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Identification of seedling resistance against leaf rust using innovative phenotyping methods

Nachweis von Keimpflanzenresistenzen gegenüber Braunrost mit Hilfe innovativer Phänotypisierungsmethoden

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Abstract

Puccinia triticina, as the causal agent of leaf rust, is one of the most important fungal diseases of wheat. Effective resistance can prevent yield losses and reduced quality. More than 80 leaf rust resistance (*Lr* genes) genes are known, most of which are vertical resistance genes vulnerable to breakdown by virulent races of leaf rust. Therefore, breeding activities are focused on quantitative resistance genes, e.g., *Lr34* and *Lr46*. In an F_2 population derived from the partially resistant cultivar Pavon F76, carrying *Lr46* at the adult plant stage, and the susceptible spring wheat variety Thatcher, seedling resistance QTLs could be detected, independent of the expected chromosomal regions for already known *Lr* genes. Using innovative phenotyping methods, e.g., microscopic evaluation and counting of uredospore pustules, three QTLs were detected on chromosomes 2B, 4D and 7D. These resistance QTLs explained more than 11% of the phenotypic variance. KASP markers can be derived from markers within the QTL peaks and are available for marker-assisted selection. The study proves again that Mendelian rules do not only describe the inheritance of phenotype. They also apply to the inheritance of the marker alleles and are therefore essential for marker selection and marker-assisted breeding.

Keywords

Leaf rust resistance, wheat cultivars, QTL mapping, seedling resistance, markers

Zusammenfassung

Puccinia triticina als Auslöser des Braunrosts, ist eines der wichtigsten pilzlichen Pathogene des Weizens. Effektive Resistenzen können vor Ertragsverlusten und verringerter Qualität schützen. Mehr als 80 Braunrostresistenzgene (*Lr*-Gene) sind bekannt, die meisten sind vertikale Resistenzgene und sind infolge des Auftretens virulenter Braunrostrassen gefährdet, ihre Wirksamkeit zu verlieren. Daher fokussieren sich Züchtungsaktivitäten auf quantitative Resistenzgene wie z. B. *Lr34* und *Lr46*. In einer F_2 Population, die aus dem resistenten Elter Pavon F76, der *Lr46* im Adultpflanzenstadium ausprägt, und der anfälligen Linie Thatcher entstanden ist, wurden bereits im Keimpflanzenstadium wirksame Resistenz-QTLs in Regionen, in denen keine bekannten Resistenzgene zu erwarten sind, nachgewiesen. Mit Hilfe innovativer Phänotypisierungsmethoden, z. B. mikroskopischen Beobachtungen und der Zählung der Uredosporen, konnten drei QTL auf den Chromosomen 2B, 4D und 7D nachgewiesen werden. Diese Resistenz-QTL erklären mehr als 11 % der phänotypischen Varianz. Von Markern innerhalb der QTL können nun KASP Marker abgeleitet werden, die für eine markergestützte Züchtung geeignet sind. Die Ergebnisse der Studie zeigen erneut, dass die Mendelschen Gesetze nicht nur auf phänotypische Merkmale anwendbar sind. Sie gelten ebenso für die Vererbung der unterschiedlichen Allele, die durch Marker nachweisbar sind und sind somit essentiell für die Markerselektion und die markergestützte Züchtung.

Stichwörter

Braunrostresistenz, Weizensorten, QTL Kartierung, Keimpflanzenresistenz, Marker

Introduction

Resistance against fungal pathogens has been shown several times to be a cost-effective and environmentally safe control strategy (Figlan et al., 2020). However, rusts, especially leaf rust (*Puccinia triticina*), frequently generate aggressive races showing different virulence/avirulence patterns. Such races overcome existing race-specific (vertical) leaf rust resistance

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(*Lr*) genes (Figlan et al., 2020). More than 80 *Lr* genes have been reported and identified in wheat cultivars and several other genetic resources (Qureshi et al., 2018). The timewise limited effectivity of race-specific *Lr* genes based on a gene-for-gene interaction was hypothesized by Flor & Comstock (1971). Most often, an incompatibility between the rust and host plant results in a hypersensitive reaction causing cell death around infection sites (Wang & Chen, 2017). Typically, race-specific *Lr* genes code for proteins with a nucleotide binding (NB) site and leucine-rich repeat (LRR) domains. Most of these genes are seedling resistance genes; however, adult plant resistance (APR) genes have also been described, are more durable, and are usually race-independent resistance genes. Currently, 8 APR *Lr* genes have been identified (Pinto da Silva et al., 2018). These *Lr* genes include quantitative resistance genes, e.g., *Lr34* and *Lr46,* of which *Lr46* confers resistance not only in typical wheat genotypes but also in durum wheat (Singh et al., 1998). *Lr46* was originally detected at the adult plant stage in the cultivar Pavon F76, which has maintained its slow rusting characteristic since its release in 1976 (Singh et al., 1998). *Lr46* results in partial immunity, in contrast to race-specific genes that confer full resistance to the host (Lowe et al., 2011). Partial immunity produces a resistance that slows the growth of leaf rust by several mechanisms, such as a reduction in haustoria formation at later plant developmental stages, a reduction in hyphal growth in intercellular spaces, a higher latent period of *P. triticina* and smaller uredospore pustules with a lower number of uredinia (Martinez et al., 2001; Herrera-Foessel et al., 2008; Lagudah, 2009; Lowe et al., 2011). *Lr46* was located on chromosome 1B of the cultivar Pavon F76 (Singh et al. 1998). The *Lr34/Yr18* slow rusting complex has been mapped previously by Suenaga et al. (2003) and Lillemo et al. (2008), and the genes involved have been cloned (Krattinger et al., 2009). Some genes have been annotated into the region of *Lr46*, e.g., a receptor-like kinase (RLK), and a transcription factor with the WRKY domain (Cobo et al., 2018). Despite the high level of similarity and comparable effects, such as quantitatively reduced infection level, leaf tip necrosis and reduced yield, between the APR genes *Lr46/Yr29* and *Lr34/Yr18* (Rosewarne et al., 2006; Lillemo et al., 2008), a similar genetic background containing these genes was not found. Furthermore, it remains unclear whether the resistance mechanism is based on an ABC transporter, as in the case of *Lr34/Yr18* (Krattinger et al., 2009). Many cultivars carry more than one leaf rust resistance gene, which was summarized by McIntosh et al. (1995) for Pavon F76 among other varieties and accessions. Based on the pedigree, Pavon F76 also carries *Lr1*, *Lr10* and *Lr13* (Singh et al., 1998). However, these *Lr* genes are broken down by most German rust populations (Serfling et al., 2011; Goyeau et al., 2006; Hanzalová et al., 2021), and an increased level of seedling resistance was not observed in those studies, so another or several other genes may be expressed in Pavon F76. The aim of this study was to apply innovative phenotyping methods to map quantitative seedling resistance in an F_2 population of a cross between the susceptible cultivar Thatcher and Pavon F76 to identify loci independent from *Lr46* and other known *Lr*-genes carried by Pavon F76. Quantitative resistance genes cannot be detected with nominal rating scales; therefore, phenotyping using fluorescence microscopy, counting of haustorial mother cells, counting of the uredospore numbers, visual ratings of macroscopic symptoms and/or resistance reactions and quantification of uredospores were performed and compared via a converted rating scale. On the basis of the segregation predicted by mendel, which applies to both the phenotype and the genotype data, markers were selected and resistance QTL determined. For mapping, a 25K SNP array and consensus maps from Wang et al. (2014) and Wen et al. (2017) together with phenotypic data were used.

Materials and Methods

Plant and fungal material

Cultivar Pavon F76 and the leaf rust-susceptible cultivar Thatcher were originally and kindly provided by GRIN-Global (Agricultural Research Service (ARS), Washington, USA; Pavon F76 deposited as PI519847, Thatcher as PI168659). Pavon F76 carries *Lr46* and is partially resistant against leaf rust at the adult plant stage. From each variety, five plants were crossed, and 27 F_1 plants were generated. After selfing F_1 plants, 154 F_2 plants were used for the study. For inoculation, *P. triticina* single spore isolate 4136, showing virulence against the resistance genes *Lr2a*, *Lr2b*, *Lr2c*, *Lr10*, *Lr11*, *Lr12*, *Lr13*, *Lr14a*, *Lr14b*, *Lr16*, *Lr17*, *Lr18*, *Lr20*, *Lr21*, *Lr22a*, *Lr22b*, *Lr23*, *Lr32*, *Lr35*, *Lr37*, and *LrB*, was collected from the cultivar Thatcher in a field trial at Quedlinburg in 2013.

Growing conditions and multiplication of spores

Seeds were sown in potting soil (Archut Fruhstorfer Type P, HAWITA, Lauterbach, Germany) in plastic trays containing 77 pots, each with a size of 5 cm × 5 cm. After sowing, the trays were placed in the greenhouse with a 16 h day and 8 h night cycle, an average temperature of 18°C to 22°C, humidity of >80% and daily manual irrigation. *P. triticina* single spore isolate 4136 was replicated on seedlings of the cultivar Thatcher, and 2 mg per investigated parent and F_2 plant were applied. Plants in the three-leaf stage were sprayed with a Tween 20 (Carl Roth, Karlsruhe, Germany) water solution (1 ml Tween 20 l^{-1}) and inoculated with uredospores mixed with talcum powder (1:20 w/w) using a powder blower. Immediately after inoculation, the plants were covered for 24 h using a plastic sheet chamber.

Rating of seedlings

Ten days after inoculation with leaf rust isolate 4136, genotypes were scored using the rating scale from McIntosh et al. (1995). This scale classified plant-leaf rust interactions as “immune” (rated as “0”), “very resistant” (rated as “;”), “resistant” (rated as “1”), “moderately resistant” (rated as “2”) “moderately resistant to moderately susceptible” (rated as “3”) and “susceptible” (rated as “4”) to leaf rust. Within the segregating F_2 population, 0, 1, and 2 were assigned as resistant, and 3 and 4 were assigned as susceptible. The resulting data were converted into a 1 to 10 scale according to Rollar et al. (2021). To improve the assessment of disease reactions

(infection type, IT) and better observe minor pustules on the leaf surface, a stereomicroscope was used.

Staining of leaves and fungal structures and microscopic analysis

To determine the disease reaction at the seedling stage, two leaf segments of approximately 1.5 cm in length from each inoculated plant from the F_2 population and the parental lines were cut 72 h after inoculation (hai) and 7 d after inoculation (dai). Samples were incubated in 2 ml reaction tubes with a mixture of ethanol, chloroform (2:1 v/v) and 10 ml of trichloroacetic acid (20% in water, v/v) overnight at room temperature. After decolorization of the leaves, the mixture was removed, and 1.5 ml of a lactophenol/ethanol (1:2 v/v) mixture was added and incubated for 2 h. Thereafter, the reaction tubes were boiled for 5 minutes. The solution was removed, 1.5 ml of an ethanol/H_2O (33.3% ethanol, 66.6% deionized water v/v) solution was added, and the reaction tubes were shaken for 15 min. The ethanol/H_2O solution was removed, and 1.5 ml of 0.05 M sodium hydroxide (NaOH) solution was added to the tubes and shaken for 15 minutes. The solution was discarded, and 1.5 ml of sterile water was added followed by 15 min of shaking to reduce the background fluorescence of the samples. After removing the water, 1.5 ml of 0.1 M Tris-HCl solution was poured into the tubes and incubated for 2 h at room temperature. The solution was removed, and Calcofluor white M2R (0.2% in sterile water, w/v) staining solution was added with an incubation time of 5 min followed by removal of the staining solution and washing of the tubes with sterile water four times at intervals of 5 min between each wash step. Leaf samples were then transferred to microscope slides and embedded in a glycerol/water solution (1:1 v/v). Haustorial mother cells (hmc) and the number of uredospores from 10 infection sites per leaf were studied using fluorescence microscopy (Axioskop 50, Carl Zeiss, Jena, Germany). Leaf cells and fungal structures were analysed using the Axiocam MRc camera system connected to the software package Axiovision 4 (Carl Zeiss AG, Jena). Stained fungal structures were visualized using filter set 02 (excitation filter G 365, beam splitter FT 395, and barrier filter LP 420). Autofluorescence within plant tissue was recorded using filter set 05 (excitation filter BP 400-440, beam splitter FT 460, barrier filter LP 470). The number of hmc was counted at 72 hai, and the number of mature uredospores was counted at 7 dai. In total, infection sites from two leaf segments from each of the 154 genotypes and the parental lines were analysed. Uredospore pustules and necrosis/hypersensitive reactions were further assessed using a stereomicroscope (Stemi2000, Carl Zeiss, Jena, Germany) coupled with an Axiocam 305 digital camera used for microscopy. The average number of uredospore pustules per mm^2 leaf area was calculated. Hmc were taken from 10 infection sites from three leaves, and the average was used as the phenotype for the QTL analysis.

DNA genotyping

Genomic DNA from 154 genotypes of the F_2 population and the parental lines was extracted from leaves of four-week-old seedlings according to Stein et al. (2001). Extracted DNA was dissolved in TE buffer (pH 8.0), and its concentration was measured using a Nanodrop 8000 spectrophotometer (Thermo Fisher, Dreieich, Germany). DNA was diluted in deionized sterile water to a concentration of 50 ng μl^{-1}. Genotyping was performed with a wheat 25K Infinium iSelect array (Traitgenetics, Seeland, Gatersleben, Germany). Genotyping data were filtered for markers showing polymorphisms between the parental lines and a call rate of 90%, resulting in 8666 markers. Chromosomal positions of markers were extracted from a consensus map based on the 90K SNP array (Wang et al., 2014; Wen et al., 2017). The annotation provides the physical position in base pairs (bp) and genetic position in centimorgans (cM). Only markers with reported physical and genetic positions on the consensus map and the reference genome, respectively (4990 markers), were used to construct the linkage map. Genotyping failed for 2 genotypes, which were thus excluded, resulting in a total of 152 genotypes.

Map construction

A genetic linkage map was constructed using JoinMap4.0 (Kyazma, Wageningen, Netherlands). SNP data were rewritten as a (maternal allele, Pavon F46), b (paternal allele, Thatcher), and h (heterozygote) in a matrix to prepare the data file. The dataset was checked for errors (missing alleles, nonusable marker designation, and wrong allele designation). The predicted Mendelian segregation of 1:2:1 was validated using the chi-squared test at a level of $\alpha = 0.05$ and a significance for segregation distortion of >3.84. Markers were positioned on linkage groups based on independent LOD threshold values of 2.0–3.0 based on the chromosome assignment of Wang et al. (2014) and/or Wen et al. (2017). Linkage analysis and marker order assignment were carried out using the regression-mapping algorithm. The genetic map was constructed using the Kosambi function (Kosambi, 1944).

QTL analysis

MapQTL 5.0 (Kyazma, Wageningen, Netherlands) was used to perform interval mapping. For the identification and selection of a significant QTL on a linkage group, a significance threshold (LOD) was calculated using a permutation test at a p-level of 0.05. The rating scale based on McIntosh et al. (1995) was nominally scaled, and to obtain a decimal scale, the rating scale was converted following Rollar et al. (2021). Traits for QTL analysis were the converted rating scale, average of number of hmc at 72 hai from 10 infection sites of each genotype, and the average number of uredospores from 10 infection sites of each genotype at 7 dai.

Potential candidate genes were identified by physical mapping of flanking markers in the reference genome of Chinese Spring (IWGSC RefSeq assembly v1.0) available on the web page https://urgi.versailles.inrae.fr/blast/?dbgroup=wheat_iwgsc_refseq_v2_chromosomes&program=blastn (Alaux et al., 2018).

Identification of candidate genes

The sequence between flanking markers was then uploaded to https://megante.dna.affrc.go.jp/home (Numa & Itoh, 2014) and blasted to the UniProtKg web page https://www.uniprot.org/blast to find genes exclusive to wheat (taxon ID 4565) (Wang et al., 2021). Possible candidate genes were defined based on their specificity to wheat and based on the lowest E value.

Results

Phenotypic results

Differences in the traits hmc and uredospore generation could be detected by microscopic analysis. Differences could also be demonstrated by the rating results using the converted scale from McIntosh et al. (1995, Fig. 1 as a third trait). The partially resistant parent Pavon F76, which had a converted rating scale value 2 at the seedling stage, was defined as moderately resistant compared to the susceptible line Thatcher, which showed a value of 10 (completely susceptible, supplementary Table S1). A chi-squared test for segregation ratios of 3:1 (resistant:susceptible), as expected for single major resistance genes, was not significant. A nearly normal distribution from resistant to more susceptible genotypes could be detected in the case of the traits hmc and uredospore generation, whereas the data recorded with the rating scale showed no continuous distribution due to the conversion from a non-continious scale into a decimal scale (Fig. 2).

A significant difference between parental lines was found for both hmc and uredospore generation, which was not surprising given the high susceptibility of Thatcher. In contrast, the resistant line showed an average of 8.16 uredospores compared to 14.24 hmc per infection site (Table 1).

Genotype means of the F_2 population for the traits hmc, the converted rating scale and the number of uredospores were determined to be between the means of the parent lines. More than 30% of the genotypes were within hmc classes 8 to 10, whereas hmc class ranges between 0 and 2 and between 16 and 18 each comprised approximately 5% of the F_2 population. The uredospore spore generation trait showed a peak between 30 and 40 uredospores (30%) (hmc and uredospores, Fig. 2). Only 2% of the genotypes shared a susceptibility phenotype at the level of the susceptible parent or higher (53.27 uredospores). The converted rating scale showed gaps because not all resistance reactions or pustule generation levels could be observed (Fig. 2C).

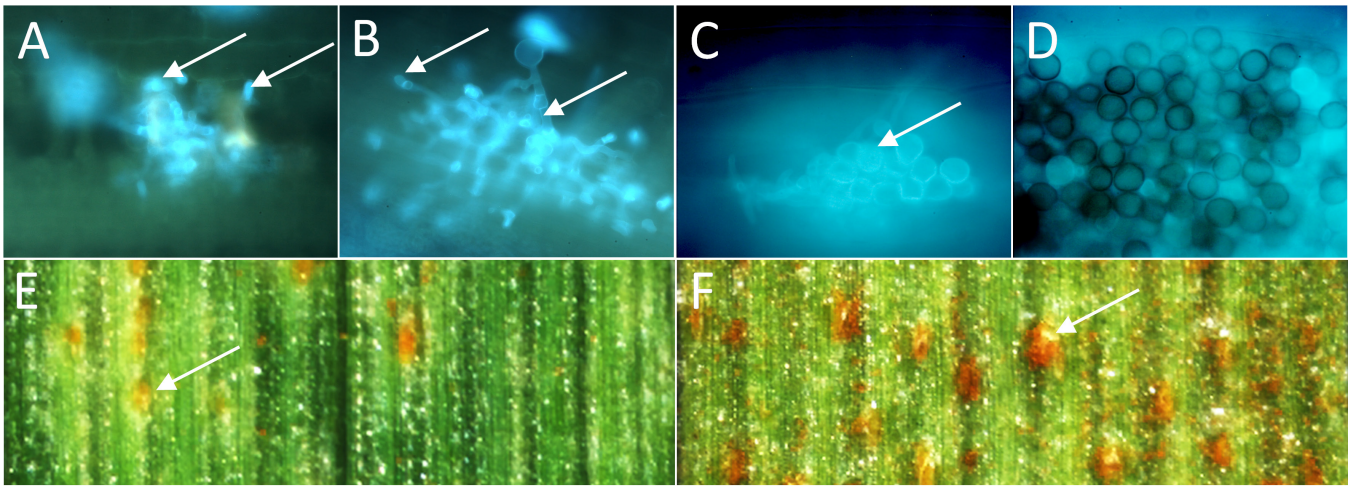

Fig. 1. Different amounts of haustorial mother cells (arrows in A and B) in the resistant parent Pavon 76 (A) and sensitive parent Thatcher (B) indicate different levels of resistance at a microscopic level 72 hours after inoculation with leaf rust. After 7 d, only a few uredospores were produced on leaves of Pavon 76 (C, arrow), whereas the entire leaf area was covered by spores in Thatcher (D). After 10 d, quantitative differences in uredospore pustules were visible on a macroscopic level (arrows E, F).

Table 1. Data for the traits number of hmc and uredospores per infection site (10 infection sites per leaf) for the F_2 population Pavon 76 × Thatcher at the seedling stage.

Generation of haustorial mother cells at 72 hai				Uredospore generation at 7 dai			
Parents		F_2 population		Parents		F_2 population	
Pavon F76	Thatcher	Mean	Max/min	Pavon F76	Thatcher	Mean	Max/min
4.16±1.82	16.02±5.71	9.48	16.24/1.81	14.24±5.86	53.27±9.40	27.18	52.05/0.75

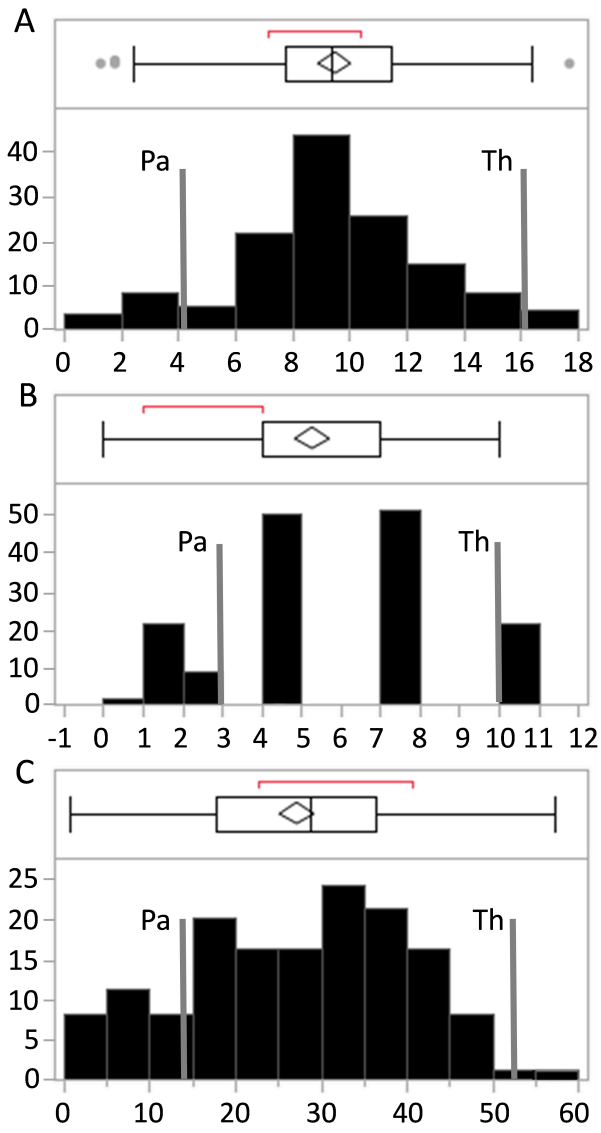

Fig. 2. Distribution of hmc (A), converted rating scale (B) and uredospore generation per infection site (C) within the F_2 population. Observations of the resistant parental line Pavon 76 (Pa) and susceptible line Thatcher (Th) are shown as grey lines

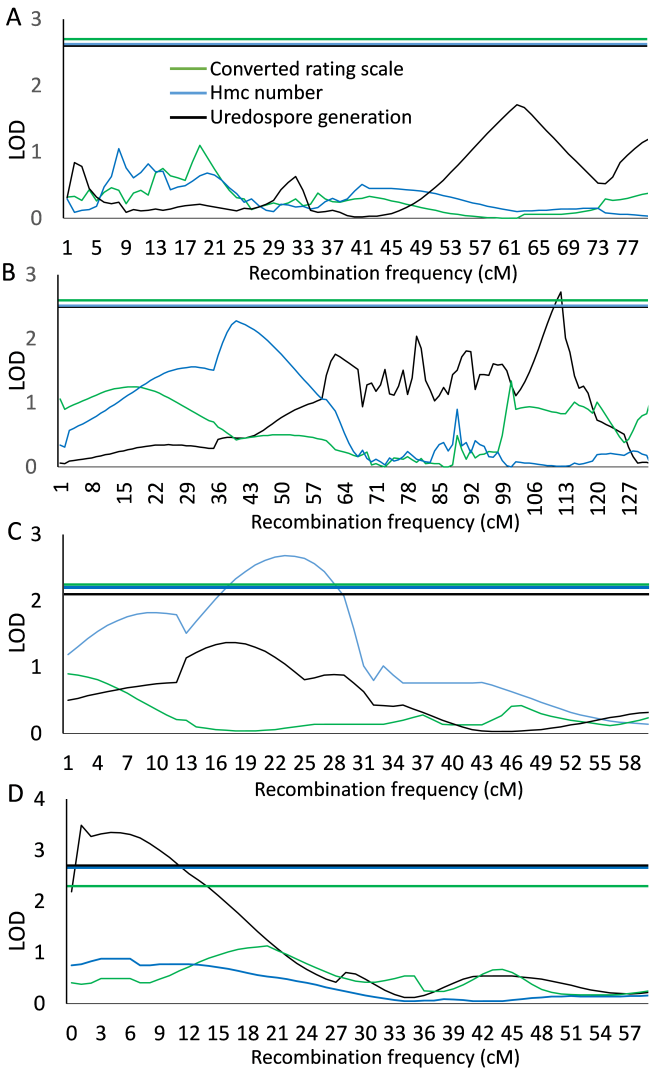

Fig. 3. Logarithm of the odds (LOD) for association of the converted rating scale (coloured in green), hmc number (coloured in blue), and uredospore number (coloured in black) traits on chromosomes 1B (A), known for Apr *Lr46*; 2B (B); 4D (C); and 7D (D). Horizontal lines display global significance thresholds for each trait (coloured as mentioned above).

Mapping and QTL analysis

The final linkage map comprised 3063 markers and had a total length of 2563.43 cM. Each chromosome had between 20 (chromosome 7D) and 421 (chromosome 5B) markers and a length of 42 to 202 cM (Table 2). The D-genome of wheat showed the lowest recombination frequency and lowest number of markers per chromosome. Surprisingly, on chromosome 6B, only 44 markers could be mapped in a range of 123.19 cM. Exact positions on single chromosomes for each marker could be identified based on linkage mapping, in contrast to BLAST, where several hits at different physical positions often matched a single marker sequence. The linkage map was also used to identify flanking markers and other markers within the QTL that were then compared with the reference genome (Table 2).

Neither QTL analysis based on either trait resulted in any QTL on chromosome 1B, which is where *Lr46* is located in Pavon 76 (Fig. 3A). A maximum LOD of 2.39, explaining 7.8% of the phenotypic variance, at 83.49 cM was slightly but not significantly associated with the reduced hmc number at 72 hai (threshold using permutation procedure = 2.5). However, no association with the converted rating scale or uredospore generation could be observed (Fig. 3A). Other possible resistance genes, such as *Lr1*, *Lr10* and *Lr13*, which are known in the resistant parent Pavon F76, are located on chromosomes 5DL, 1AS and 2BS. On chromosome 2B, reduced uredospore pustule generation (LOD = 2.75, threshold = 2.4, and explained variance = 8.1; Fig. 3B) could be detected in a physical region between 763 and 767 Mbp (now named QTL_2B). On chromosome 5D, no region was associated with any resistance reaction (Fig. 3C). The leaf rust isolate 4326 was virulent against *Lr10* and *Lr13*. In addition to the QTL on chromosome 2B, two other QTLs could be detected on chromosomes 4D (now named QTL_4D, Fig. 3C) and 7D (now named QTL_7D, Fig. 3D). The QTL on chromosome 4D showed a peak at 24.19 cM, where 7 markers were physically located in a 95 Mbp segment between 455 Mbp and 455 Mbp. A single QTL for hmc

Table 2. Average genetic distance between markers, the maximum distance within each chromosome, and the number of markers per chromosome

Chromosome	Number of markers	Average distance (cM)	Maximum distance (cM)	Length (cM)
1A	141	1.32	33.17	184.13
1B	185	0.49	20.31	83.16
1D	93 + 45[a]	0.86	30.34	79.24
2A	237	0.59	30.94	138.59
2B	148	0.92	32.63	135.32
2D	13 + 18[a]	7.08	19.75	73.12
3A	221	1.47	20.68	164.86
3B	177	0.73	29.30	126.50
3D	25 + 3[a]	1.77	41.83	42.25
4A	215	1.27	17.80	147.00
4B	155	0.65	10.24	100.40
4D	14 + 6[a]	3.17	16.54	60.19
5A	223	1.55	29.30	201.67
5B	421	0.97	20.70	161.99
5D	18 + 10[a]	4.656	24.58	98.49
6A	169	0.66	14.66	109.62
6B	31 + 14[a]	0.50	25.03	123.19
6D	43 + 37[a]	0.43	25.54	190.64
7A	121	2.77	17.74	168.15
7B	271	2.06	20.55	113.06
7D	16	3.87	20.48	61.86
Sum/*Average*	2937/3070[a]	*1.799*	*23.910*	2563.43

[a] Unmapped on consensus maps but successfully added by linkage mapping

number could be identified on chromosome 7D, and QTLs on chromosomes 2B and 7D were found for reduced generation of uredospores. QTL_7D showed the highest LOD of 3.62, was located between 58 Mbp and 59 Mbp, included 7 associated SNP markers, and explained up to 11.5% of phenotypic variance (Fig. 3D). Detailed information about markers and QTLs are available in supplementary Table S2 and Fig. S.

Identification of candidate genes

For the identified QTLs, markers could be physically mapped according to the genetic map in the peak region of each QTL. The identification of candidate genes using BLAST exclusive to wheat was very helpful for excluding already sequenced *Lr* genes, e.g., *Lr34* and *Lr67*. Within QTL_2B, a protein kinase and mRNA coding for a transporter gene (GO term transmembrane transporter activity) was identified (Table 3). Within QTL_4D, a calcineurin B-like protein kinase-like gene, which is involved in ATP binding protein phosphorylation, was identified. On chromosome 7D, within QTL_7D, an mRNA coding for an uncharacterized protein from which the largest domain belongs to the protein kinase-like domain superfamily was identified (Table 3).

Discussion

Leaf rust infections reduce the yield and grain quality of wheat worldwide. Resistant cultivars are essential for the control of leaf rust and help to reduce the use of fungicides. A prerequisite for breeding resistant varieties is the genetic characterization of resistance, and the identification and use of closely linked or gene-specific molecular markers can speed up the breeding process (Gil et al., 2019).

More than 80 *Lr* genes have been described, but only a few resistance genes are quantitative, non-race-specific and effective at adult plant development stages. One of these quantitative resistance genes, *Lr*46, which can be found in cv. Pavon F76 was first detected by Singh et al. (1998) on chromosome 1B and described as slow rusting resistance. This position was confirmed in several studies, such as Martinez et al. (2001) and William et al. (2003). However, cv. Pavon F76 also carries the *Lr* genes *Lr1*, *Lr10* and *Lr13*, which can be detected by virulence/avirulence studies (McIntosh et al., 1995). These *Lr* genes are broken down by virulent races, including isolate 4136. Using this isolate at the seedling stage, no QTL was detected on chromosome 1B. Since *Lr*46 is considered an APR gene, effective differentiation and recognition of APR at the seedling stage is impossible. However, the parental lines of our population were highly differentiated at the seedling stage, and a nearly normal distribution of infection levels could be observed. Thus, leaf rust resistance, independent of *Lr1*, *Lr10*, *Lr13* and *Lr46*, must have caused reduced leaf rust symptoms. Due to the seedling stage approach established in the present study, the QTLs identified here correspond to early expression, and there are *Lr* genes, which are quantitatively expressed at the seedling stage (Zetzsche et al., 2019; Kthiri et al., 2018). Quantitative expression of the resistance genes was detected in this

Table 3. Potential candidate genes in respective QTLs

QTL	Description (hit to T. aestivum)	Largest domain hit	E-value	GO term	GO code	Start*	End*	Peak markers
QTL_2B	G-type lectin S-receptor-like serine/threonine-protein kinase B120 (LOC123042568)	Bulb-type lectin domain superfamily	0	Protein kinase activity	GO:0004672	1221953	1228220	Lr46_F2_7882/ BS00080318_51
				ATP binding	GO:0005524			
	Protein DETOXIFICATION 31-like (LOC123042573)	Multi antimicrobial extrusion protein	0	Drug transmembrane transporter activity	GO:0015238	1612641	1617572	
				Drug transmembrane transport	GO:0006855			
				Transmembrane transport	GO:0055085			
	CBL-interacting protein kinase 22-like (LOC123047257)	Calcium/calmodulin-dependent/calcium-dependent protein kinase	0	Protein kinase activity	GO:0004672	2949832	2951133	
				ATP binding	GO:0005524			
				signal transduction	GO:0007165			
QTL_4D	Cysteine-rich receptor-like protein kinase (*Stb16q*) genes	Cysteine-rich receptor kinase	0	Protein kinase activity	GO:0004672	52798	54702	Lr46_F2_6718/ D_GDS7LZN02F0 W89_230
				ATP binding	GO:0005524			
				protein phosphorylation	GO:0006468			
QTL_7D	Uncharacterized protein slr1919-like (LOC123165622)	Protein kinase-like domain superfamily	0	No GO term	No GO code	51405	58488	Lr46_F2_7165/ RAC875_ c29314_291

study for the traits hmc number and uredospore generation. Our study showed that these phenotyping methods were suitable for detecting quantitative resistance genes at the seedling stage. Phenotyping based on countable infection structures of the pathogen has been shown to be suitable to predict resistance genes that are effective at later plant development stages, including APR (Beukert et al., 2021). Surprisingly, the conversion of a nominal rating scale (Rollar et al., 2021) resulted in no detection of QTLs in our study. QTLs for a reduced generation of uredospores could be identified on chromosomes 2B (QTL_2B) and 7D (QTL_7D). On chromosome 2B, QTL_2B was detected in a physical region that is not known as the genetic background for the *Lr* genes *Lr13*, *Lr16* and *Lr35*. *Lr13* was mentioned by McIntosh et al. (1995) as race-specific and derived originally from the Exchange and Frontana varieties; *Lr16* was derived from the Selkirk variety; and *Lr35* was introgressed from *Triticum speltoides* (Seyfarth et al., 1999). These varieties or genetic resources are not part of the Pavon F76 pedigree (Vicam-71//Ciano-67/Siete-Cerros-66/3/Kalyasona/Bluebird, Zeven, 1976). Seedling resistance traits and QTLs independent of known *Lr* genes were found on chromosomes 1A, 2B, and 7D. To identify possible candidate genes involved in defence reactions, the QTLs obtained in our study were compared to similar QTLs found in previous studies based on chromosome location, genetic position and physical position (Table 4). The region of QTL_2B is more than 600 Mbp from known *Lr* genes, and resistance gene *Lr35* is located on chromosome 4DS. On chromosome 4D, *Lr67* has been described and sequenced, and it is located at 412.7 Mbp. Further QTLs identified in a MAGIC population from elite cultivars were found at 455.8 Mbp (Rollar et al., 2021). These QTLs overlap with the QTLs from our study (Table 4). The *Lr* genes *Lr19* and *Lr34* are reported to be located on chromosome 7D. QTL_7d is located approximately 1 Mbp away from the resistance gene *Lr34* but more than 500 Mbp away from the probable position of *Lr19* (Table 4). Due to the genetic map and the closely linked markers within each QTL, a search using the physical reference genome could narrow down the physical regions to <100000 bp (QTL_4D) and < 1 Mbp (QTL_7D) or <4 Mbp (QTL_2B).

Within QTL regions, mRNAs coding for two protein kinases and transporter proteins could be identified. Protein kinase 22 is a CBL-interacting protein kinase that is involved in calcium signalling as part of the response to stresses and oxidative bursts or hypersensitive reactions (Xiao et al., 2013; Du et al., 2009). Lectin-receptor-kinases are also emerging as potential components and regulators of PRR (pattern recognition receptors), are known as the first level of defence that recognize pathogen-associated molecular patterns (PAMPs) and are differentially expressed in leaf rust interactions with host plants carrying *Lr57* (Yadav et al., 2016). One QTL signal was identified exclusively for hmc on chromosome 4D and could be physically narrowed down to approximately 0.5 Mbp. This QTL showed a direct hit to a hypothetical protein that is also known as cysteine-rich receptor-like protein kinase (*Stb16q*) and in barley as a seronin/threonine kinase. This gene has been mentioned in several studies as conferring broad spectrum resistance against *Septoria tritici* blotch (Saintenac et al., 2021); it blocks fungal development at the level of infectious hyphae resulting from few successful

Table 4. Overview of known *Lr* genes or QTLs in proximity to the detected QTLs.

Chr.	QTL and Lr genes on chr.	Traits	References	Physical position (Mbp)*	Genetic position (cM)	LOD (peak) explained variance (%)
2B	QTL_2B	Uredospores 7 dai	Qiu et al., 2020	763.84 to 767.02	109.93 to 111.06	2.75
	QTL peak		Kassa et al., 2017	763.84	110.49	8.10
	Lr13		Pinto da Silva, 2018	153.00 to 159.00		
	Lr16			6.26		
	Lr35			2BS		
4D	QTL_4D	Hmc number 72 hai	Rollar et al., 2021	455.25 to 455.34	23.86 to 24.19	2.68
	QTL peak		Rollar et al., 2021	455.34	24.19	10.30
	QTL			455.80		
	Lr67			412.70		
7D	QTL_7D	Uredospores 7 dai	Fatima et al., 2020	58.50 to 59.09	0.24 to 0.73	3.62
	QTL peak		Krattinger et al., 2009	58.86	0.72	11.50
	Lr19			605.88 to 608.09		
	Lr34			48.90 to 51.00		

*Blasted to the reference genome (Chinese Spring)

penetration events in the substomatal cavity of primary-infected stomata (Battache et al., 2022). This is also a crucial stage of rust fungus infection. An effective defence reaction at this infection stage would inhibit the generation of hmc and haustoria (for a review of the infection process, see Bolton et al., 2008). On chromosome 4D, Rollar et al. (2021) found several QTLs and *Lr67* in crosses with Chinese genotypes. However, the sequence of this resistance gene was more than 12 Mbp away from the QTL found in our study. The most significant reliable QTL showed a LOD of more than 3.6 on chromosome 7D and explained more than 11% of the phenotypic variance. Genotypes with the resistance allele showed reduced uredospore production. Several resistance genes are located on chromosome 7D, including the quantitative resistance *Lr34*, which codes for an ABC transporter (Krattinger et al., 2009). The mRNA coding for an uncharacterized protein slr1919 has also been functionally characterized as an ABC transporter (Dadshani, 2018), but it shows a weak relationship with the sequence of *Lr34* (86% identity in a range of 29 bp from >100000 bp).

In conclusion, in this study, three leaf rust resistance QTL regions were found on 3 chromosomes. We found QTLs on chromosomes where other seedling resistance genes have been found, such as *Lr13*, *Lr16*, and *Lr35* on chromosome 2B; *Lr67* on chromosome 4D; and *Lr19* and *Lr34* on chromosome 7D. We were able to rule these genes out and detect previously unknown resistance alleles. Through the use of innovative phenotyping methodology, we show that already described and analysed genotypes can carry unknown resistance genes with minor effects that could be used for breeding to increase the resistance level. The SNP markers identified in our study can be integrated into the breeding process for quantitative resistance in the future.

Supplementary information

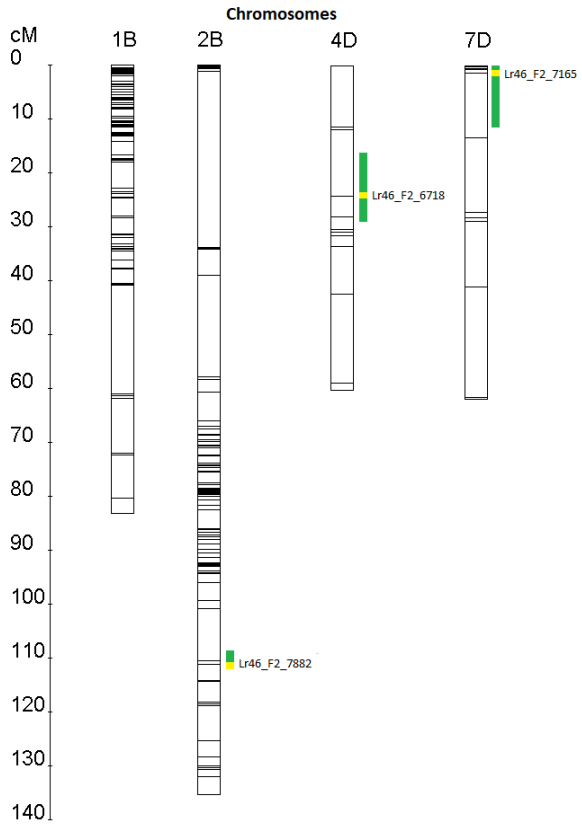

Fig. S. Linkage map of the chromosomes 1B (known for Apr *Lr46*) and 2B, 4D and 7D. QTL regions are indicated by green areas to the right of the chromosomes. The region with the highest LOD is highlighted in yellow, the marker within is shown.

Conflicts of interest

The authors declare that they do not have any conflicts of interest.

The supplementary Tables S1 and S2 for this article can be found online at https://doi.org/10.5073/JfK.2022.11-12-07.

Table S1. Phenotypic data which have been used as traits for the calculation of QTL. Haustorial mother cells are abbreviated as hmc, hours after inoculation as hai. Missing data are represented by asterisks

Table S2. Analysis shown for the chromosomes and traits with significant QTL. QTL regions are marked in green, peaks are marked in yellow. Logarithm of the odds is abbreviated by "LOD", the variance in phenotype explained is abbreviated by "% Expl.", genotypic information coefficient is abbreviated by "GIC", number of iteratrions needed to reach the tolerance criterium by "# Iter", estimated value of the distribution of the quantitative trait associated with the A, H or B genotype by "mu_A", "mu_H", "mu_B".

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