Genome-Wide Association Study Reveals Genetic Architecture of Septoria Tritici Blotch Resistance in a Historic Landrace Collection

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**Genome-wide association study reveals genetic architecture of septoria tritici
blotch resistance in a historic landrace collection**

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Abstract

Septoria tritici blotch (STB), caused by the fungus *Zymoseptoria tritici*, is a major constraint in global wheat production. The lack of genetic diversity in modern elite wheat cultivars largely hinders the improvement of STB resistance. Wheat landraces are reservoirs of untapped genetic diversity, which can be exploited to find novel STB resistance genes or alleles. Here, we characterized 188 Swiss wheat landraces for resistance to STB using four Swiss *Z. tritici* isolates. We used a genome-wide association study (GWAS) to identify genetic variants associated with the amount of lesion and pycnidia production by the fungus. The majority of the landraces were highly resistant for both traits to the isolate 1E4, indicating a gene-for-gene relationship, while higher phenotypic variability was observed against other isolates. GWAS detected a significant SNP on chromosome 3A that was associated with both traits in the isolate 1E4. The resistance response against 1E4 was likely controlled by the *Stb6* gene. Sanger sequencing revealed that the majority of these ~100-year-old landraces carry the *Stb6* resistance allele. This indicates the importance of this gene in Switzerland during the early 1900s for disease control in the field. Our study demonstrates the importance of characterizing historic landrace collections for STB resistance to provide valuable information on resistance variability and contributing alleles. This will help breeders in the future to make decisions on integrating such germplasms in STB resistance breeding.

Keywords: wheat, landrace, disease resistance, septoria tritici blotch, *Zymoseptoria tritici*, germplasm collection.

Introduction

Wheat (*Triticum aestivum* L.) is a staple crop that plays an important role in meeting the growing demand for food of a growing global population (UN, 2015). However, the current rate of global wheat yield increase is insufficient to ensure food security (Ray et al. 2013). This is partly due to continuous threats of diseases on wheat production caused by rapidly evolving pathogens. Septoria tritici blotch (STB) is considered as a major fungal foliar disease of wheat worldwide, especially in Europe, causing yield losses of up to 50% in severe epidemics (Jørgensen et al. 2014; Fones and Gurr, 2015; Savary et al. 2019). STB is caused by the hemibiotrophic fungus *Zymoseptoria tritici*, which has a mixed reproductive system producing airborne sexual ascospores and asexual fruiting bodies called pycnidia (McDonald and Mundt 2016). The fungus causes necrotic lesions on leaves that reduce the photosynthetic activity, leading to yield loss. Pycnidia produced within lesions generate millions of spores that are dispersed mainly by rain splash to nearby plants and wheat fields. Several cycles of sexual and asexual reproduction during a growing season (Karisto et al. 2018) facilitates the maintenance of high genetic diversity with high effective population size and significant gene flow (Zhan, Pettway, and McDonald 2003). Cultivation of resistant wheat varieties and applications of fungicides are the major approaches used to minimize yield loss. However, the fungus can rapidly adapt to these control measures owing to its high evolutionary potential, monocropping with single resistant cultivars, and intensive use of fungicides (Cowger, Hoffer, and Mundt 2000; Estep et al. 2015; Hayes et al. 2016). Because fungicide applications are costly and detrimental to the environment (Fones and Gurr, 2015), finding novel STB resistance genes or alleles, and introgression into modern cultivars remains the most effective and environmentally sound approach to control STB.

STB resistance can be both quantitative (i.e., controlled by several small effect loci) and qualitative (i.e., controlled by a major gene) under field conditions depending on the cultivar-isolate interactions (Brown et al. 2015). To date, 21 qualitative STB resistance genes and 167 genomic regions harboring quantitative trait loci (QTL) conferring STB resistance have been identified and mapped (Brown et al., 2015; Yates et al. 2019). Qualitative resistance is mostly active against *Z. tritici* isolates carrying corresponding avirulence genes. Although such genes are often prioritized in breeding programs due to their large phenotypic effects, their resistance is easily overcome by

147 rapidly evolving pathogens (Cowger, Hoffer, and Mundt 2000; Ponomarenko et al. 2011). Owing
148 to high levels of genetic variability in plant pathogen populations, resistance genes are often under
149 diversifying selection (Jacob, Vernaldi, and Maekawa 2013). For example, a differential resistance
150 pattern of different alleles of *Pm3* (a wheat powdery mildew resistance gene) was observed against
151 a range of powdery mildew isolates (Bhullar et al. 2009, 2010). Recently, *Stb6*, a major STB
152 resistance gene that is predominant in modern and old wheat cultivars, has been cloned and found
153 to possess a single functional resistance allele and several susceptible alleles (Saintenac et al.
154 2018). *Stb6* encodes a wall-associated receptor kinase (WAK) and follows a gene-for-gene
155 relationship with the corresponding avirulence gene *AvrStb6* (Zhong et al. 2017). However, it
156 remains unknown whether wheat gene bank materials such as landraces harbor any novel *Stb6*
157 resistance alleles and how such alleles would contribute to STB resistance. Given the advancement
158 in modern genomics, it has become feasible to genotype large collections of gene bank materials
159 to discover genes of interest and rescue lost allelic forms of genes controlling key agronomic traits
160 (Milner et al. 2019; Liu et al. 2021). Together with precision disease phenotyping and genetic
161 information, phenotypic contributions of different alleles can be dissected and novel alleles can be
162 identified to introgress into modern cultivars. In essence, the knowledge of allelic diversity in gene
163 bank materials provides historical insights into resistance gene deployment and evolution and
164 might improve our understanding of host-pathogen interactions.

165
166 Domestication, early landrace selection and modern breeding have significantly reduced the
167 genetic diversity in today's elite wheat gene pool (Tanksley and McCouch 1997; Reif et al. 2005;
168 Wang et al. 2014). This exposes the vulnerability of modern wheat varieties to emerging diseases
169 and sudden epidemics as they carry few resistance genes with low durability (e.g. wheat blast and
170 stem rust; Islam et al. 2016; Singh et al. 2015). In contrast, wheat gene banks conserve hundreds
171 of thousands of accessions (including wild relatives and landraces), which represent a reservoir of
172 genetic diversity for various traits (Milner et al. 2019; Hajjar and Hodgkin 2007). The value of
173 wheat landraces in harboring novel resistance genes or alleles has been demonstrated for different
174 diseases (Cavanagh et al. 2013; Mengistu et al. 2016). However, efforts to utilize wheat landraces
175 for finding novel sources of STB resistance are largely lacking. The Swiss gene bank conserves
176 more than ~7,500 wheat accession. Despite STB being a major disease in Central Europe, it is

unclear whether Swiss wheat landraces carry any novel STB resistance genes or alleles that can be utilized in STB resistance breeding.

Most of the reported *Stb* genes have been identified using bi-parental QTL mapping, which provided valuable information for resistance gene pyramiding. However, the allelic variation of identified genes is restricted to the two parental genotypes involved in generating the progenies. Genome-wide association mapping (GWAS) provides a wider opportunity to study the allelic diversity of genes as it can be done on natural populations or germplasm collections (Hall, Tegström, and Ingvarsson 2010). GWAS identifies the genomic position of a gene more precisely due to historical recombination events accumulated over generations in natural populations (Yu and Buckler 2006). Relatively few GWAS studies have been done on European wheat cultivars to find novel sources of STB resistance (Kollers et al. 2013; Miedaner et al. 2013; Vagndorf et al. 2017; Yates et al. 2019). In this study, we characterized a panel of 188 historic wheat landraces from the Swiss Gene Bank for resistance to STB. We performed GWAS to identify novel genetic variants based on the amount of leaf area covered by necrotic lesions and the density of pycnidia within lesions using four Swiss *Z. tritici* isolates. We characterized the allelic diversity of the *Stb6* gene, which may provide valuable information to wheat breeders while using these landraces for STB resistance breeding.

Materials and Methods

Plant and fungal material

A collection of 188 bread wheat (*Triticum aestivum* ssp. *aestivum*) and spelt (*Triticum aestivum* spp. *spelta*) accessions (**Supplementary Table S1**) was selected for this study from a genetically characterized panel of 795 wheat landraces (Müller et al. 2018). These landraces were obtained from the Swiss National Gene Bank (www.bdn.ch) and represent the Swiss wheat breeding history from the early 1900s to date. The panel contains a mixture of local varieties, modern varieties, and breeding lines. Two control wheat genotypes, Chinese Spring (a landrace carrying isolate-specific resistance) and Drifter (a highly susceptible modern cultivar), were used in the disease phenotyping experiment. We used four isolates of *Z. tritici*, namely 1E4, 1A5, 3D1, and 3D7 for

the infection assay. These isolates were collected from two nearby Swiss wheat fields in 1999 (Zhan et al. 2005) and have been fully sequenced (Croll, Zala, and McDonald 2013; Lendenmann et al. 2014). Previous studies identified the fungal avirulence genes *AvrStb6* (Zhong et al. 2017) and *Avr3D1* (Meile et al. 2018) in isolates 1E4 and 3D1, respectively.

Preparation of fungal inoculum

The isolates were retrieved from long-term glycerol storage by following standard laboratory protocols. Simply, 40 µl of concentrated spore suspension from each isolate were added into 100-ml Erlenmeyer flasks containing 50 ml of yeast sucrose broth (YSB, 10 g/L sucrose, 10 g/L yeast extract amended with 50 µg/ml kanamycin sulfate to control other microbial growth). Blastospore production was facilitated by keeping the flasks at 18°C on a continuous shaker at 120 rpm. After five days of growth, blastospores were collected by filtering the liquid media through two layers of sterile cheesecloth. Subsequently, blastospores were pelleted for each isolate by centrifugation (1,575 g, 15 min, 4°C), traces of residual growth media were removed by washing with sterile water and spores were resuspended in sterile water. The spores were counted and adjusted to a final concentration of 5×10^6 spores/ml using KOVA counting slides (Hycor Biomedical, Inc., Garden Grove, CA, USA).

Disease phenotyping and data collection

Two grains of each landrace were sown in an individual Jiffy square pot filled with peat substrate Jiffy GO PP7 (Jiffy Products International, Moerdijk, the Netherlands). Each pot is a part of a Jiffy pot strip and eight strips of Jiffy pots were arranged in a tray to accommodate 96 wheat landraces. Grains were subjected to a cold pre-treatment for five days to break the dormancy and synchronize germination. After the pre-treatment, trays were kept in a greenhouse chamber at 22°C (day) and 18°C (night) with 70% relative humidity (RH) and 16-hr photoperiod. Inoculation with the fungal spores was performed on 14-day-old seedlings with a fully expanded second leaf. The final volume of each spore suspension used for inoculation was adjusted to 75 ml by adding sterile water supplemented with 0.1% of the TWEEN 20 surfactant. The third leaf and subsequent leaves were trimmed before inoculation, and new leaves were trimmed until data collection to facilitate a more

uniform distribution of spores and better light penetration into the inoculated leaf. We used an airbrush spray gun (1A Profi Handels GmbH, Wiesbaden, Germany) to inoculate each tray containing 96 wheat genotypes uniformly until run-off. The spraying was done in a confined area for each isolate separately to minimize any chance of cross-contamination. The trays were covered with plastic wrapped around a metal frame after inoculation to provide 100% RH and transferred back into the greenhouse chamber. Three independent biological replicates were obtained by repeating the experiments three times over three consecutive weeks. Spraying of all four isolates was performed on a single day.

Plastic covers were removed three days post-inoculation (dpi) and plants were kept at the same environmental conditions as before. The second leaf of each infected landrace for each isolate was harvested at 21 dpi. Each second leaf was excised and mounted on A4 paper for scanning as described previously (Karisto et al. 2018). Each A4 sheet containing eight inoculated leaves was scanned using a flatbed scanner (CanoScan LiDE 220) for automated image analysis (AIA; Karisto et al. 2018). The AIA provided quantitative data on the percent of leaf area covered by lesions (PLACL) and pycnidia density per cm² of the lesions on each leaf. Importantly, AIA facilitates separation of resistance that affects pathogen damage from the resistance that affects pycnidia production (a quantitative measure of pathogen reproduction).

Genome-wide association analysis

For each isolate, we implemented a linear model using the *lm()* function from the package “stats” in R-studio (R Core Team, 2019) to obtain the least square (LS) means for PLACL and pycnidia density for each landrace. Prior to linear modeling, raw data were log-transformed to fulfill the normality assumptions. We reported un-transformed data in figures and texts for clarity. We used the un-transformed data to perform a global Kruskal-Wallis test (Sokal and Rohlf 2012) using the *kruskal.test* function in R-studio to observe the difference among the four isolates for both traits. We performed genome-wide association mapping (GWAS) on PLACL and pycnidia density for each isolate individually using the log-transformed LS-means and 9,284 bi-allelic single nucleotide polymorphisms (SNP) at 5% minor allele frequency obtained from Müller et al. (2018). The Illumina Infinium 15K wheat SNP array (TraitGenetics GmbH, Gatersleben, Germany) containing 13,006 SNPs was used to genotype the landraces and obtain the final SNP dataset

(Müller et al. 2018). All the GWAS analyses were performed using the R package GAPIT v.3.0 (Tang et al. 2016). A mixed linear model (MLM) was selected for the GWAS as it improves the control of false positives by including fixed and random effects (Yu et al. 2006). A kinship matrix was estimated by following VanRaden's method (VanRaden 2008) implemented in GAPIT. We also conducted principal component analysis (PCA) and added it to the GWAS model as a fixed effect. Both PCA and kinship matrix are useful in controlling false positive signals due to population structure and cryptic genetic relatedness in GWAS. However, the inclusion of principal components did not improve the GWAS model as suggested by the Bayesian information criterion (BIC; (Schwarz 1978), hence these were excluded. Thus, the MLM model with a kinship matrix as a random effect was selected as the most appropriate for our GWAS analysis. Considering the relatively small sample size ($n = 188$), we used an MLM instead of a compressed MLM, which groups individuals according to phenotypes following the implementation in GAPIT (Zhang et al. 2010). The following mixed linear model was used: $y = Xb + Zu + e$, where y is the vector of phenotypes, X and Z are the known design matrices, b is the unknown vector of fixed effects (SNPs), u is the vector of random effects (based on the kinship matrix) and e is the vector of residual effects. We used the stringent Bonferroni threshold ($\alpha=0.05$; $-\log_{10}(P) = 5.27$) to detect significant marker-trait associations. Manhattan plots and qq-plots were generated by using the R package qqman (Turner, 2014). The variation in the phenotypic traits explained by significant SNPs was determined using a linear model with the *lm()* function implemented in the "stats" package in R. Phenotypic trait values were used as response variables and the SNPs as fixed effects.

Molecular screening and sequencing of the Stb6 alleles

Two-week-old second leaves from 188 landraces and Chinese Spring (control) were lyophilized, grounded into powder and genomic DNA was extracted using the DNeasy Plant Mini DNA extraction kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. The *Stb6* gene contains four exons in total (Saintenac et al. 2018) and can be amplified with two separate polymerase chain reactions (PCR). We used the primers 8311F19 (forward primer, CGCGGTTCCAGTCACATCAC) and 8311F3 (reverse, CCGTTTAGCTCGTGTTGTGC) to amplify the exon 1 with a PCR product length of ~1.6 kilobase pair (kb). The remaining exons 2-

4 were amplified using the primers 8311F16 (forward, GCGACATGGTAGCTCAATCAAA) and 8311R16 (reverse, TTCCTTCCATGGTCGGTAACTT) with a PCR product length of ~2.0 kb. The primer sequences were obtained from Saintenac et al. (2018). PCR amplification of the first exon was performed in a total volume of 20 μ L. The PCR mix contained 1 μ L (10 μ M) of each forward and reverse primer, 2.5 μ L (20 ng/ μ L) DNA, 5.5 μ L DNase/RNase free water and 10 μ L of AmpliTaq Gold 360 Master Mix (Applied Biosystems). The PCR cycle conditions were: 10 min of initial denaturation at 95°C followed by 40 cycles of 95°C for 30s, annealing at 63°C for 30s, 72°C for 45s and final extension at 72°C for 5 min. PCR amplification of the remaining exons was performed with the Phusion® High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific) keeping other parameters unchanged. The PCR cycle parameters were: 1 min of initial denaturation at 98°C followed by 40 cycles of 98°C for 10s, annealing at 69°C for 30s, 72°C for 1 min, and final extension at 72°C for 5 min. PCR products were visualized on 1% agarose gels. PCR reactions were purified to remove unincorporated nucleotides and primers using Macherey-Nagel™ NucleoSpin™ Gel and PCR Clean-up Kit (Macherey–Nagel, Oensingen, Switzerland). The purified PCR reactions were sent to GATC Biotech SARL (Konstanz, Germany) for Sanger sequencing with the above-mentioned primers.

Forward and reverse Sanger sequences of 178 landraces were trimmed to remove low-quality sequence, assembled, aligned to produce nucleotide sequence alignment and translated into protein sequences using the genomic analysis software Geneious, v 9.1.8 (Biomatters, Auckland, New Zealand) and the implemented MUSCLE algorithm. Multiple sequence alignment of landrace sequences along with previously identified *Stb6* alleles (Saintenac et al. 2018) was performed to identify sequence polymorphisms and the existence of any novel alleles. Phylogenetic analysis was performed using the DNA sequences from the landraces together with previously reported *Stb6* alleles and the homeologs of the *Stb6* gene to analyze their genetic relatedness. We performed a BLAST search of the *Stb6* resistance allele against the wheat reference genome in Chinese Spring (RefSeq v1.0) provided by the International Wheat Genome Sequencing Consortium (IWGSC; Appels et al. 2018) and used the homoeologous genes on chromosome 3B and 3D in the phylogenetic tree. A distantly related WAK kinase gene from *Aegilops tauschii* (XM_020327419.1) was used as an outgroup. The rooted phylogenetic tree was constructed using the ‘Neighbor-joining’ method and ‘Jukes-Cantor’ for nucleotide distance measure and a bootstrap

performed with 1,000 replications in the Geneious software. In cases of high dissimilarity (i.e. high degree of polymorphism in the conserved protein kinase region) between the reported *Stb6* alleles and landraces alleles, we performed an additional PCR with the reverse complements of the reverse primer of Exon 1 and the forward primer of the remaining exons to join the two ends. The amplified PCR product was ~2.1 kb long and PCR conditions were kept the same as with the Phusion® High-Fidelity PCR Master Mix with HF Buffer (Thermo Scientific). We used the *Stb6* resistance allele as a control group and visualized the amplifications on 1% agarose gels.

Results and Discussion

Phenotypic variation for resistance to STB among Swiss wheat landraces

We obtained quantitative data on the amount of leaf necrosis and pycnidia density from the interactions between 188 wheat landraces and four *Z. tritici* strains using AIA to characterize the resistance response to STB. The landraces showed a considerable degree of phenotypic variability for both traits for the four isolates (**Fig. 1a, b, Supplementary Table S1**). There were significant differences ($P < 2.2e^{-16}$) among the four isolates for PLACL and pycnidia density (**Fig. 2a, b**). Using the non-transformed data, PLACL and pycnidia density with the isolate 1A5 ranged from 2% to 99% and 0 to 30 with means of 66% and 8.3, respectively. Similar ranges of PLACL and pycnidia density were observed with the other three isolates. The majority of the landraces showed high resistance to the isolate 1E4 with a mean PLACL of 28% and a pycnidia density of 17. Around 48% of the landraces showed very high resistance (PLACL<10%) to isolate 1E4, suggesting the presence of a major gene following a gene-for-gene relationship with the corresponding *Avr* (possibly *AvrStb6*). In contrast, the majority of the landraces were moderately to highly susceptible to the isolates 3D1 (mean PLACL of 63 and pycnidia density of 14) and 3D7 (mean PLACL of 74 and pycnidia density of 38). In terms of infectivity, the isolate 3D7 appeared the most damaging overall to Swiss wheat landraces. However, a greater proportion of the landraces with high PLACL were able to suppress pathogen reproduction as indicated by the overall low pycnidia density and its low correlation with PLACL in 1A5 ($r = 0.2$, $P = 0.005$) and 3D1 ($r = 0.38$, $P = 1.1e^{-07}$) compared to 1E4 and 3D7 (**Supplementary Fig. S1**). This may indicate the different genetic resistance mechanism for PLACL and pycnidia density. A field-based study comprising 335 elite wheat cultivars indicated that host resistance affecting leaf necrosis and host resistance affecting

pathogen reproduction were under different genetic control (Karisto et al. 2018; Yates et al. 2019). Even after successful colonization of the leaf, nutrient availability and genetic resistance can largely suppress pathogen reproduction. Such components of host resistance can be beneficial in reducing in-field pathogen inoculum load that causes epidemics. The higher phenotypic variation for both traits in the three isolates except 1E4 suggests that host resistance is governed by a complex genetic architecture. Additionally, we identified two landraces, 1011 (PLACL < 9% and pycnidia density = 0) and 1030 (PLACL < 6% and pycnidia density = 0) that were highly resistant for both traits to all four *Z. tritici* isolates (**Supplementary Table S1**). These landraces are promising candidates for identifying novel STB resistance genes through bi-parental QTL mapping.

Genome wide association study identifies Stb6 as the major STB resistance locus

We conducted a mixed linear model GWAS on PLACL and pycnidia density for each of the four isolates separately. There was considerable genetic variation among the landraces as depicted by the kinship matrix (**Supplementary Fig. S2**). The spelt group clustered together, indicating high genetic similarity. We included the kinship matrix as a random effect in the GWAS model to control *p*-value inflation caused by such genetic clustering and cryptic relatedness. We identified a significant SNP (*Excalibur_c34649556*) over the stringent Bonferroni threshold (*p*-value < 7.80×10^{-09} , minor allele frequency = 0.12) on wheat chromosome 3A at 26,461,994 bp associated with PLACL and pycnidia density in the isolate 1E4 (**Fig. 3a, b**). Using the genome browser of IWGSC RefSeq v1.0, we found that the SNP is located ~255 kb upstream of the major STB resistance gene *Stb6*. Linkage disequilibrium (LD) in wheat is large in comparison to other cereals due to its self-pollinating nature (Cavanagh et al. 2013; Wang et al. 2014; Pang et al. 2020). Pang et al. (2020) showed that the average genome-wide LD block size in wheat is around 4.2 Mb. In another study, LD decayed at more than 7 Mb in different populations including landraces and modern cultivars (Liu et al. 2019). In the current landrace population, LD decayed after 1.94 Mb in bread wheat and after 6.34 Mb in spelt wheat landraces (Müller et al. 2018). Hence, we consider it likely that the resistance to 1E4 was controlled by the *Stb6* gene. However, the significant SNP explained only 18% and 8% of the phenotypic variation for PLACL and pycnidia density, respectively. This suggests that there may be other small effect QTLs contributing to the resistance

response, which GWAS could not detect due to the low SNP density. A recent study identified a QTL in close proximity to the *Stb6* gene that provided partial resistance to STB in a durum wheat panel (Ballini et al. 2020). GWAS analysis for both traits in the other three isolates did not detect any significantly associated SNP. This suggests that host resistance towards these isolates was controlled by many small effect loci (i.e. quantitative resistance). Quantitative host resistance to STB has been observed previously in field-based studies (Kidane et al. 2017; Muqaddasi et al. 2018; Yates et al. 2019). The majority of the landraces carrying the “T” allele of the significant SNP were highly resistant for both traits (**Fig. 4a, b**) suggesting that it is the favorable allele. However, several landraces carrying this allele were susceptible to 1E4 for both traits, raising the possibility that these landraces contain an alternate susceptible allele or that there was recombination in the 255 kb region separating the SNP and *Stb6*.

Widespread prevalence of *Stb6* resistance allele among Swiss wheat landraces

To validate the presence of the *Stb6* gene, we amplified the full-length coding sequence using gene-specific primers from Saintenac et al. (2018) and Sanger sequenced the two PCR products in 178 landraces (**Supplementary Fig. S3**). Ten landraces failed to amplify a PCR product, perhaps due to the absence of a *Stb6* allele. We identified 118 landraces (66%) that carried the functional reference resistance *Stb6*-1 allele (GeneBank accession number KY485190.1) and 24 landraces (13%) that carried the reference susceptible *Stb6*-3 allele first described in the susceptible wheat cultivar Courtot (KY485192.1). The remaining 36 landraces carried seven novel *Stb6* alleles (*Stb6*-18 to *Stb6*-24) that were not previously reported by Saintenac et al. (2018). We categorized the landraces into several groups based on single nucleotide polymorphisms (SNP) compared to the reference *Stb6*-1 allele (**Fig. 5**). The two alleles *Stb6*-21 and *Stb6*-24 were identified in single landraces whereas the remaining unique alleles were identified in several landraces. We created a neighbor-joining tree with all the published *Stb6* alleles, including alleles (*Stb6*-18 to *Stb6*-24) from this study and two *Stb6* homoeologous sequences, to evaluate the genetic relatedness among them (**Fig. 6**). The *Stb6*-18 and *Stb6*-19 alleles were closely related to the homoeologous sequences on chromosome 3B and 3D, respectively and clustered independently from all other *Stb6* alleles. These two alleles were characterized by an unusually high number of SNPs in the 3' region encoding the protein kinase domain, while the 5' region of the gene encoding the extracellular

domain showed a high degree of sequence similarity to *Stb6-1* (**Fig. 5**). Because the *Stb6* alleles were amplified by two independent PCR reactions, we tested if the two amplicons of *Stb6-18* and *Stb6-19* were from the same genomic region. To do so, we developed a PCR probe that bridges the two independent PCR amplicons. While this probe amplified in *Stb6-1* containing landraces, we obtained no amplification product in the *Stb6-18* and *Stb6-19* containing landraces (**Supplementary Fig. S4**). This suggests that these two alleles are not real. The most likely explanation is that the *Stb6* gene in these landraces is truncated and only contains the 5' region. The 3' region might have been amplified from the homoeologous chromosomes. The alleles *Stb6-20* and *Stb6-24* contained seven and ten synonymous SNPs compared to the reference *Stb6-1* allele, respectively and their predicted protein sequence showed 100% similarity to the deduced protein sequence of the previously reported *Stb6-7* (KY485196.1) allele. The alleles *Stb6-21*, *Stb6-22* and *Stb6-23* carried few synonymous SNPs compared to the reference *Stb6-1* allele and showed comparable susceptibility to the susceptible allele *Stb6-3* (KY485192.1) (**Fig. 7**). Our analysis of gene bank material did not find any novel resistance alleles, confirming previous results that there is only one functional *Stb6* resistant allele (*Stb6-1*). Five landraces carrying the resistance *Stb6-1* allele showed moderate to high susceptibility (PLACL > 40%) towards 1E4 and several landraces produced moderate numbers of pycnidia within lesions (**Fig. 7**). This can result from the presence of a suppressor gene that is inhibiting the resistance response of *Stb6*. Suppressor genes often interact with a specific resistance gene and promote susceptibility (Kema et al. 1995; Ren et al. 1997). The presence of suppressor genes hindering the resistance gene action have been reported for STB (Arraiano et al. 2007) and different rust diseases in wheat (Zeller and Hsam, 1996; Nelson et al. 1997; Wu et al. 2015). Further experiments are required to validate the presence of a suppressor gene interacting with the resistance gene in these landraces.

The high frequency of *Stb6* among these ~100 year-old-landraces is intriguing. Previous studies demonstrated that *Stb6* is a very old gene and widely present in worldwide wheat cultivars (Chartrain, Brading, and Brown 2005). During the initial phases of Swiss wheat breeding programs in the early 1900s, wheat germplasms were frequently exchanged between Switzerland and its neighboring countries (Dr. Fabio Mascher, Agroscope, personal communication). This might have contributed to the widespread distribution of *Stb6*, although its origin among the Swiss landraces still remains unknown. Importantly, the majority of the landraces (including 28 spelt wheat out of

31 tested) carried the resistance *Stb6* allele. In contrast, such a high frequency of the resistance allele of *Lr34* (leaf rust resistance gene) was not observed in landrace populations worldwide (Krattinger et al 2009; Kolmer et al. 2008). Although STB became an important disease in Europe and started to draw attention of wheat breeders in the 1970s, it already inflicted heavy to moderate yield loss in Switzerland before 1950 (Zogg, Horber and Salzmann, 1950). Given the conspicuous nature of STB symptoms and the ability to cause significant yield loss, we can assume that farmers in the past could easily identify the disease and observe the resistance response of different landraces. Since *Stb6* provides some field resistance (Chartrain, Brading, and Brown 2005), we can speculate that the more resistant landraces carrying *Stb6* were continuously selected and exchanged among Swiss farmers, and were incorporated into breeding programs that resulted in the high frequency of the resistance allele. Moreover, the widespread distribution of the resistance *Stb6* allele may explain the lack of improvement for STB resistance in earlier times. As the knowledge of the particular gene was lacking, breeders might have been crossing wheat genotypes both carrying the same *Stb6* allele and failed to improve STB resistance by transgressive segregation.

Although this study did not identify a new resistance-conferring *Stb6* allele, we demonstrate the value of ‘digital’ gene bank information for gene discovery in historic wheat collections. The knowledge of allelic distribution from this study will help breeders to make decisions on integrating these materials into breeding programs to improve STB resistance. For example, the two highly resistant landraces identified in this study can be incorporated into QTL mapping studies to elucidate their mechanism of resistance to all four isolates. Also, crossing between cultivars containing the *Stb6-1* resistance allele and highly resistant landraces lacking the *Stb6-1* allele might result in transgressive segregation and improve STB resistance. Additionally, such landraces can be used in developing near-isogenic lines in the genetic background of modern cultivars to precisely determine the phenotypic effect of resistance genes and perform map-based cloning. As our disease phenotyping was limited to four Swiss isolates, increasing the diversity in the isolates used for inoculation or evaluating these landraces under natural field conditions may help to detect other resistance loci.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

AD, DC, BAM and SK conceived the idea of this study. AD conducted the experiment, collected phenotypic data, performed data analysis and wrote the first manuscript draft. BAM provided funding. All co-authors edited and approved the final version of the manuscript.

Data availability

Raw phenotypic data can be provided upon request.

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Figure legends

Fig. 1 Frequency distribution of resistance scores to septoria tritici blotch. **(a)** Percent leaf area covered by lesions (PLACL) and **(b)** Pycnidia density per cm² of lesion among 188 Swiss wheat landraces inoculated with four *Zymoseptoria tritici* isolates. Raw data are shown

Fig. 2 Boxplot showing multiple comparisons among four *Zymoseptoria tritici* isolates for **(a)** percent leaf area covered by lesions (PLACL) and **(b)** pycnidia density per cm² of lesion from 188 Swiss wheat landraces. The box boundaries indicate the 25th (lower) and 75th (upper) percentile, and the line within the box indicates the median value. The whiskers above and below the box represent the highest and lowest values, respectively

Fig. 3 Genome-wide association mapping (GWAS) for resistance to septoria tritici blotch. Manhattan plots showing SNP marker association p -values for **(a)** percent leaf area covered by lesions (PLACL) and **(b)** pycnidia density per cm² of lesion among 188 Swiss wheat landraces for the *Zymoseptoria tritici* isolate 1E4. SNP markers are shown as dots colored according to their associated chromosomes. The red line indicates the Bonferroni threshold at 5% significance level ($-\log_{10}(P) = 5.27$). The SNP above the Bonferroni threshold is labelled with a specific SNP identifier

Fig. 4 Boxplots showing resistance values for **(a)** percent leaf area covered by lesions (PLACL) and **(b)** pycnidia density per cm² of lesion among 188 Swiss wheat landraces carrying either the resistance (T) or susceptible (C) allele at the SNP

Excalibur_c34649_556. The box boundaries indicate the 25th (lower) and 75th (upper) percentile, and the line within the box indicates the median value. The whiskers above and below the box represent the highest and lowest values, respectively

Fig. 5 Schematic representation of sequence alignment of *Stb6* alleles. The DNA sequence alignment of different allelic forms of the *Stb6* gene isolated from 178 Swiss wheat landraces together with the resistance *Stb6*-1 allele (KY_485190.1) and the susceptible *Stb6*-3 allele (KY_485192.1) as references is shown. Only the nucleotide sequence alignments are shown. The domain regions of the reference allele are illustrated at the bottom in the yellow box. The unit scale indicates the nucleotide position in base pairs. The black lines inside the grey rectangles indicate nucleotide polymorphisms compared to the reference allele *Stb6*-1. Numbers within brackets on the right side indicate the number of landraces carrying the allele and the number of nucleotide polymorphisms, respectively. The number in the parenthesis on the left side is a representative landrace identifier from each group of alleles

Fig. 6 Phylogenetic relationships using Neighbor-Joining method among the identified *Stb6* alleles. Phylogenetic analysis was performed using *Stb6* allele sequences identified among Swiss wheat landraces together with previously reported *Stb6* allele sequences (black colored) from Saintenac et al. (2018). Two homoeologous sequences of the resistance *Stb6*-1 allele (KY_485190.1) on wheat chromosome 3B and 3D were from the Chinese Spring RefSeq v1.0. A distantly related wall-associated kinase (XM_020327419.1) in *Aegilops tauschii* was used as an outgroup. Bootstrap values (1,000 replications) are shown at the

branch nodes. The alleles identified in Swiss wheat landraces are labelled in green and blue

Fig. 7 Boxplot showing the phenotypic distribution of (a) percent leaf area covered by lesions (PLACL) and (b) pycnidia density per cm² of lesion for each of the alleles found among 178 Swiss wheat landraces. The resistant and susceptible landrace groups correspond to the reference resistance allele *Stb6*-1 and susceptible allele *Stb6*-3, respectively. The box boundaries indicate the 25th (lower) and 75th (upper) percentile, and the line within the box indicates the median value. The whiskers above and below the box represent the highest and lowest values, respectively

Supplementary information

Supplementary Table S1 List of 188 Swiss wheat landraces with their unique identifier, landrace number, name, cultivation year, and a website to obtain full information and disease resistance scores for percent leaf area covered by lesions (PLACL) and pycnidia density per cm² of lesion for each of the *Zymoseptoria tritici* isolates and their *Stb6* allelic form. *Stb6*-1 and *Stb6*-3 corresponds to the landraces carrying the exact reference resistance (KY485190.1) and susceptible (KY485192.1) alleles, respectively

Supplementary Fig. S1 Phenotypic correlation between percent leaf area covered by lesions (PLACL) and pycnidia density per cm² of lesion among 188 Swiss wheat landraces for four *Zymoseptoria tritici* isolates. Raw data were used to calculate the correlations

Supplementary Fig. S2 Kinship analysis in GAPIT using the SNP dataset on 188 Swiss wheat landraces. Top left, the distribution of estimated kinship values follows a normal distribution (turquoise curve). The heatmap shows the pairwise kinship values between each pair of landraces. The darker colour (i.e., red) indicates that two landraces have a higher genetic relationship. The dendrogram was estimated based on the kinship values

Supplementary Fig. S3 Representative electrophoresis gel picture of molecular screening for *Stb6* alleles in Swiss wheat landraces. Molecular screening was performed using previously reported *Stb6* gene specific primers 8311F19, 8311F3, 8311F16 and 8311R16, 100bp DNA ladder, and control (Chinese Spring carrying *Stb6* resistance gene). 1–14 are sample

landraces. The landraces showing the amplified product of ~1.6 kilobase pair fragment confirm the presence of the first exon of the *Stb6* gene.

Supplementary Fig. S4 Sample electrophoresis gel picture for confirming the presence of novel alleles of *Stb6* gene in Swiss wheat landraces. Molecular screening was performed using the reverse complement of the reverse primer 8311F3 that amplifies the first exon and reverse complement of the forward primer 8311F16 that amplifies the remaining exons, 1 kilobase pair DNA ladder, resistant controls carrying the *Stb6* resistance allele (KY_485190.1). 1–5 are sample landraces showing high sequence dissimilarity to the reference *Stb6* resistance allele

Figures

Figure 1

Frequency distribution of resistance scores to septoria tritici blotch. (a) Percent leaf area covered by lesions (PLACL) and (b) Pycnidia density per cm² of lesion among 188 Swiss wheat landraces inoculated with four *Zymoseptoria tritici* isolates. Raw data are shown

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Schematic representation of sequence alignment of Stb6 alleles. The DNA sequence alignment of different allelic forms of the Stb6 gene isolated from 178 Swiss wheat landraces together with the resistance Stb6-1 allele (KY_485190.1) and the susceptible Stb6-3 allele (KY_485192.1) as references is shown. Only the nucleotide sequence alignments are shown. The domain regions of the reference allele are illustrated at the bottom in the yellow box. The unit scale indicates the nucleotide position in base pairs. The black lines inside the grey rectangles indicate nucleotide polymorphisms compared to the reference allele Stb6-1. Numbers within brackets on the right side indicate the number of landraces

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