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Communication

Genome-Wide Association Mapping of Resistance to *Warrior (-)* Yellow Rust Race at the Seedling Stage in Current Central and Northern European Winter Wheat Germplasm

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Abstract: To evaluate genetic variability and seedling plant response to a dominated *Warrior (-)* race of yellow rust in Northern and Central European germplasm, we used a population of 229 winter wheat cultivars and breeding lines for genome-wide association study (GWAS). A wide variation in yellow rust disease severity (based on infection types 1-9) was observed in this panel. Four breeding lines TS049 (from Austria), TS111, TS185, and TS229 (from Germany) and one cultivar TS158 (KWS Talent) from Germany were found to be resistant to *Warrior (-) FS 53/20* and *Warrior (-) G 23/19*. GWAS identified five significant SNPs associated with yellow rust on chromosomes 1B, 2A, 5B, and 7A for *Warrior (-) FS 53/20*, while one SNP on chromosome 5B was associated with disease for *Warrior (-) G 23/19*. For *Warrior (-) FS 53/20*, we discovered a new QTL for yellow rust resistance associated with the marker *Kukri_c5357_323* on chromosome 1B. The resistant allele G at the marker locus *Kukri_c5357_323* on chromosome 1B and the susceptible allele T at the marker locus *Excalibur_c17489_804* on chromosome 5B showed the largest effects (1.21 and 0.81, respectively) on the severity of yellow rust detected in *Warrior (-) FS 53/20* and *Warrior (-) G 23/19*. Among 144 putative genes within the flanking sequence of the significant SNPs detected by GWAS, the function of the best candidate genes was determined as protein kinase activity and oxidoreductase activity. Our results provide the basis for knowledge-based resistance breeding in the face of the enormous impact of the *Warrior (-)* race on wheat production in Europe.

Keywords: *Warrior (-)* race; stripe rust; GWAS; *Yr29/Lr46* gene; European wheat; SNP marker

1. Introduction

In 1894, Eriksson and Henning recognized yellow rust (also known as stripe rust) as a distinct rust disease in which the pathogen *Puccinia striiformis* Westend. (*Pst*) can attack wheat, rye, barley, and 59 grass species [1,2]. The fungal pathogen produces yellow to orange uredinia mainly on leaf blades, but also on leaf sheaths, stems, glumes, awns, and young grains of susceptible plants. When leaves are covered by uredinia, photosynthesis is severely limited and continuous production of urediniospors deprives host plants of water and nutrients, reducing plant growth, number of ears and grains per ear, and test weight. The disease can result in an average loss of 13% of grain yield [3] and up to 100% in fields planted with highly susceptible cultivars under extreme yellow rust-friendly weather conditions [4,5]. Chlorosis or necrosis (hypersensitive reaction) is a disease symptom in resistant plants [6]. The intensity of the reaction depends on the degree of plant resistance and the three main weather conditions, including wind, moisture, and temperature. Yellow rust has been considered primarily a disease of cooler climates (2 °C - 15 °C) and higher and northern elevations, but recent epidemics of the disease indicate that fresh strains show greater adaptation to higher temperatures and countries near the equator [2].

Genetic resistance to *Pst* in wheat is based on the effect of genes (major, minor), number of genes (monogenic, polygenic), inheritance of genes (qualitative, quantitative), and molecular basis of genes (NBS-LRR type resistance, non-NBS-LRR type resistance) [7].

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However, depending on race specificity, growth stage, and temperature sensitivity, resistance types to yellow rust can be divided into race-specific resistance for all growth stages and non-race-specific resistance for adult plant stage [7]. The evolution of new races of *Pst* through mutation and somatic and sexual recombination results in a significant change in the virulence of the pathogen, making it more capable of overcoming genetic resistance and plant defence mechanisms.

The most economical and effective method of controlling yellow rust is genetic resistance, combining both minor and major resistance genes. Breeding for rust resistance has used both race-specific and race-nonspecific or partial resistance genes [4]. However, high genetic variation in the pathogen population and rapid selection of new virulent races have forced breeders to focus on pyramiding strategies that combines multiple race-specific and/or race-nonspecific resistance genes to increase the durability of resistant cultivars [8]. Therefore, identification of sources of effective resistance genes and their molecular characterization is an ongoing process to ensure genetic diversity in breeding programs. The main objective is therefore to develop advanced lines or cultivars with high and stable yield potential that also have durable rust resistance.

In 2011, '*Warrior*', a new virulent yellow rust strain from the region near the Himalayas [9], appeared simultaneously in several European countries and spread rapidly across much of the continent. According to observations by the Julius Kühn Institute (JKI, Germany), the '*Warrior (-)*' race, which belongs to the *PstS10* group, dominates the European yellow rust population [10]. It appears that only a few resistance genes, including *Yr5*, *Yr10, Yr15*, and *Yr27*, are still effective against these races in Europe (K. Flath 2022, JKI, personal communication). Despite the enormous impact of the *Warrior (-)* race on wheat production in Europe, little is known about the genetic control of resistance to this race, which provides the basis for knowledge-based resistance breeding [11]. Quantitative trait loci (QTL) analysis using classical bi-parental linkage mapping and genome-wide association studies using large diversity panels have successfully identified genomic regions associated with yellow rust resistance and contributed to a better understanding of the genetic basis of disease resistance in both seedling and adult stage wheat [8,11–16]. In addition, the identification of diagnostic molecular markers associated with yellow rust genes improves the ability to rapidly incorporate resistance into breeding material through marker-assisted selection (MAS). With the advent of high-throughput sequencing and SNP genotyping techniques on universal bead arrays [17] and the availability of the wheat reference genome [18], the search for candidate genes and development of functional markers for use in MAS has been greatly facilitated.

In this study, we used an association panel of winter wheat consisting of 229 commercial cultivars and advanced breeding lines selected from Central and Northern European wheat breeding programs. Our objectives were to (1) conduct an association mapping study using genome-wide SNP markers to identify chromosomal regions associated with *Warrior (-)* yellow rust resistance at the seedling stage, (2) identify sources of effective resistance alleles and associated QTL for use in breeding programs, and (3) determine the relationship between QTL identified in this study and known genes and QTL for yellow rust resistance in previous studies.

2. Results

2.1. Phenotypic evaluation for Warrior (-) yellow rust

A wide variation was observed in yellow rust disease severity (based on infection types 1-9) in the association panel, ranging from very resistant to very susceptible (Figure 1). In the seedling test of lines against *Pst* pathotype *Warrior (-) FS 53/20* (mean IT scores=6.15±0.09), 16% of genotypes were resistant and 33% were susceptible, while 51% of genotypes showed moderate resistance. A different trend was observed for the *Pst* pathotype *Warrior (-) G 23/19* (mean IT scores=6.23±0.10), in which 46% of all genotypes showed different degrees of susceptibility and only 9% resistant reaction types, while 45% showed moderate resistance.

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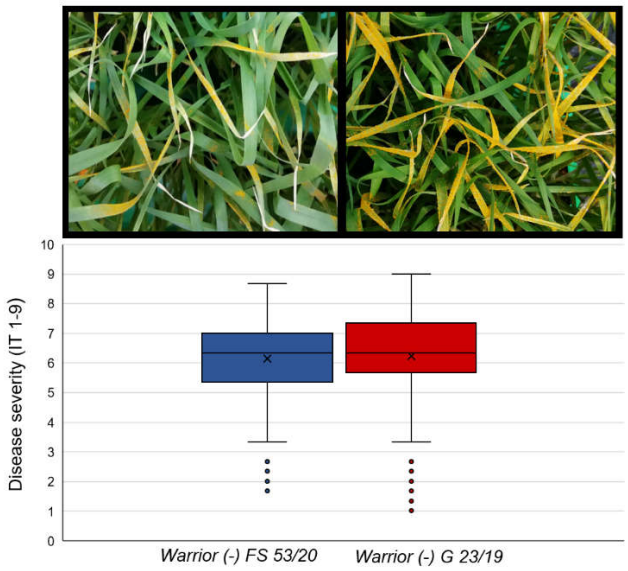

Figure 1. Yellow rust severity (IT 1-9) scored for responses of 229 winter wheat genotype to *Pst* pathotypes *Warrior (-) FS 53/20* (left) and *Warrior (-) G 23/19* (right). Typical uredinia of yellow rust caused by each *Pst* pathotype are shown on the top of box plots.

Four breeding lines TS049 (from Austria), TS111, TS185 and TS229 (from Germany) and one variety TS158 (KWS Talent) from Germany were found to be resistant to *Warrior (-) FS 53/20* and *Warrior (-) G 23/19* (Table S1). The frequency distribution of infection type for resistant and susceptible genotypes based on mean values is shown in Figure 2.

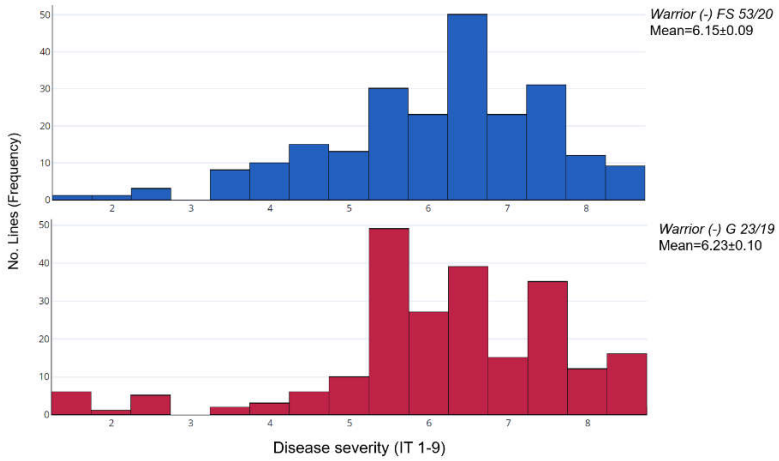

Figure 2. Frequency distribution of yellow rust disease severity (based on infection type) for association panel evaluated with *Pst* pathotypes *Warrior (-) FS 53/20* and *Warrior (-) G 23/19.*

ANOVA also revealed significant differences ($P < 0.01$) within the group of genotypes tested against *Warrior (-) FS 53/20* (mean square=167.83) and *Warrior (-) G 23/19* (mean square=28.93). A low positive correlation (r^2=0.38) was observed between the yellow rust responses to the two *Pst* pathotypes recorded based on the mean values of the genotypes.

2.2. GWAS for resistance to Warrior (-) yellow rust at seedling stage

GWAS using a mixed linear model identified five significant SNPs associated with yellow rust on chromosomes 1B, 2A, 5B, and 7A for *Warrior (-) FS 53/20*, while one SNP was associated with the disease on chromosome 5B for *Warrior (-) G 23/19*, based on the mean values obtained from winter wheat panel evaluation at seedling stage (Table 1, Figure 3).

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Table 1. SNP markers associated with yellow rust severity (based on infection type) in the winter wheat panel evaluated at the seedling stage for the means of each *Pst* pathotype. The marker alleles associated with increased resistance are bolded.

Pathotype	SNP	Chromosome	Position (bp)	R^2	Allele	Effect	*P*-value
Warrior (-) FS 53/20							
	Kukri_c5357_323	1B	637621766	0.051	**G**/T	1.21	0.0008
	Tdurum_contig13879_352	1B	680162719	0.051	**A**/G	0.91	0.0009
	BS00098033_51	2A	778724092	0.055	**C**/T	1.16	0.0005
	CAP12_c703_150	5B	550377847	0.053	**C**/T	0.75	0.0001
	BS00062869_51	7A	17454693	0.060	**A**/G	1.14	0.0004
Warrior (-) G 23/19							
	Excalibur_c17489_804	5B	670829789	0.052	C/**T**	0.81	0.0008

The percentage of explained phenotypic variance (R^2) of the associated markers ranged from 5% to 6%, whereas the effect size of these markers ranged from 0.75 to 1.21 (Table 1). The strongest association (R^2 = 6%, *P* value = 0.0001) was found for SNP marker *BS00062869_51* on chromosome 7A for *Warrior (-) FS 53/20* (Table 1). The resistant allele G at marker locus *Kukri_c5357_323* on chromosome 1B and the susceptible allele T at marker locus *Excalibur_c17489_804* on chromosome 5B showed the greatest effects (1.21 and 0.81, respectively) on the severity of yellow rust detected for *Warrior (-) FS 53/20* and *Warrior (-) G 23/19*, respectively (Table 1). The QQ plots evaluating the performance of the mixed linear models showed a high correlation effect of the GWAS model (Figure 3).

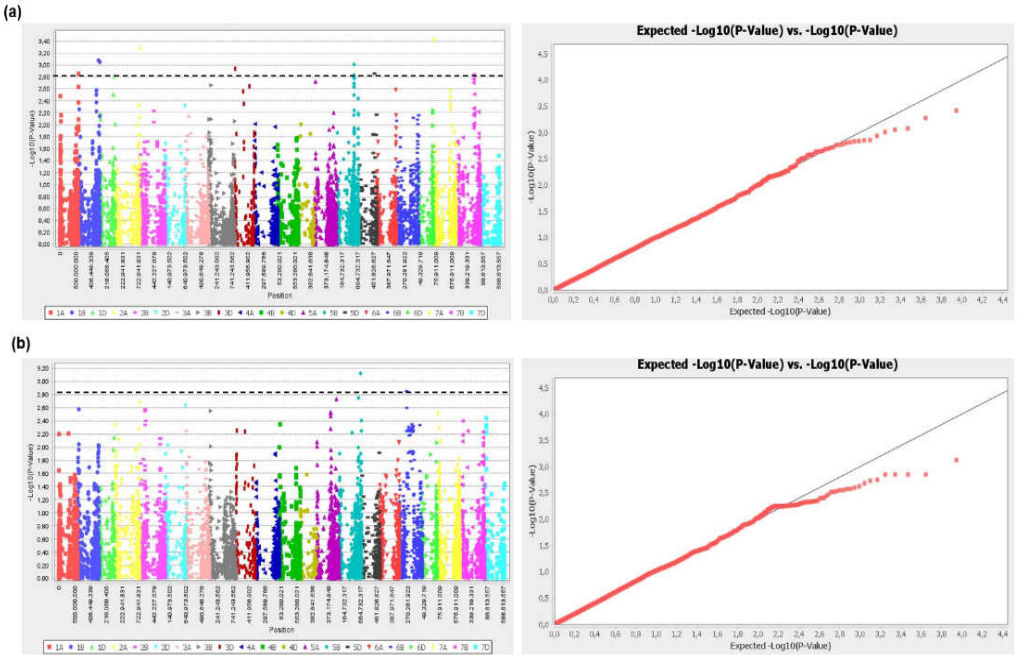

Figure 3. Manhattan plots showing the association of single nucleotide polymorphisms in the 229 genotypes (left) and QQ plot comparing the performance of the mixed linear model (right) used in the genome-wide association study for yellow rust resistance at the seedling stage for *Pst* pathotypes (a) *Warrior (-) FS 53/20* and (b) *Warrior (-) G 23/19*. The horizontal black line represents the genome-wide significance threshold.

2.3. Determining candidate genes

The search for genes within 2 Mb upstream and downstream of the significant associated SNPs resulted in identification of 111 and 33 putative candidate genes for yellow rust resistance to *Warrior (-) FS 53/20 and Warrior (-) G 23/19*, respectively (Table S2). Of

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these, five genes (*TraesCS1B02G411400*, *TraesCS1B02G472200*,_*TraesCS2A02G590600*, *TraesCS5B02G372300* and *TraesCS7A02G039100*) for resistance to *Warrior (-) FS 53/20* and one gene (*TraesCS5B02G504900*) for resistance to *Warrior (-) G 23/19* at the seedling stage were selected as the best candidates. The function of the best candidate genes was determined as protein kinase activity and oxidoreductase activity (Table S2).

3. Discussion

To expand the spectrum of resistance to yellow rust, the development and use of resistant cultivars with new alleles is a forward-looking way to control this disease economically and ecologically through breeding methods. In the present study, we focused on analyzing the response of wheat seedlings to two single spore isolates of *Warrior (-)* yellow rust. The 6 SNP loci (Table 1) associated with the severity of yellow rust infection (based on the average of infection type in the experiments) represented QTL with small effects (R^2 less than 10%). Such low-effect QTL for resistance to yellow rust at the seedling stage were also identified in the U.S. elite spring wheat lines [19], the German MAGIC winter wheat population [20], and a Chinese wheat landrace association panel [5]. About 50% of the lines in our study showed moderate resistance to both *Warrior (-)* races at the seedling stage, indicating the presence of a relatively large number of genes with a moderate effect. About 10% of the lines showed a high level of resistance, which could be caused by major resistance genes against both pathotypes or by a combination of many resistance genes. However, these major resistance genes remained undetected in GWAS, possibly due to low allele frequencies. According to the Global Rust Reference Center (https://agro.au.dk/forskning/internationale-platforme/wheatrust/yellow-rust-tools-maps-and-charts/races-changes-across-years), *Warrior (-)* was the dominant yellow rust pathogen in European countries from 2014 to 2019, apart from the *Amboise* race in 2020-2021, all of which belong to the *PstS10* group. According to the results of the RustWatch project (European Early Warning System for Wheat Rust Disease, https://ec.europa.eu/eip/agriculture/en/find-connect/projects/rustwatch-european-early-warning-system-wheat-rust), up to four races have been found for this pathogen. In addition, while some known major genes conferring resistance to *Warrior (-)* races, such as *Yr5, Yr8, Yr110, Yr15, and Yr27*, are still effective in the European wheat population, other genes such as *Yr1, Yr2, Yr3, Yr4, Yr6, Yr7, Yr9, Yr17, Yr25, Yr32, YrSp* and *YrAvS* have been overcome by this pathotype. In addition,. Although a steady increase in seedling resistance of European winter wheat materials has been reported [21], breeding progress in seedling resistance of these materials could not be attributed to single resistance genes (R genes), most likely because of the multitude of pathogen virulence and R genes affecting the susceptibility of a single cultivar [21].

To find the sources of effective resistance alleles and associated QTL for use in breeding programs in this panel, SNPs identified by GWAS were compared with known yellow rust resistance genes and QTL reported in previous studies based on available physical locations and genetic maps of flanking markers. Based on QTL obtained from the average of infection types from three replicate trials for *Warrior (-) FS 53/20*, physical overlaps were observed for the associated marker *Tdurum_contig13879_352* with eight QTL *QYrex.wgp-1BL_Express* [22], *QYr.sun-1B_Kukri* [23], *QYr.sun-1B_CPI133872* [24], *QYr.sun-1B_Wollaroi* [25], *QYr.ucw-1B* [8], *QYr.jic-1B_Guardian* [26], *QYr.tam-1B_Quaiu* [27], and *QYr.cim-1BL_Francolin* [28], as well as a single gene *Yr29/Lr46* on chromosome 1B reported by [29]. The QTL *Qyr.yellow-1BL* and *QYr-1BL.2*, previously described by Pradhan et al. [30] and Zhang et al. [31], respectively, coincided with the genomic region containing *Yr29*. *Yr29* shares the same features as *Yr18*, which is associated with a weak compatible infection type at the seedling stage. However, seedlings with *Yr18* exposed to low temperatures (e.g., 6-9 °C) show typical partial resistance with longer latency, smaller lesions, and less sporulation. This phenomenon is likely to be important for slowing winter and early spring epidemics in winter wheat. These genes all confer nonrace-specific and presumably

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durable resistance and are effective against multiple pathogens, making them a very important resistance genes in wheat [12].

The SNPs on chromosomes 2A, 5B, and 7A are physically located in the same genomic region as five previously published QTL: *QYr.inra_2AL.2_Camp Remy* [32]; *QYr.sun-5B_Janz* [23]; *QYr.caas-5BL.3_SHA3/CBRD* [33] and *QYr.cim-7AS_Avocet* [34]. Finally, for *Warrior (-) FS 53/20*, we discovered a new QTL for yellow rust resistance associated with the marker *Kukri_c5357_323* at the physical position of 637.621 Mb, distal to the previously reported QTL on chromosome 1B. For *Warrior (-) G 23/19*, two overlapping QTL *QYr.sun-5B_Wollaroi* [27] and *QYr.ui-5B_IDO444* [35] were found in the examined physical region of the associated marker *Excalibur_c17489_804* on chromosome 5B.

Among 144 putative genes within the flanking sequence extending 2 Mb upstream and downstream of the significant SNPs detected by GWAS, we identified two major classes of genes encoding protein kinase activity and oxidoreductase activity as the major candidates for controlling resistance to yellow rust at the seedling stage in our study (Table S2). Plants have developed a complex defense system against various pathogens. Once pathogens overcome mechanical barriers to infection, plant receptors initiate signaling pathways that control the expression of defense response genes. Pathogen infection triggers the production of peroxidases to generate ROS, which are essential for various aspects of the defense response [36]. Oxidoreductases are a class of enzymes consisting of glucose oxidase to scavenge ROS production under biotic and abiotic stress conditions [37]. In addition, protein kinase-like domains are involved in apoptosis, signalling, and regulatory pathways and are frequently reported for disease resistance genes [38]. Tehseen et al. [14] also reported two putative genes with protein kinase-like domains associated with SNPs on chromosome 1D and 3A controlling resistance to yellow rust in bread wheat landraces. This analysis of candidate genes can serve as a basis for using the SNP sequences to convert them into functional markers for breeding purposes in future studies.

We compared the results of our previous study [16], using the same association panel at the adult plant stage in the field, with the results of the present study, which were obtained from seedling stage evaluation in the greenhouse. The correlation coefficients between the seedling and adult plant stages were low (-0.01 to 0.25) because most lines were susceptible at the seedling stage but resistant in the field trials. Such a low correlation (0.19 and 0.46) was also found in the studies by Yao et al. [5] and Rollar et al. [20]. The discrepancy could be due to the interaction between host and pathogen at the seedling stage in the greenhouse and at the adult plant stage in the field. The seedling plants responded to two actuell isolates of the dominant *Warrior (-)* race in the greenhouse. However, there might be a small difference in the virulence spectrum of this race in the field that causes different behavior of the lines. In addition, seedling tests are usually conducted to postulate major genes and identify resources with new genes that are then used in systematic breeding. They capture gene-by-gene relationships and overlook quantitative effects, whereas field tests capture both types of resistance, all-stage and adult plant resistance (APR) genes.

While several studies [5,15,19,20] have identified similar QTL controlling resistance to yellow rust at both seedling and adult stages, suggesting the presence of resistance genes for all stages, others [39,40], including our study, have not found common QTL or representative SNP loci between these two developmental stages. This divergence may be because many other loci contribute more to resistance in the fields in response to a population of pathotypes, while in seedling tests there is only a limited response to a few races. However, the combination of the QTL identified in this study on chromosome 1B at the seedling stage and the QTL on chromosome 7DS at the adult stage [16] in the genomic region of *Yr29/Lr46* and *Yr18/Lr34*, respectively, which are likely to have epistatic effects, could be suggested for the development of durable resistance to yellow and leaf rust diseases in future research.

4. Materials and Methods

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4.1. Seedling stage yellow rust assessment

We selected a population of 229 winter wheat cultivars and breeding lines for GWAS to capture broad genetic variability in Europe (Table S1). Using breeder knowledge and the coefficient of determination algorithm [41], we collected genotypes from Germany, Austria, Norway, Sweden, Denmark, Poland, and Switzerland comprising 157, 50, 14, 4, 3, 1, and 1 genotype(s), respectively [16].

Seedling screening against *Warrior (-)* yellow rust was performed under controlled conditions in the greenhouse at the Bayerische Landesanstalt für Landwirtschaft, Institut für Pflanzenbau und Pflanzenzüchtung, Freising, Germany. The association mapping panel was screened for seedling resistance to two *PstS10* pathotypes, *Warrior (-) FS 53/20* and *Warrior (-) G 23/19*, kindly provided by Dr. Kerstin Flath, JKI, Germany. Pathotypes were selected based on their virulence and prevalence according to RustWatch project (https://ec.europa.eu/eip/agriculture/en/find-connect/projects/rustwatch-european-early-warning-system-wheat-rust) for wheat. Eight to 12 seeds of five genotypes were planted in 14 cm x 14 cm x 9 cm pots. The mixture in the pots consisted of soil, compost, and sand in a 1:1:1 ratio. Seedlings were grown in a growth chamber at 14-16 °C night/day temperature for two weeks. Plants were inoculated at the two-leaf stage by preparing the inoculum immediately before use and suspending urediospores in a solution of water and ~1ppm surfactant (Tween20) or 0.1% agarose. The solution was sprayed onto the seedlings, which were then incubated for 24 hours in the dark and in moist plastic cages with relative humidity close to 100% at 14-16 °C. Plants were then maintained at a similar temperature of 14-16 °C, 16 hours of light at 15 lux, and 8 hours of darkness until disease development. Yellow rust was evaluated 18-21 days after inoculation at the time of clearly visible disease symptoms on the leaves of the susceptible standard cultivar 'Akteur' using a scale of 1-9 infection types [42]. Seedling infection types (ITs) were rated 0-4 (R) as resistant, 5-6 (MR) as moderately resistant, and 7-9 (MS) as moderately to highly susceptible [15]. Evaluation of lines in response to each pathotype was repeated three times from October 2021 to April 2022, and the mean value recorded for IT scores was used for analyses.

4.2. Statistical analysis

Descriptive statistics, analysis of variance (ANOVA), and correlation analysis were performed using "PROC GLM" and "PROC CORR" of the SAS statistical package v.9.4 (SAS Institute, Cary, USA).

4.3. SNP genotyping, population structure and LD analyses

Genomic DNA extraction, SNP genotyping, population structure, and LD analyses were performed as described in Shahinnia et al. [16]. Briefly, genotyping was performed using the 25 K Infinium iSelect array (TraitGenetics, Seeland OT Gatersleben, Germany), and the physical location of markers was determined by BLAST using the published Chinese Spring genome sequence (IWGSC RefSeq v1.0). For population structure, LD, and genetic association analysis, 8,812 informative and polymorphic SNP markers (Table S3) with an average minor allele frequency of 0.26 were used. Linkage disequilibrium (LD) between markers was estimated for the association mapping panel using observed and expected allele frequencies in TASSEL 5.2.78 [43], as reported in Shahinnia et al. [16]. The genetic structure of the population was determined using the Bayesian clustering program STRUCTURE, and the results of STRUCTURE were analyzed in STRUCTURE HARVESTER [44,45]. This population has been divided into two subpopulations with 92 Austrian breeding lines and cultivars (group 1) separated from the other 137 genotypes (group 2) from Germany, Norway, Sweden, Denmark, Poland, and Switzerland, previously [16].

4.4. Association mapping

Marker-trait was carried out using a mixed linear model that accounts for population structure (**Q**) and kinship matrix (**K**). The model can describe as follows: $\mathbf{y} = \mathbf{X\beta} + \mathbf{Zu} + \mathbf{e}$, where $\mathbf{y}$ is the vector of observations, $\boldsymbol{\beta}$ is a vector containing fixed effects for genetic

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markers and population structure (**Q**), **u** is a vector of random additive genetic effects from multiple background QTL with **u** ~ N(0, σ_G^2**K**), **X** and **Z** are the known design matrices, and **e** is a vector of random residuals with **e** ~ N(0, σ_G^2**I**). To provide adjusted *P*-values, false discovery rate (FDR) was calculated using a threshold of <5% with the "q-value" package in R [46]. To evaluate the performance of the models and appropriate thresholds, QQ plots were drawn in TASSEL. Associations of SNP markers with yellow rust severity were represented by drawing Manhattan plots. The physical location of significantly associated markers was compared with previously published *Yr* genes and QTL using the Catalogue of Gene Symbols for Wheat (https://wheat.pw.usda.gov/GG3/WGC) [47] and an integrated map for chromosomal locations of loci associated with responses to *Pst* from Bulli et al. [13] and Maccaferri et al. [8].

4.5. Putative candidate gene identification

The flanking sequence spanning 2 Mb upstream and downstream of the significant SNP position was used to query against the Chinese Spring wheat reference genome IWGSC RefSeq v1.1 (https://urgi.versailles.inra.fr/download/iwgsc/IWGSC_RefSeq_Annotations/v1.1/). Putative genes within the physical interval of 2 Mb were identified and their associated functions were compared to choose the best possible candidates. Functional annotations of candidate genes and gene ontology terms were obtained from EnsemblPlants using the biomaRt package [48].

Supplementary Materials: Table S1: List of 229 winter wheat cultivars and breeding lines assembled from the Northern and Central European countries including Germany, Austria, Norway, Sweden, Denmark, Poland, and Switzerland. Mean values of yellow rust scores (infection type 1-9) against two *Pst* pathotypes, *Warrior (-) FS 53/20* and *Warrior (-) G 23/19*, evaluated in the panel are presented.; **Table S2:** Functional annotation of putative candidate genes within 2 Mb (1 Mb upstream and downstream) physical interval of the peaks of significant SNPs associated with yellow rust disease resistance in wheat at the seedling stage. The genes indicated in bold were tagged directly by the sequences of significant markers.; **Table S3:** List of 8,812 SNP markers and their chromosomal position used for GWAS analysis in the association panel of 229 genotypes.

Author Contributions: Conceptualization, L.H. and V.M.; methodology, F.S., V.M. and L.H.; software, F.S.; validation, F.S. and L.H.; formal analysis, F.S.; investigation, F.S.; resources, L.H.; data curation, F.S., V.M.; writing—original draft preparation, F.S.; writing—review and editing, F.S., V.M. and L.H.; visualization, F.S.; supervision, L.H.; project administration, L.H.; funding acquisition, L.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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