregating. The segregation pattern confirmed a monogenic inheritance of leaf rust resistance (X2 = 1.875, p = 0.342) and the resistance locus was tentatively designated as LrAp.

In situ hybridisation studies. To identify the genomic origin of the Ae. peregrina introgression in IL pau16061, i.e. whether from the Up or Sp genome, GISH was performed with genomic DNA from the U- and S-genome diploid species Ae. umbellulata and Ae. speltoides, respectively, as probes. Utilising S-genome DNA as probe, a strong signal was detected e distally in one pair of sub-metacentric chromosomes (Fig. 2a). GISH using U-genome DNA also detected a weak signal on the distal region of the long arm of a pair of chromosomes with satellites (Fig. 2b) indicating that IL pau16061 carries introgression from both the Up and Sp genomes of
Figure 2. GISH and FISH on the metaphase cells of IL pau16061 (a–d), resistant (e,g) and susceptible RILs (f, h). For GISH, the DNA was labeled with Biotin-16-dUTP and signals were detected with fluorescein-avidin-DN, which fluoresces yellow-green. Chromosomes were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and pseudocolored red. Arrowheads point to the hybridisation signals. For FISH, two probes labelled with FITC and Rhodamine were used to generate chromosome specific hybridisation signals. Chromosomes were counterstained with DAPI. (a) GISH on IL pau16061 using total genomic DNA from Aegilops speltoides; (b) GISH on IL pau16061 using total genomic DNA from Ae. umbellulata; (c,d) Sequential FISH of the same metaphase spreads using probes Oligo-pSc119.2-2 and Oligo-pTa535-2 labelled with 6-carboxyfluorescein (6-FAM) or 6-carboxytetramethylrhodamine (Tamra). Chromosomes have been identified from the banding pattern generated by the hybridization signals as reported by Tang et al.47; (e) GISH using S genome DNA as probe on a resistant RIL; (f) GISH using S genome DNA as probe on a susceptible RIL; (g) Sequential FISH using Oligo-pSc119.2-2 and Oligo-pTa535-2 probes on the same cell in the R RIL as in (e); (h) FISH using Oligo-pSc119.2-2 and Oligo-pTa535-2 probes on the same cell in the S RIL as in (f).
on two different pairs of chromosomes. To identify the chromosomes carrying the U and S genome introgressions, sequential FISH was conducted on the same metaphase spreads on which U and S genome introgressions were detected with GISH. S genome introgression was confirmed to be on the long arm of wheat chromosome 2B (Fig. 2c) while the U genome introgression was identified to be on the long arm of wheat chromosome 6B (Fig. 2d). GISH using S-genome probe was also conducted on 11 homozygous resistant (HR) and 11 homozygous susceptible (HS) RILs to identify whether the U or S genome introgression carries leaf rust resistance. Strong hybridisation signals were detected in both the HR (Fig. 2e) and HS lines (Fig. 2f), indicating that the S-genome introgression does not carry leaf rust resistance. Sequential FISH also confirmed the recombinant chromosome with GISH signals to be 2B in both HR and HS RILs (Fig. 2g,h). That the U genome introgression confers leaf rust resistance was further confirmed with FISH because a very strong hybridisation site with probe Oligo-pTa535-2 at the very distal end of 6BL was missing in IL pau16061 (Fig. 2c,d) and resistant RILs (Fig. 2g) but present in the susceptible RILs (Fig. 2h). Loss of a wheat specific signal at the telomere of 6BL indicated that the distal end of 6BL in IL pau16061 and resistant lines was replaced by a U-genome introgression. In conclusion, it is U-genome introgression in 6BL that confers the resistance to leaf rust in IL pau16061.

NLR enrichment, sequencing and assembly. For mapping of LrAp, MapRenSeq was employed, the sequential strategy of which is outlined in Supplementary Fig. 1. We performed short read NLR enrichment on IL pau16061, the recurrent susceptible parent WL711, and Fₙ RB and SB. Quantitative PCR on the enriched libraries indicated a 500 to 1,000-fold increase in NLRs relative to other genes. The enriched libraries were paired-end sequenced on the Illumina HiSeq2500 platform. De novo assembly of short reads using CLC resulted in 3,586 NLR contigs (287 of which could be annotated as complete) after running through NLR–parser (Supplementary Table S1). Paired-end read (125 bp) data of susceptible parent and bulks ranged from 1.0 to 1.2 Gb for these libraries, which corresponded to an average NLR coverage of ~100×.

Mapping LrAp with NLR markers. RenSeq BAM files of the resistant parent, susceptible parent and bulks were imported into the Savant genome browser and 3,586 NLR containing contigs were manually inspected to identify polymorphisms. We identified five contigs with SNPs with an allele frequency indicative of linkage to LrAp, i.e. a majority of SNPs derived from the susceptible parent were detected in the SB and a majority of SNPs derived from the resistant parent against the IWGSC RefSeq v.1.0 assembly gave best hits with the two scaffolds of chromosome 6B. Out of five contigs, four contigs, namely ctg_1473_3, ctg_2659_1, ctg_1087_1 and ctg_765_1, showed a best BLAST hit on 6BL scaffold 119265 at positions 713.06 Mb, 713.14 Mb, 713.78 Mb and 713.96 Mb, respectively, while contig_5109_1 gave a best hit on scaffold 151695 at position 715.97 Mb (Supplementary Table S2).

The linkage analysis of the RenSeq markers and phenotypic data indicated that LrAp was cosegregating with these loci indicating the location of all five markers on the alien segment which did not recombine with its wheat counterpart (Fig. 3). Most proximal polymorphic marker Ren_1473 was physically mapped at 713.09 Mb and distal most marker at 715.97 Mb with reference to the wheat RefSeqv1.0 (IWGSC 2018) delineating alien segment to this 2.91 Mb region at the telomeric end of long arm of wheat chromosome 6B. Distal end of chromosome 6BL would be considered as a hotspot for disease resistance genes as 3.00 Mb region encompass 52 genes out of which 34 genes have a signature NB_ARC domain of resistance genes while comparing with high confidence gene pairs21, most likely it is a compensating translocation from chromosome 6UPL.

Discussion

In wheat breeding, the identification, characterisation, mapping and deployment of new major rust resistance genes remains a crucial activity in the constant battle against evolving pathogen populations. Previously, transfer of one stripe rust and two leaf rust resistance genes from Ae. peregrina accession pau3519 to cultivated wheat have been reported11. The present study describes the cytogenetic characterisation and genetic mapping of the leaf rust resistance gene in the wheat-Ae. peregrina introgression line IL pau16061 using exome capture of NLRs (MapRenSeq). Inheritance studies showed that the leaf rust resistance to pathotype 77-5 in IL pau16061 is conferred by a single major dominant gene (LrAp). LrAp mediated resistance is expressed at both the seedling and adult plant stages making it an all stage resistance (ASR) gene. Using GISH and FISH on IL pau16061, we identified that the leaf rust resistance is conferred by an introgression on the long arm of wheat chromosome 6B from the Ugenome of Ae. peregrina. Because the introgression lines were developed through induced homoeologous pairing12, most likely it is a compensating translocation from chromosome 6UPL.

Various studies have used high-throughput genotyping combined with bulked segregant analysis (BSA) to identify molecular markers associated with traits of interest23. For example, combined RNA-Seq with BSA to identify SNPs associated with the stripe rust resistance gene Yr15 introgressed into wheat from T. dicoccoides24. However, RNA-Seq is biased by the tissue sampled, the time of sampling and limited by the sequencing depth. This problem is further confounded in the case of NLRs which are often clustered in complex arrays, display extreme sequence and copy number variation between different accessions due to diversifying selection23 and are expressed at relatively low levels24,26.

To mitigate these obstacles, we combined BSA mapping with NLR exome capture and sequencing (dubbed MapRenSeq) to define NLRs associated with LrAp. This strategy was previously used in the wild diploid potatoes Solanum berthaultii and S. ruiz-ceballosii to map Rpi-ber2 and Rpi-rzc1 conferring resistance to late blight19. By
capturing and sequencing long NLR fragments using Pacific Biosciences SMRT technology, improved the resolution of NLR assembly in MapRenSeq. This allowed the fine mapping and cloning of the late blight resistance gene *Rpi-amr3i* in the wild diploid potato *S. americanum*. In another variation of the technology, RenSeq was applied to multiple independently derived ethyl methanesulfonate (EMS) mutants of the stem rust resistance genes *Sr22* and *Sr45* introgressed into hexaploid wheat, which allowed the direct identification of single candidate genes without fine mapping.

In the present study, we used MapRenSeq to characterise the alien segment carrying the *LrAp* gene conferring leaf rust resistance in wheat IL pau16061. The approach allowed the development of five co-segregating NLR_SNP markers to *LrAp*. The linked contigs could be positioned by sequence homology search onto two scaffolds, Ta6BL-151695 and Ta6BL-119265, on the long arm of chromosome 6B of the IWGSC RefSeq v1.1 assembly. *LrAp* thus has been introgressed from the *U* genome of *Ae. peregrina* into the long arm of 6B. Both molecular cytogenetic studies and MapRenSeq based mapping unequivocally identified the introgression to be on the wheat chromosome arm 6BL. Previously, due to insufficient SSR marker density at terminal regions we were unable to identify any linked marker with *LrAp*\(^1\). However, MapRenSeq allowed direct access to NLR markers on the terminal alien
segment. All the five NLR contigs polymorphic in RB and SB did not recombine with LrAp in 160 RILs indicating one of these might be carrying a putative candidate gene for LrAp. Delineating LrAp introgression will now allows this hypothesis to be tested by mutagenising IL. pau16016 to obtain leaf rust susceptible mutants and sequencing these by MuTRemSeq to clone LrAp. Telomeric region of the wheat chromosome 6B was observed to be a hot spot of disease resistance genes as almost 52 disease resistance genes have been annotated in this region in wheat RefSeqv1.1 (https://urgi.versailles.inra.fr/).

To date, only two other leaf rust resistance genes have been transferred from *Ae. peregrina* to wheat, namely *Lr59* into chromosome arm 1AL, and *LrP* linked with a stripe rust resistance gene, *YrP*, on wheat chromosome arm 5DS by our group. The original *Lr59*-containing translocation has group 6 chromosome homoeology at the distal end 6BS telomere (*T1AS·1LP-6SP-6BS*)32. The gene *LrP* targeted in the present study is clearly different and new since no other published leaf rust resistance gene has been transferred from 6UP of *Ae. peregrina* onto chromosome 6B of wheat. Only two *L* genes have been reported on the long arm of 6B viz. *Lr3* from *T. aestivum*34 and *Lr9* from the U genome of *Ae. umbellulata*33. The relationship, if any, between *LrP* and *Lr9* will require further studies.

The identification of a co-segregating markers for *LrP* in the present study will facilitate its marker-assisted selection and pyramiding with other major genes such as *Yr15*, *Yr5*, *Lr76/Yr70*, *Lr57/Yr40*, *Lr37/Yr17/Sr38*, *Yr36* and minor genes like *Lr34/Yr18/Sr57* to increase the potential for durable leaf rust resistance.

**Methods**

**Plant materials.** IL pau16016 was derived from the cross *T. aestivum* cv. Chinese Spring *Fh1*/*Ae. peregrina* pau3519/2/*W*WL711 (NN). Leaf and stripe rust susceptible recipient parent WL711 (NN), has the *kr* (crossability) alleles and is a near-isogenic line of *T. aestivum* cv. *WL*711. The strategy for developing introgression lines has been previously described in detail31. To study the inheritance pattern of rust resistance, IL pau16016 was crossed with WL711 (NN). The F1 was self-pollinated and a population of 160 F5:6 RILs was developed for genetic mapping of leaf rust resistance.

**Leaf rust screening.** IL pau16016 and WL711 was screened against 10 *P. triticina* (*Pt*) pathotypes prevalent in the Indian subcontinent. Seedlings of RILs along with parental lines were screened against the *Pt77-5*36 in a glasshouse in the School of Agricultural Biotechnology, Punjab Agricultural University, Ludhiana, Punjab, India. The pathotype 77-5 is highly virulent, having overcome a number of leaf rust resistance genes in common wheat cultivars36. Seven-day-old seedlings were inoculated with urediniospores mixed with talc, incubated in humidified chambers for 16h in the dark and then moved to a glasshouse maintained at 18–20 °C. Seedling reactions were recorded 14 days after inoculation and were classified according to the ‘0–4’ infection type (IT) scale38, with ITs ranging from ‘0’ to ‘2’ considered as resistant and ITs ranging from 3 to 5+ considered as susceptible. After recording the ITS, the seedlings were transplanted in the field in 1-meter rows to assess leaf rust severity at the adult plant stage. The susceptible cultivar WL711 was planted all around the experimental area. To maintain rust pressure, a mixture of urediniospores of leaf rust pathotypes 77-2, 77-5 and 104-2 was sprayed over the WL711 infector lines. Disease severity was recorded as the percentage of leaf area covered by rust following a modified Cobb’s scale39.

**Cytogenetic characterisation of IL pau16016 and homozygous resistant and homozygous susceptible lines.** Genomic *in situ* hybridisation (GISH) was performed on IL pau16016 following the procedure of Zhang et al. (2001)40. Total genomic DNA from *Ae. umbellulata* (University of Sydney Cytogenetics collection C64.108) and *Ae. speltoides* (C64.69) were both labeled with Biotin-16-dUTP (Roche Diagnostic Australia, Castle Hill, NSW, Australia) using nick translation. Unlabeled total genomic DNA of wheat cv. Chinese Spring was used as a blocker. The probe to blocker ratio was ~1:80 and 1:120 for *Ae. umbellulata* and *Ae. speltoides*, respectively. Signals were detected with Fluorescein Avidin DN (Vector Laboratories, Burlingame, CA). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Life Science, Carlsbad, CA) and pseudo colored red. Slides were analyzed with a Zeiss Axiol Imager epifluorescence microscope. Images were captured with a Retiga EXi CCD (charge-coupled device) camera (QImaging, Surrey, BC, Canada) operated with Image-Pro Plus version 7.0 software (Media Cybernetics Inc., Bethesda, MD) and processed with Photoshop version CS6 software (Adobe Systems, San Jose, CA).

Sequential Fluorescence *in situ* hybridisation (FISH) was performed on the same metaphase spreads using FITC- and Rhodamine- labelled probes, Oligo-pSc119.2-2 and Oligo-pTa535-2, respectively, for the identification of chromosomes according to Zhang et al. (2019)41.

**DNA extraction and quantification.** Genomic DNA from young leaves of IL pau16016 (resistant parent), WL711 (susceptible parent) and the RILs was extracted using the CTAB method42 and quantified with Nanodrop and the Quant-IT PicoGreen dsDNA Assay kit (Life Technologies, Carlsbad, CA, USA). Two bulks were produced; resistant bulk (RB) and susceptible bulk (SB), comprising an equimolar concentration of DNA from the 20 most resistant RILs and the 20 most susceptible RILs, respectively.

**Illumina library preparation, enrichment and sequencing.** A multiplexed NLR capture experiment was conducted on DNA of the resistant parent, susceptible parent, and RB and SB RILs. Short read libraries were prepared on these samples using the NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Inc., Ipswich, MA, USA) following the manufacturer’s instructions. Three to four samples were pooled prior to capture. Target NLR capture was carried out using a custom MYcroarray MYbaits kit v2.0 (MI, USA)18. Approximately 500 ng of the prepared libraries were hybridised in hybridisation buffer (10x SSPE, 10x Denhardt’s solution, 10 mM EDTA, 0.2% SDS) to the biotinylated RNA baits for 16-24 h at 65°C. After hybridisation, bound DNA was recovered using magnetic streptavidin-coated beads and amplified through 8 cycles of PCR. Libraries
were purified with AMPure XP beads and quantitative PCR was performed to establish the level of enrichment in NLRs relative to other non-target genes. The enriched libraries were paired-end sequenced on the Illumina HiSeq 2500 platform at the Earlham Institute (previously The Genome Analysis Center, Norwich Research Park, UK). The raw data is available from NCBI, Study Numbers SRR848566 (IL. pae16061), SRR7867988 (WL711), SRR8369790 (resistant bulk) and SRR8382591 (susceptible bulk). The resistant parent was de novo assembled using CLC assembly cell (http://www.clcbio.com/products/clc-assembly-cell/) and default parameters. All contigs were run through NLR-parser to select those harboring NLR sequences.

**Genetic mapping of LrAp and chromosome assignment.** Quality controlled reads of resistant parent, susceptible parent, RB and SB were mapped onto the assembled resistant parent contigs using Bowtie Wheeler’s Algorithm (BWA) with default settings. Mapping data were visualised in the Savant Genome Browser (www.genomesavant.com). To identify linked polymorphisms, we looked into all NLR contigs for SNPs between the resistant and susceptible parents. Positive contigs with an alternate allele frequency of >70–80% in the susceptible parent and SB reads with an average coverage above 50 were identified. Regions with an average coverage less than 10% of the median overall coverage were considered as a presence/absence variation. Positive contigs were subsequently used in BLASTn searches (>80% identity) against the International Wheat Genome Sequencing Consortium (IWGSC) RefSeq v1.0 (IWGSC 2018), to identify the most likely position in the wheat genome.

**Primer design.** Primers were designed with Primer3 version 4.1.0. The allele specific primers were designed in such a way that they encompass the parental polymorphism in the 3’ end. The reverse primers were selected 40-50bp downstream specific to the required genome. For each SNP, two allele specific forward oligos with standard FAM or HEX compatible tails (FAM tail: 5’ GAAGTGGACAGTTGCATGCT 3’; HEX tail: 5’ GAAGTGGAGTGCAAGGGATT 3’) and one common reverse primer were ordered. Primer mix was prepared by mixing 46 µL ADH-O, 30 µL common primer (100µM) and 12 µL of each allele specific primer (100µM). An assay of 5.07 µL volume included 2.5 µL of 2x KASP master mix (LGC, UK), 0.07 µL of primer mix and 30 ng of gDNA. PCR was performed as described by Ramirez et al. (2015)44. Optically clear plates (384-well) were read on a Tecan Safire plate reader. Fluorescence was observed at 36°C for 30 s. For allele discrimination Clustercaller software v3.4.1.36 (LGK, UK) was used and three genotyping groups, FAM (homozygote), HEX (homozygote) and mixed (heterozygote), were observed.

**Statistical analysis and map construction.** Chi-squared tests were performed to establish goodness of fit of observed segregation with the expected genetic ratio in the F5 RIL population and to detect marker-trait linkages. The positioning of markers was done manually based on recombination between genotypic and phenotypic data and on the basis of recombination frequency. Genetic distances were computed in centiMorgan (cM).

**Data availability**
The datasets generated during and/or analysed during the current study are available at the NCBI, as under SRR848566 (IL. pae16061), SRR7867988 (WL711), SRR8369790 (resistant bulk), and SRR8382591 (susceptible bulk).

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Competing interests
The authors declare no competing interests.

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