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QTL Mapping and Transcriptome Analysis to Identify Differentially Expressed Genes Induced by Septoria Tritici Blotch Disease of Wheat

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Abstract: Resistance to Septoria tritici blotch (STB) is an economically important trait in many wheat-breeding programs across the world. Several quantitative trait loci (QTL) for STB resistance were identified in wheat but due to the dynamic pathogen population it is necessary to continuously identify new resistance genes/QTL and determine the underlying resistance mechanism. In this work, we integrated QTL mapping and transcriptome profiling to identify candidate genes underlying QTL associated with STB resistance in bread wheat at the seedling stage. The results revealed four QTL on chromosomes 1BS, 1BL, 3AS and 3DL for STB resistance. Among these, two QTL on 2BL and 3DL were mapped for chlorosis, necrosis and pycnidia while the other two on 1BS and 3AS were associated with necrosis and pycnidia. Among the four identified QTL, genes were identified in three QTL (1BS, 2BL and 3DL). In total, 238 differentially expressed genes (DEGs) were localized in 1BS, 16 DEGs in 2BL and 80 DEGs in 3DL QTL region respectively. F-box protein, NBS-LRR disease resistance genes and receptor-like protein kinase were the most over-represented. The results emphasize the importance of integrating QTL and transcriptome analysis to accelerate the identification of key genes underlying the traits of interest.

Keywords: septoria tritici blotch; *Stb* genes; wheat; transcriptomics; QTL analysis

1. Introduction

Septoria tritici blotch(STB), caused by the ascomycete fungus *Zymoseptoria tritici* (also known as *Mycosphaerella graminicola*), is currently one of the most devastating foliar diseases on wheat crops globally. STB is a major concern among European wheat producers, especially in humid, temperate areas, such as north-western Europe [1]. In Europe, airborne ascospores of *Z. tritici* discharged from either distant or local infected wheat debris and deposited in young wheat plants in the fall are the primary source of STB [2]. Rain splash dispersed pycnidiospores accelerate the spread of STB during the growing season in the field [3]. Spores germinate on the leaf surface and the fungus invades exclusively through the stomata [4]. STB has a prolonged symptomless phase of up to three weeks following infection and sudden switch to symptomatic phase leading to leaf chlorosis which develops into necrotic irregularly-shaped blotches in which fungal asexual sporulation structures (pycnidia) develop [5,6].

Management of STB still relies heavily on fungicides. In Europe, 70% of all fungicides are used for control of *Z. tritici* [7]. The high input of fungicides in combating this disease has led to a high percentage of fungal strains having resistance to strobilurin and azole-based fungicides [8–10]. Recently, succinate dehydrogenase inhibitors (SDHI) specific non-target site resistance in the field population of *Z. tritici* was reported [11]. The European Commission (EC) is promoting low pesticide-input farming in the member states promoting the implementation of Integrated Pest Management (IPM) strategies to farmers [12]. One of the key IPM alternatives for reducing the use of pesticides to control STB is to grow less susceptible varieties [13], and the deployment of *Z. tritici* resistance genes for breeding wheat cultivars remains the most cost-effective and environmentally friendly approach to control STB [14,15].

To date, 22 major *Stb* resistance genes and several QTL have been identified and mapped [15–18]. However, the majority of these genes have narrow spectra of specificity towards *Z. tritici* isolates, due to its interaction with additive and epistatic effects [19,20]. Furthermore, several sexual cycles of *Z. tritici* during the wheat growing season make the pathogen population highly diverse and complicates disease management [2,21]. Known individual *Stb* genes, except *Stb19* that has not been tested, are not currently effective against *Z. tritici* populations in Europe [22]. Therefore, identification of new *Stb* genes and their stacking resulting in durable and effective STB management are considered to be key aspects to manage STB in applied breeding [6,15].

Recently, the International Wheat Genome Sequencing Consortium announced an annotated reference hexaploid wheat genome in the 21 chromosome-like sequence assemblies, giving access to 107,891 high confidence genes (IWGSC, 2018). *Z. tritici* IPO323 isolate genome was released in 2011, whose assembly is 39.69 Mb in length, representing 21 chromosomes [23]. These resources and the advanced omics techniques can accelerate the identification of new major resistance genes in the host and avirulence genes in the pathogens. Numerous genes in *Z. tritici* [24,25] or pathogen-wheat complex [26] were discovered and annotated under various stage of infection by RNA sequencing (RNA-Seq) analysis. Rud et al. [26] detected expression of at least 80% of predicted loci and identified over 3000 *Z. tritici* genes and 7000 wheat genes as differentially expressed during the development of infection. Stewart et al. [25] identified four new and re-annotated thirty-five candidate genes located in a quantitative trait locus (QTL) for pycnidia density and percentage leaf area covered by lesions (PLACL) on chromosome 7 in *Z. tritici*.

The aim of the present work was to evaluate the possibility to identify candidate genes for STB resistance during different infection stages (chlorosis, necrosis and pycnidia) in a bi-parental doubled haploid (DH) wheat population by combining both QTL and transcriptomics analysis.

2. Results

2.1. Phenotypic Analysis

The phenotypic analysis revealed broad segregation for disease severity levels in chlorosis (CHL), necrosis (NEC) and pycnidia (PYC) in the bi-parental population (Figure 1). Both parental genotypes Stigg and Nimbus produced consistent resistant and susceptible reactions to the pathogen in all infection phases (CHL, NEC and PYC). A significant difference ($p < 0.01$) was found among genotypes in AUDPC of CHL, NEC and PYC. Pearson correlations between means of AUDPC revealed that the highest correlation was observed between CHL and NEC ($r = 0.88$, $p < 0.001$), followed by that between NEC and PYC ($r = 0.83$, $p < 0.001$), and the lowest between CHL and PYC ($r = 0.62$, $p < 0.001$), respectively.

2.2. Genotyping and Linkage Mapping

A total of 17,267 SNPs were scored for the population, and 2963 high-quality SNPs retained after filtering. Cleaning for redundancy resulted in 402 non-redundant SNPs for linkage mapping, and 376 SNPs were mapped successfully while 26 were unlinked. In total, 62 LGs were generated,

representing all 21 wheat chromosomes. The LGs cover a total genetic distance of 1603 cM, with an average genetic distance of 4.3 cM between markers.

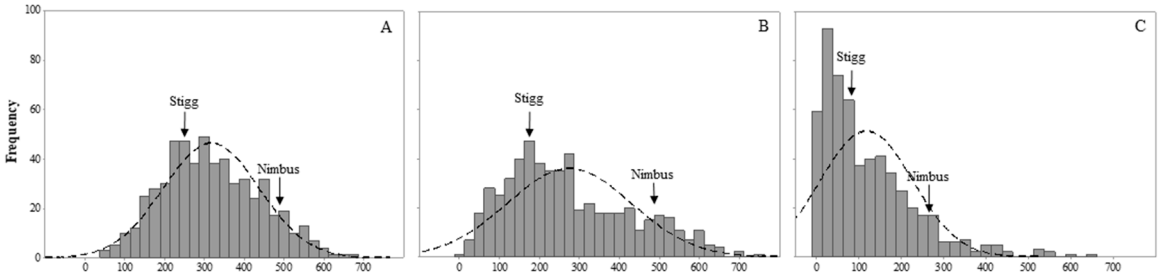


Figure 1. Phenotype distribution of area under disease progress curve (AUDPC) for STB disease (**A**) chlorosis (CHL), (**B**) necrosis (NEC), (**C**) pycnidia (PYC).

2.3. QTL Mapping

Two QTL were mapped for chlorosis symptom and were located on chromosomes 2BL and 3DL. These two QTL were also associated with resistance to necrosis symptoms and pycnidia. For the latter two traits, additional QTL were mapped on 1BS and 3AS (Table 1). QTL regions for the three traits coincided on each of the four chromosome arms (Figure 2), implying the same underlying genetic mechanism. The 2BL QTL showed the strongest phenotypic effects in this study, explaining the phenotypic variation of 13.9–22.7% (Table 1). Except for the 3AS QTL with resistance allele derived from the susceptible parent Nimbus, all other QTL have their resistance alleles contributed by the resistant parent Stigg.

Table 1. QTL for STB related traits in the 'Nimbus' x 'Stigg' population. QTL are listed if they were over the LOD threshold of 3 (in bold) in at least one environment or over the threshold of 2 in multiple environments. CHL—chlorosis, NEC—necrosis, PYC—pycnidia.

Trait	Chr.	Position	Left Marker	Right Marker	Phenotypic Variation Explained (%)			R Source
					Exp.1	Exp.2	Mean	
CHL	3DL	0–1.2	CAP12_rep_c3953_177	Excalibur_c13336_670	9.5		9.3	Stigg
	2BL	11–19.1	Excalibur_c47996_509	Kukri_c3501_1175	**13.9**		**16.3**	Stigg
Accumulated percentage of variation explained					23.4	0	25.6	
NEC	3DL	0–1.2	CAP12_rep_c3953_177	Excalibur_c13336_670	**9.4**	8.5	**10.5**	Stigg
	1BS	0–9.4	AX-94509078	AX-94436250	**12.2**	5	7.2	Stigg
	3AS	1.2–2.4	tplb0053a24_2194	AX-94704180	8.9	10.2	5.9	Nimbus
	2BL	0–19.1	AX-94869203	Kukri_c3501_1175	**16.3**	**15.7**	**18.2**	Stigg
Accumulated percentage of variation explained					46.8	39.4	41.8	
PYC	3DL	0–1.2	CAP12_rep_c3953_177	Excalibur_c13336_670	**6.7**	5.5	**7.3**	Stigg
	1BS	0–1.3	AX-94509078	AX-95162604	**13.2**	4.7	**11.4**	Stigg
	3AS	1.2–2.4	tplb0053a24_2194	AX-94704180	**8.3**		**7.1**	Nimbus
	2BL	0–19.1	AX-94869203	Kukri_c3501_1175	**15.3**	**22.7**	**17.9**	Stigg
Accumulated percentage of variation explained					43.5	32.9	43.7	

Exp.1—experiment 1, Exp.2—experiment 2.

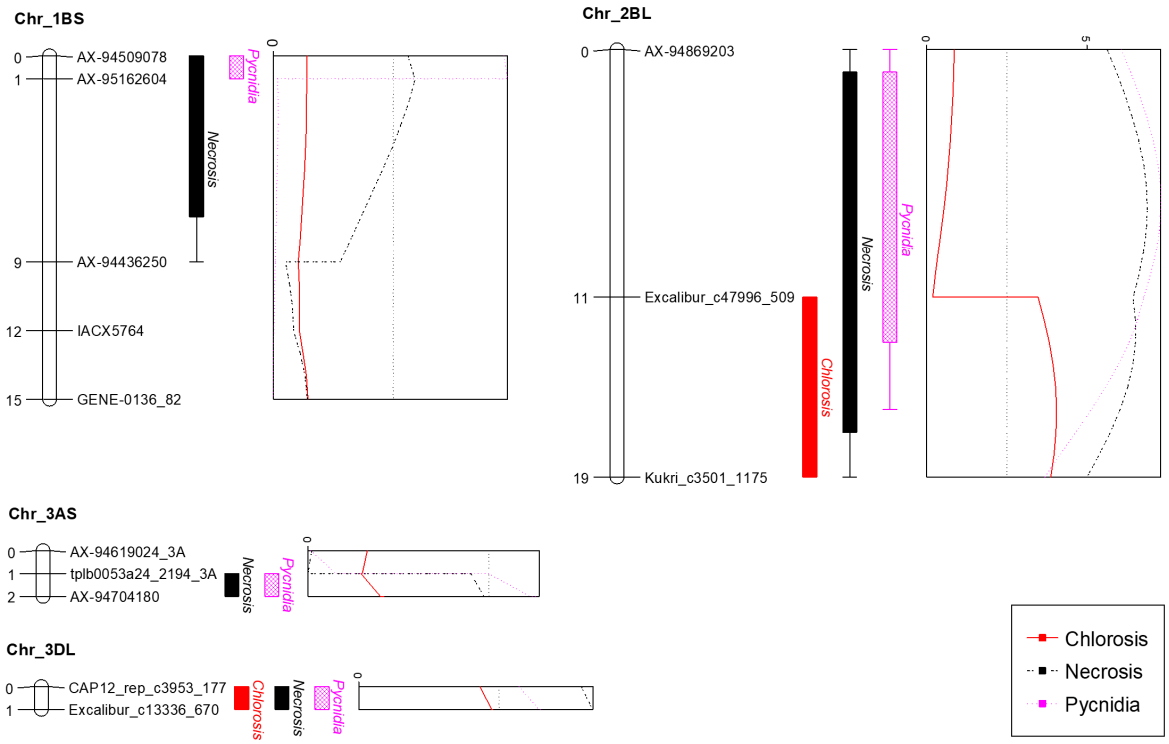


Figure 2. QTL profiles for the three components of STB, i.e., chlorosis, necrosis, and pycnidia on chromosomes 1BS, 2BL, 3AS and 3DL in the Nimbus x Stigg population. Genetic distances are shown in centimorgans to the left of the chromosomes. A threshold of 2.5 is indicated by a dashed vertical line in the LOD graphs.

The identified QTL showed a significant additive effect, with lines with all four QTL showing the lowest mean values for the three traits (Figure 3). It is noteworthy that although chlorosis QTL were only identified on 2BL and 3DL, introgressing QTL on 1BS and 3AS is beneficial to reduce the disease severity further.

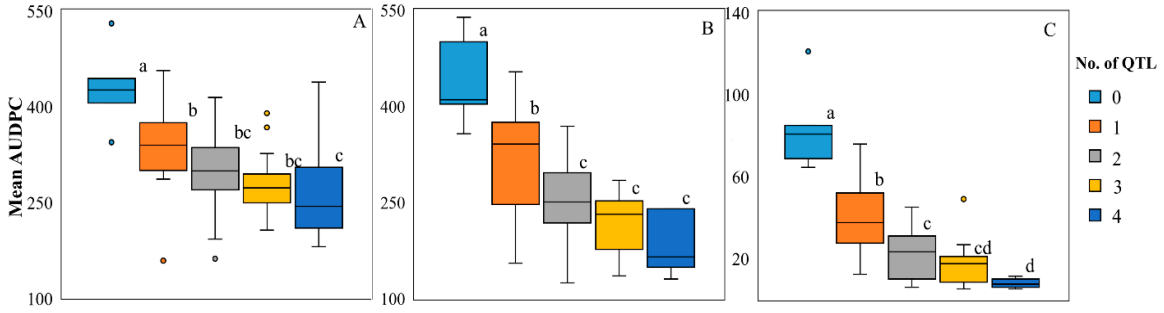


Figure 3. Phenotypic effects of pyramiding different numbers of QTL on chlorosis (**A**), necrossis (**B**) and pycnidia (**C**). Different letters indicate significant difference at alpha = 0.01.

2.4. Gene Expression and Ontology

In total, 754 genes were differentially expressed at 10 dpi and 1770 at 12 dpi in the resistant and susceptible groups, respectively (Figure 4, Supplementary Table S1). The transcriptome analysis on resistant and susceptible lines at 10 and 12 dpi revealed up-regulation of 450 and 1287 genes and down-regulation of 304 and 483 genes, respectively.

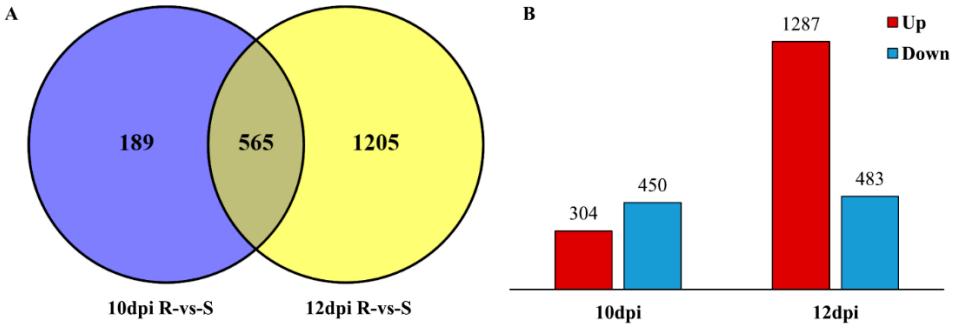
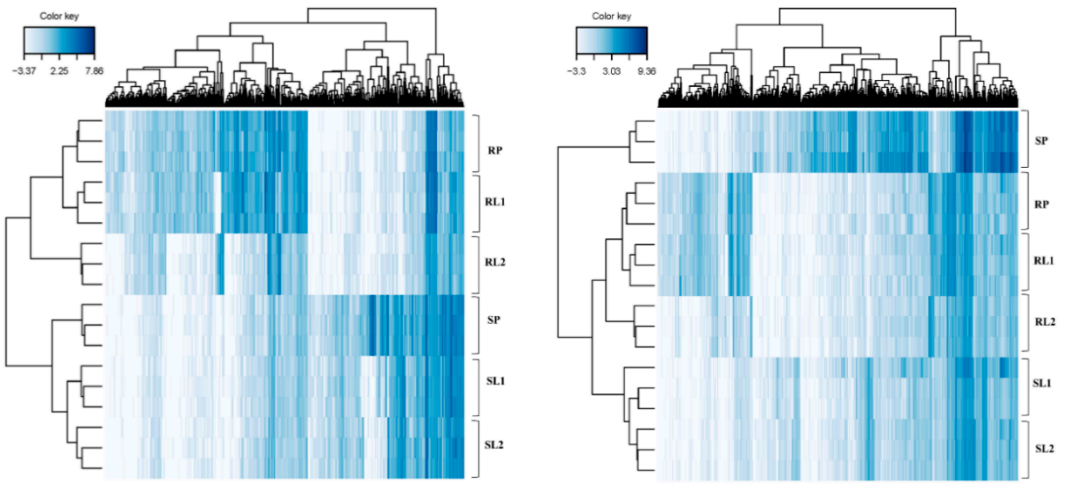


Figure 4. Differential gene expression analysis of the resistant/susceptible winter wheat genotypes after infection with STB at 10 and 12 dpi. (**A**) Venn diagrams showing the numbers of common and distinct differentially expressed genes (≥2-fold, FDR ≤ 0.05). (**B**) The summarized numbers of up-regulated and down-regulated genes.

The heat map shows two distinct clusters of differentially expressed transcripts at 10 dpi, where a clear separation was observed between the samples from resistant and susceptible lines (Figure 5A). At 12 dpi, three distinct clusters were identified (Figure 5B) where all the resistant lines gathered in one cluster while the susceptible lines separated in two different clusters. A total of 35 gene ontology (GO) terms were strongly enriched at 10 dpi and 26 terms of them were representing molecular functions, 8 biological processes and 1 cellular component categories. At 12 dpi, the number of enriched GOs were 97 and from which 51 were associated with molecular functions, 44 biological processes and 2 cellular components (Supplementary Table S2). The gene ontology analysis of the differentially expressed genes revealed that the top three affected molecular functions at 10 dpi were ADP binding, adenyl ribonucleotide binding and binding; the top three affected biological processes are protein phosphorylation, post-translational protein modification and cellular protein modification process (Figure 6A, Supplementary Table S2). The top three enriched GO at 12 dpi in molecular functions were hydrolase activity- hydrolyzing O-glycosyl compounds, hydrolase activity-acting on glycosyl bonds and ADP binding; in biological processes were response to water, cell wall macromolecule metabolic process aspartate family amino acid biosynthetic process (Figure 6B, Supplementary Table S2). Only one affected cellular component (cytosol) was identified at 10 dpi while at 12 dpi two components (extracellular region and apoplast) were identified. (Supplementary Table S2).



(**A**) (**B**)

Figure 5. Heat map of different gene expression profiles (≥1.5-fold, FDR ≤ 0.05) of resistant/susceptible genotypes after infection with STB at (**A**) 10 dpi and (**B**) 12 dpi. SP—susceptible parent, RP—resistant parent, SL1—susceptible line 1, SL2—susceptible line 2, RL1—resistant line 1 and RL2—resistant line 2.

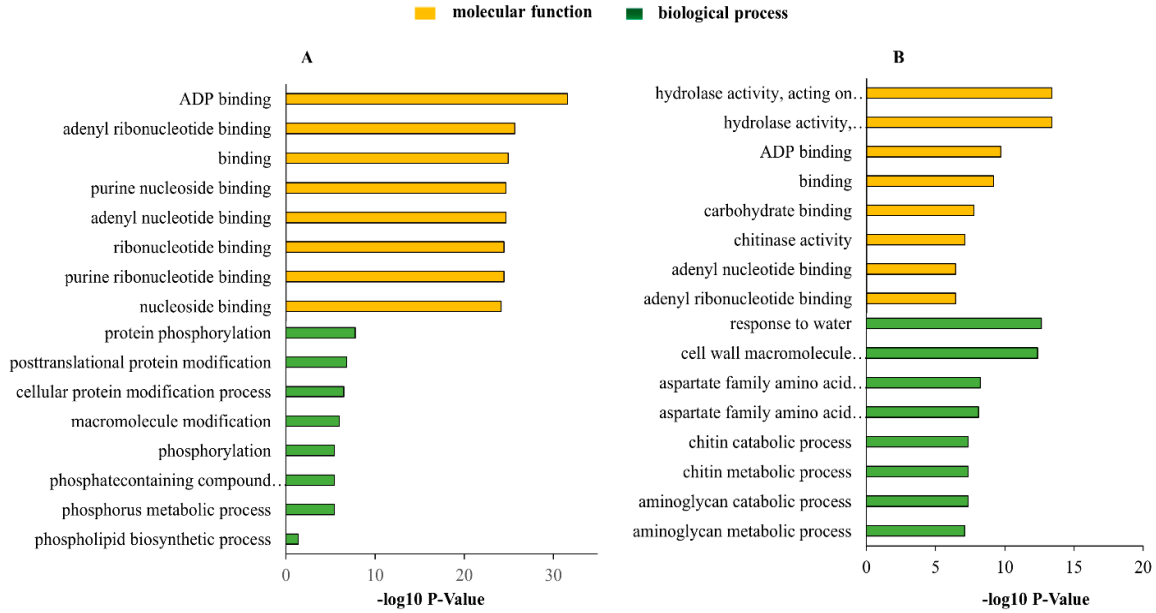


Figure 6. Functional annotation analysis of differentially expressed genes (FDR < 0.05) at (**A**) 10 and (**B**) 12 days after infection with STB.

2.5. Quantification of the Fungal Sequence Reads

No significant difference in fungal sequence reads was found between the samples from resistant and susceptible genotypes at 10 dpi (Figure 7A). However, the number of fungal reads in 12 dpi was significantly ($p < 0.01$) lower in the samples from the resistant genotypes in comparison to susceptible genotypes (Figure 7B).

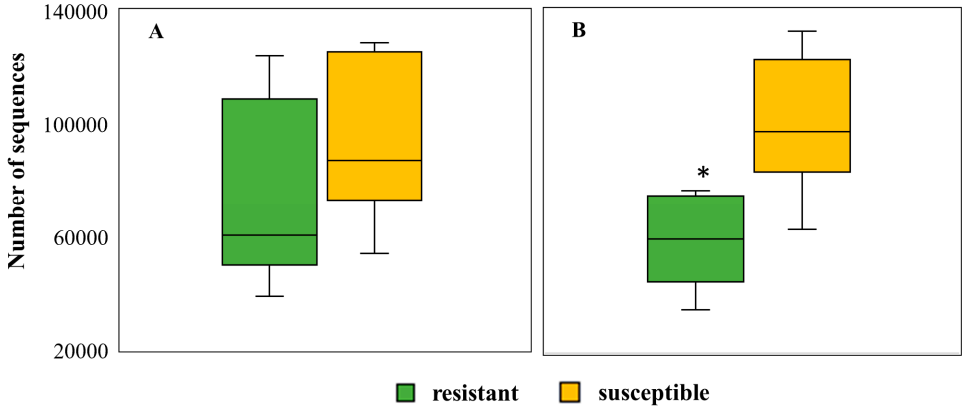


Figure 7. Comparison of the sequence reads mapped to the reference genome of *Zymoseptoria tritici* between the resistant and susceptible genotypes. A comparison was made between (**A**) all resistant lines/parent and susceptible lines/parent at 10 dpi, (**B**) all resistant lines/parent and susceptible lines/parent at 12 dpi, (2 sample t-test, * $p < 0.01$).

2.6. Co-Localization of DEGs in QTL Regions

Of the 310 markers flanking the four QTL, only 31 were not successfully mapped on their corresponding chromosomes in IWGSC RefSeq v1.0 genome (Supplementary Table S3). Accordingly, the 1BS QTL fell into a physical range of 1,202,951 to 420,357,704, the 2BL QTL between 789,437,020 and 799,254,343, the 3AS QTL between 7,436,985 and 8,686,717, and the 3DL QTL between 593,442,231 and 614,366,132 (Supplementary Table S3). Based on the result, significantly (≥2-fold, FDR ≤ 0.05) DEGs (334) were only localized in 1BS, 2BL and 3DL QTL intervals. No significantly DEGs were identified

in 3AS QTL region. Several DEGs related to F-box protein, NBS-LRR disease resistance protein-like protein, receptor-like protein kinase and protein kinase family protein were most over-represented in all three QTL regions (Figure 8).

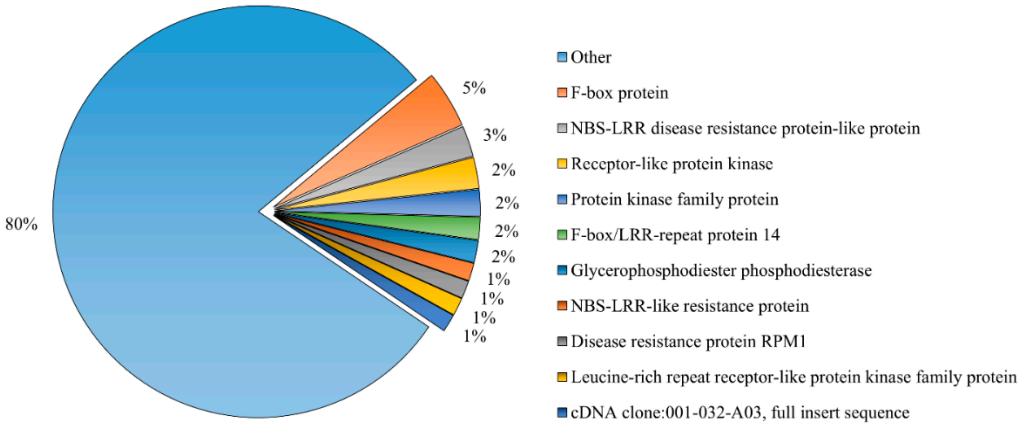


Figure 8. Pie chart demonstrating the over-represented DEGs that were localized in 1BS, 2BL and 3DL QTL regions.

In total, 238 DEGs were localized in 1BS QTL region (Supplementary Table S4). Among them, 42 DEGs were up/down regulated at 10 dpi, 40 genes at 12 dpi and 156 overlapping genes at both time points in the resistant lines compared to the susceptible ones (Supplementary Table S4). The top three up/down-regulated genes at 10 dpi were PGR5-like protein 1A, phloem protein 2-like protein and basic helix-loop-helix (bHLH) DNA-binding superfamily protein and at 12 dpi protein kinase-like, cDNA clone: J013058P10, full insert sequence and NBS-LRR-like resistance protein (Figure 9A). Only sixteen significantly up/down-regulated genes were localized in the 2BL QTL region (Supplementary Table S4). In total, four genes at 10dpi, one gene at 12 dpi and eleven overlapping genes at both time points were identified as up and down-regulated. Protein NRT1/PTR FAMILY 5.5, NBS-LRR-like resistance protein and tetraacyldisaccharide 4'-kinase were identified as the top three up-regulated and NBS-LRR disease resistance protein homologue, CsAtPR5 and cystic fibrosis transmembrane conductance regulator were identified as the top down-regulated at both time points (Figure 9B). Overall, 80 significantly up/down-regulated genes were identified in the 3DL QTL region (Supplementary Table S4). The top three significantly up-regulated genes in this QTL during both time points were protein kinase family protein, disease resistance protein (NBS-LRR class) family and the top three significantly down-regulated genes were CBS domain-containing protein-like and receptor-like protein kinase, pectin acetylesterase and polyubiquitin (Figure 9C).

3. Discussion

Host plant resistance is considered as the most sustainable approach for decreasing yield losses caused by different pathogens in many crops, including wheat. Integrating analysis of linkage mapping and putative genes responsible for resistance can facilitate the improvement of wheat through the identification and utilization of molecular markers for effective marker-assisted selection [27]. This type of integrated analysis assisted in decreasing the number of candidate genes in different plant species, such as maize [28], Arabidopsis [29] and wheat [30]. In the present study, a similar method was used to identify candidate genes for STB resistance during the different infection stages of STB (chlorosis, necrosis and pycnidia) in a bi-parental DH wheat population.

Based on the QTL mapping results, QTL on 3DL and 2BL were the most stable, being significant for all three STB induced symptoms. The most significant QTL on 3DL reported so far is *Stb16q*, being flanked by *barc125* and *barc128* [20], which has a projected region on 3DL of Chinese Spring RefSeq v1.0 from 174.8 to 532.9 Mb. On the other hand, the 3DL QTL in this study was found in the

region from 593.4 to 614.4 Mb, being outside yet not far from the *Stb16q* region. Therefore, it remains inconclusive as for whether the 3DL QTL in this study represents *Stb16q* or a novel QTL. On 2BL, Stb9 and a few mega-QTL have been mapped, of which the former is mapped at the distal region [17]. According to Chartrain et al. [31], Stb9 was encompassed by several SSRs of which wmc332 and gpw1214 represent the outmost markers, delimiting the gene in the physical region between 739.4 and 786.6 Mb. In our study, however, the QTL region is between 789.4 and 799.3 Mb, demonstrating a similar situation for the 3DL QTL, i.e., no solid conclusion could be drawn either as to whether this QTL is new. QTL on 1BS and 3AS were significant for only necrosis and pycnidia. The former was mapped in a large chromosome region in this study, spanning the entire 1BS and part of 1BL. According to Brown et al. [17], several genes and QTL have been mapped onto 1BS, including Stb2, Stb11, StbWW, MQTL2, etc. Limited by the low mapping precision in the current study we cannot make sure if the 1BS QTL represents any of the above-mentioned genes or QTL, unfortunately. The QTL on 3AS was mapped at the distal region from 7.4 to 8.7 Mb. On 3AS, there have been several genes and QTL mapped, including StbSm3, Stb6, MQTL12 etc., of which the first was located at the most distal region [17]. StbSm3 was identified in a common wheat landrace Salamouni, being flanked by SSR markers barc321 and barc12 [32] that delimiting a physical range between 11.7 and 15.5 Mb, being different from the region of our 3AS QTL, yet they are close to each other, and we cannot conclude with confidence that ours is a new QTL.

Several candidate genes with putative roles in resistance to STB in wheat were identified in the present work. The F- box proteins were identified as one of the most represented genes in all three QTL regions (Supplementary Table S4, Figure 8). They play an essential role in plant immune responses through their involvement in hormone pathways such us salicylic acid (SA) and jasmonic acid (JA) [33]. These hormones play major roles in regulating plant defence responses against numerous pest and pathogens [34]. SA is usually involved in the activation of defence responses against biotrophic and hemi-biotrophic, whereas JA is generally associated with defence against necrotrophic pathogens [35]. The lifestyle of the *Z. tritici* is a hemibiotrophic and thus both hormones are playing an important role in defence against STB. Zamani et al. [36] have shown that the exogenous application of SA has significantly reduced the STB disease symptoms on the wheat leaves. It was also reported that the fungus during the switch to necrotrophic stage induced changes in expression of wheat jasmonic acid biosynthesis genes and large-scale activation of other plant defence responses [26].

Disease resistance protein (NBS-LRR class) family genes were the second most over-represented among the DEGs in all three QTL regions (Supplementary Table S4, Figure 9). These disease resistance genes (R genes) encode proteins containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRRs) [37]. As an example, around 150 putative NBS-LRR genes have been identified in Arabidopsis [38], 400 in rice [39] genomes. The members of NBS-LRR family confer resistance to different pathogens in various plant species [40–43].

The third most over-represented genes within the QTL intervals in the present study were genes encoding receptor-like kinases (RLK) (Supplementary Table S4, Figure 9). Plants deploy a large number of surface RLKs as pattern recognition receptors to detect stresses such as herbivore feeding or to the presence of pathogens through detection of non-self molecules [44]. Several RLKs have recently been associated in plant defence responses [45]. Recently, a major resistance gene (*Stb6*) for STB was cloned which encodes a conserved wall-associated receptor kinase-like protein [46]. Ma, et al. [47] performing a comparative transcriptomics revealed that the early asymptomatic stage during the infection by *Z. tritici* is characterized by strong upregulation of genes encoding RLKs.

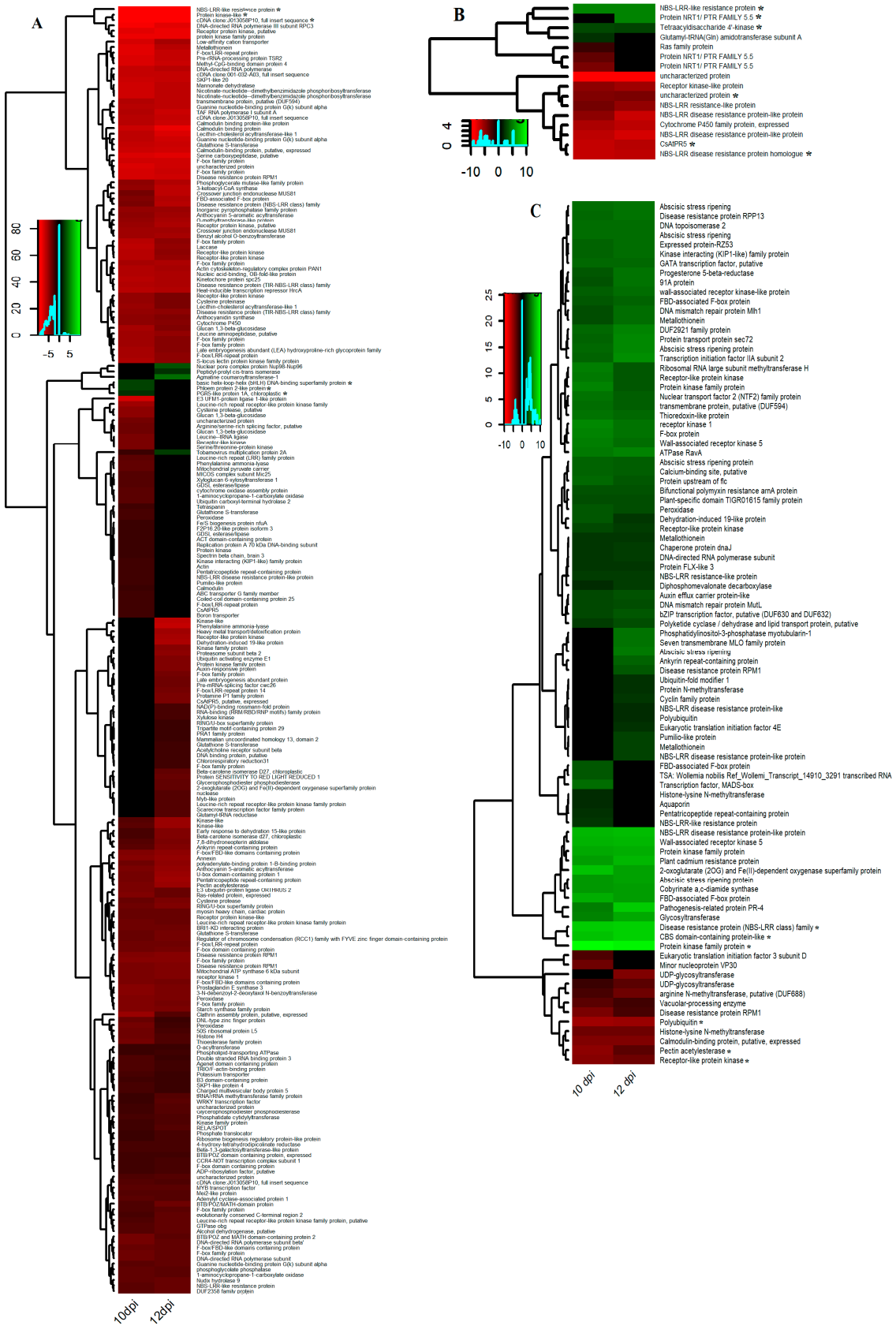


Figure 9. Heatmap of different gene expression profiles (≥2-fold, FDR ≤ 0.05) after infection with STB at 10 and 12 dpi. The DEGs were localized in (**A**) 1BS, (**B**) 2BL and (**C**) 3DL QTL intervals. The star indicates the expression of the six highly up and down regulated genes in the QTL intervals.

4. Materials and Methods

4.1. Plant Materials and Experimental Design

A set of 84 bi-parental doubled-haploid (DH) lines derived from a cross between winter wheat cultivars Stigg (resistant to STB) and Nimbus (susceptible to STB) were used to evaluate STB resistance in the greenhouse condition. Planting of wheat materials and the experimental design were done as described previously [48]. Briefly, the seeds were germinated on the moist filter paper in the Petri dishes at 4 °C in dark for 4 days followed by germination at room temperature for another two days. After germination, the seeds were sown in plastic pots (8 × 8 × 8 cm, 2 seeds per pot) filled with commercial pot soil Blomjord Exclusive (Emmaljunga Torvmull AB, Sweden) and grown in a greenhouse condition at 22 °C (day) and 18 °C (night) with a 16 h photoperiod. The experiment was performed in three randomized complete blocks design and the entire experiment was repeated twice.

4.2. Inoculation and Disease Assessment

Inoculum preparation and inoculation condition were performed according to Odilbekov, et al. [48]. Twenty-one days after the planting, both sides of marked second and third leaf of the seedlings were brushed with the conidial suspension using a flat watercolour paintbrush (bristle length 15 mm). Inoculated plants were allowed to dry for one hour and transferred to the plastic tent at a relative humidity (RH) of >95% for 48 h. Thereafter, the plants were returned to the greenhouse conditions. Disease severity was visually assessed at 13, 15, 17, 20 and 22 days after inoculation as a percentage (from 0 to 100%) of chlorotic (CHL), necrotic (NEC) pycnidial coverage area (PYC). The symptoms development for each line over the assessment period was calculated by using the area under the disease progress curves (AUDPC). Differences in AUDPC were investigated with ANOVA (PROC GLM), and Minitab software (Version 17.1.0) was used for statistical computations.

4.3. Genotyping

The leaf tissue for DNA extraction was collected from a six-week-old seedling and stored at −80 °C until further processing. The DNA extraction and genotyping of the samples was performed at TraitGenetics GmbH, Germany (http://www.traitgenetics.com/en/) and 20K SNP (single nucleotide polymorphism) wheat marker array was used for genotyping. Marker filtering was applied to eliminate (1) monomorphic markers, (2) highly distorted markers with segregation ratio beyond the range 0.5–2.0, and (3) markers with missing data point higher than 20%. A further cleaning step was performed with the BIN functionality of the ICIMapping v. 4.1 software (www.isbreeding.net) to remove redundant markers.

4.4. Linkage and QTL Mapping

Linkage groups (LGs) were generated with the JoinMap v.4 software [49] using LOD values from 3 to 10 and the Maximum Likelihood algorithm for ordering markers within each LG. Chromosome anchoring of LGs was performed according to the Illumina 90 K SNP map in Wang et al. [50]. QTL mapping was performed with the MapQTL v. 6.0 software [51]. Interval mapping (IM) was first run to detect potential QTL for each trait, and then multiple QTL mapping (MQM) for each QTL was performed using the closest linked markers identified in IM as a cofactor. QTL were regarded as significant and reported if they exceed the LOD threshold of 3 in at least one experiment or over the threshold of 2 in both experiments. The software MapChart ver. 2.3 [52] was used to draw LGs and LOD profiles.

4.5. RNA Isolation, Sequencing and Data Analysis

Six different genotypes (2 resistant, 2 susceptible and 2 parents) were selected for RNA-seq analysis based on phenotypic results. The growing condition of the plants, inoculum and inoculation were

performed as described above. The experiment was arranged in three replicates and samples were collected at 10 and 12 days post inoculation (dpi). At each time point, two leaflets were collected from each individual plant pooled and the collected samples (totally 36) were immediately frozen in liquid nitrogen and stored at −80 °C until further processing. Total RNA was isolated from 100 mg of frozen tissue with the RNeasy Plant Mini kit (Qiagen) including DNase treatment (RNase-free DNase set, Qiagen) based on the manufacturer's instructions. RNA quality and concentration were estimated in Experion™ Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, CA, USA). cDNA library construction and sequencing of the samples were done at the SciLifeLab (Stockholm, Sweden). Paired-end mRNA reads were generated using Illumina high-throughput sequencing machine and initial Quality Control (QC) check was performed with FastQC v0.11.7 [53]. All types of ribosomal RNAs (rRNAs) were removed using Sortmerna-v2.1b [54], and some minor percentage of remaining adapters were trimmed with Trimmomatic-v0.36 [55] as a minimum length of 20 bases along with other default settings. After merging reads from different libraries (only technical replicates), second round of QC check was performed on independent samples and for better visualization combined MultiQC v1.6 [56] tool was used. Sample reads were aligned to the published reference Wheat genome IWGSC RefSeq v1.0 genome [57] using STAR-v2.5.4a [58] spliced transcript aligner. Samtools-v1.5 [59] was used to sort reads from the alignment, transcript abundance was estimated (feature counts/TPM) with eXpress-v1.5.1 [60], especially total read counts were used for further analysis. Differential expression analysis of the genes (DEGs) was performed using the EdgeR package [61]. DEGs between the resistant and susceptible genotypes were determined based on a false discovery rate (FDR < 0.05) and relative fold change value (≥2-fold). The functional annotations of the differentially expressed genes were assessed with AgriGo [62] with the *Triticum aestivum* database. The annotations were further summarized using Revigo (Gibas et al. 2011). In order to verify the fungal transcript activity, available fungal reference genome *Z. tritici* (GCF_000219625.1_MYCGR_v2.0) [23] was used to align mRNA sequencing reads from all experiments. Performed alignment using STAR-v2.5.4a aligner, QC check and other filtering steps performed similar to the above analysis (with wheat reference genome). Transcript abundance was estimated (feature counts/TPM) with eXpress-v1.5.1 [60], and total read counts were measured for different experiments.

4.6. Genes in QTL Physical Positions

Attempts were made to locate the physical positions of all significant QTL via BLASTing sequences of their flanking markers against IWGSC RefSeq v1.0 genome. Considering the small population size, variation in physical position within a cluster of co-segregating markers was too big to be neglected, thus the physical confidence interval of a QTL was not only determined by the flanking markers listed in Table 1, but also their co-segregating markers, whichever showed wider ranges. We have localized significantly DEGs within these QTL wider ranges of co-segregating markers and extracted their gene annotation from IWGSC RefSeq v1.0 genome used for DE analysis. Genes from the four QTL regions were further filtered based on significantly DEGs from two different experiments (1) R-vs-S_10dpi, (2) R-vs-S_12dpi that were conducted for this study.

5. Conclusions

In this study, we identified four QTL for STB resistance of which two QTL were mapped for chlorosis, necrosis and pycnidia, and the other two were only associated with necrosis and pycnidia. The result suggests that integrating QTL mapping and transcriptome analyses is a powerful approach to narrow down and identify the correct candidate genes. The DEGs localized in the QTL regions serve as future candidate genes for enhancing STB resistance in wheat by using molecular approaches.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/9/510/s1, Table S1: List of differentially expressed genes identified by RNA-Seq at 10 and 12dpi, Table S2: Gene Ontology enrichment analysis, Table S3: Physical positions of markers flanking the four QTL mapped in this study, Table S4: List of differentially expressed genes localized on 1BS, 2BL and 3DL QTL regions.

Author Contributions: A.C. planned and designed the project. T.H. developed the DH bi-parental population. F.O. and R.A. performed the experiments in the greenhouse and laboratory. A.C., F.O., X.H. and G.V.S. analyzed the data. All authors interpreted the results and contributed to the writing.

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References

1. Fones, H.; Gurr, S. The impact of Septoria tritici blotch disease on wheat: An EU perspective. *Fungal Genet. Biol.* **2015**, *79*, 3–7. [CrossRef] [PubMed]
2. Suffert, F.; Sache, I.; Lannou, C. Early stages of Septoria tritici blotch epidemics of winter wheat: Build-up, overseasoning, and release of primary inoculum. *Plant. Pathol.* **2011**, *60*, 166–177. [CrossRef]
3. Eriksen, L.; Munk, L. The occurrence of *Mycosphaerella graminicola* and its anamorph *Septoria tritici* in winter wheat during the growing season. *Eur. J. Plant. Pathol.* **2003**, *109*, 253–259. [CrossRef]
4. Kema, G.; Annone, J.; Sayoud, R.; Van Silfhout, C.; Van Ginkel, M.; De Bree, J. Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem. I: Interactions between pathogen isolates and host cultivars. *Phytopathology* **1996**, *86*, 200–212. [CrossRef]
5. Steinberg, G. Cell biology of *Zymoseptoria tritici*: Pathogen cell organization and wheat infection. *Fungal Genet. Biol.* **2015**, *79*, 17–23. [CrossRef]
6. Kettles, G.J.; Kanyuka, K. Dissecting the molecular interactions between wheat and the fungal pathogen *Zymoseptoria tritici*. *Front. Plant. Sci.* **2016**, *7*, 508. [CrossRef] [PubMed]
7. Torriani, S.F.; Melichar, J.P.; Mills, C.; Pain, N.; Sierotzki, H.; Courbot, M. *Zymoseptoria tritici*: A major threat to wheat production, integrated approaches to control. *Fungal Genet. Biol.* **2015**, *79*, 8–12. [CrossRef]
8. Fraaije, B.; Cools, H.; Fountaine, J.; Lovell, D.; Motteram, J.; West, J.; Lucas, J. Role of ascospores in further spread of qoi-resistant cytochrome b alleles (g143a) in field populations of *Mycosphaerella graminicola*. *Phytopathology* **2005**, *95*, 933–941. [CrossRef]
9. Cools, H.J.; Fraaije, B.A. Update on mechanisms of azole resistance in *Mycosphaerella graminicola* and implications for future control. *Pest. Manag. Sci.* **2013**, *69*, 150–155. [CrossRef]
10. Cheval, P.; Siah, A.; Bomble, M.; Popper, A.D.; Reignault, P.; Halama, P. Evolution of qoi resistance of the wheat pathogen *Zymoseptoria tritici* in northern France. *Crop. Prot.* **2017**, *92*, 131–133. [CrossRef]
11. Yamashita, M.; Fraaije, B. Non-target site sdhi resistance is present as standing genetic variation in field populations of *Zymoseptoria tritici*. *Pest. Manag. Sci.* **2018**, *74*, 672–681. [CrossRef] [PubMed]
12. European Commission. Development of Guidance for Establishing Integrated Pest Management (IPM) Principles. Available online: https://ec.europa.eu/environment/archives/ppps/pdf/final_report_ipm.pdf (accessed on 3 September 2019).
13. Hillocks, R.J. Farming with fewer pesticides: Eu pesticide review and resulting challenges for UK agriculture. *Crop. Prot.* **2012**, *31*, 85–93. [CrossRef]
14. Miedaner, T.; Zhao, Y.; Gowda, M.; Longin, C.F.H.; Korzun, V.; Ebmeyer, E.; Kazman, E.; Reif, J.C. Genetic architecture of resistance to Septoria tritici blotch in European wheat. *BMC Genom.* **2013**, *14*, 858. [CrossRef] [PubMed]
15. Ghaffary, S.M.T.; Chawade, A.; Singh, P.K. Practical breeding strategies to improve resistance to Septoria tritici blotch of wheat. *Euphytica* **2018**, *214*. [CrossRef]
16. Yang, N.; McDonald, M.C.; Solomon, P.S.; Milgate, A.W. Genetic mapping of *stb19*, a new resistance gene to *Zymoseptoria tritici* in wheat. *Appl. Genet.* **2018**, *131*, 2765–2773. [CrossRef] [PubMed]
17. Brown, J.K.M.; Chartrain, L.; Lasserre-Zuber, P.; Saintenac, C. Genetics of resistance to *Zymoseptoria tritici* and applications to wheat breeding. *Fungal Genet. Biol.* **2015**, *79*, 33–41. [CrossRef]
18. Dreisigacker, S.; Wang, X.; Cisneros, B.A.M.; Jing, R.; Singh, P.K. Adult-plant resistance to Septoria tritici blotch in hexaploid spring wheat. *Appl. Genet.* **2015**, *128*, 2317–2329. [CrossRef]
19. Arraiano, L.; Brown, J. Identification of isolate-specific and partial resistance to Septoria tritici blotch in 238 European wheat cultivars and breeding lines. *Plant Pathol.* **2006**, *55*, 726–738. [CrossRef]

20. Ghaffary, S.M.T.; Faris, J.D.; Friesen, T.L.; Visser, R.G.F.; van der Lee, T.A.J.; Robert, O.; Kema, G.H.J. New broad-spectrum resistance to Septoria tritici blotch derived from synthetic hexaploid wheat. *Appl. Genet.* **2012**, *124*, 125–142. [CrossRef]
21. Goodwin, S.B. Resistance in wheat to septoria diseases caused by *Mycosphaerella graminicola* (*Septoria tritici*) and *Phaeosphaeria* (*Stagonospora*) *nodorum*. In *Disease Resistance in Wheat*; Sharma, I., Ed.; CABI: Wallingford, UK, 2012; pp. 151–159.
22. Plissonneau, C.; Hartmann, F.E.; Croll, D. Pangenome analyses of the wheat pathogen *Zymoseptoria tritici* reveal the structural basis of a highly plastic eukaryotic genome. *BMC Biol.* **2018**, *16*, 5. [CrossRef]
23. Goodwin, S.B.; M'barek, S.B.; Dhillon, B.; Wittenberg, A.H.; Crane, C.F.; Hane, J.K.; Foster, A.J.; Van der Lee, T.A.; Grimwood, J.; Aerts, A. Finished genome of the fungal wheat pathogen *Mycosphaerella graminicola* reveals dispensome structure, chromosome plasticity, and stealth pathogenesis. *PLoS Genet.* **2011**, *7*, e1002070. [CrossRef] [PubMed]
24. Palma-Guerrero, J.; Ma, X.; Torriani, S.F.; Zala, M.; Francisco, C.S.; Hartmann, F.E.; Croll, D.; McDonald, B.A. Comparative transcriptome analyses in *Zymoseptoria tritici* reveal significant differences in gene expression among strains during plant infection. *Mol. Plant. Microbe Interact.* **2017**, *30*, 231–244. [CrossRef] [PubMed]
25. Stewart, E.L.; Croll, D.; Lendenmann, M.H.; Sanchez-Vallet, A.; Hartmann, F.E.; Palma-Guerrero, J.; Ma, X.; Mcdonald, B.A. Quantitative trait locus mapping reveals complex genetic architecture of quantitative virulence in the wheat pathogen *Zymoseptoria tritici*. *Mol. Plant. Pathol.* **2018**, *19*, 201–216. [CrossRef] [PubMed]
26. Rudd, J.J.; Kanyuka, K.; Hassani-Pak, K.; Derbyshire, M.; Andongabo, A.; Devonshire, J.; Lysenko, A.; Saqi, M.; Desai, N.M.; Powers, S.J. Transcriptome and metabolite profiling of the infection cycle of *Zymoseptoria tritici* on wheat reveals a biphasic interaction with plant immunity involving differential pathogen chromosomal contributions and a variation on the hemibiotrophic lifestyle definition. *Plant Physiol.* **2015**, *167*, 1158–1185. [PubMed]
27. Biselli, C.; Bagnaresi, P.; Faccioli, P.; Hu, X.; Balcerzak, M.; Mattera, M.G.; Yan, Z.; Ouellet, T.; Cattivelli, L.; Valè, G. Comparative transcriptome profiles of near-isogenic hexaploid wheat lines differing for effective alleles at the 2DL Fhb resistance QTL. *Front. Plant. Sci.* **2018**, *9*, 37. [CrossRef] [PubMed]
28. Marino, R.; Ponnaiah, M.; Krajewski, P.; Frova, C.; Gianfranceschi, L.; Pè, M.E.; Sari-Gorla, M. Addressing drought tolerance in maize by transcriptional profiling and mapping. *Mol. Genet. Genom.* **2009**, *281*, 163–179. [CrossRef] [PubMed]
29. Xu, E.; Vaahtera, L.; Horak, H.; Hincha, D.K.; Heyer, A.G.; Brosche, M. Quantitative trait loci mapping and transcriptome analysis reveal candidate genes regulating the response to ozone in *Arabidopsis thaliana*. *Plant Cell Environ.* **2015**, *38*, 1418–1433. [CrossRef]
30. Dhokane, D.; Karre, S.; Kushalappa, A.C.; McCartney, C. Integrated metabolo-transcriptomics reveals fusarium head blight candidate resistance genes in wheat QTL-fhb2. *PLoS ONE* **2016**, *11*, e0155851. [CrossRef]
31. Chartrain, L.; Sourdille, P.; Bernard, M.; Brown, J.K.M. Identification and location of *stb9*, a gene for resistance to Septoria tritici blotch in wheat cultivars courtot and tonic. *Plant Pathol.* **2009**, *58*, 547–555. [CrossRef]
32. Cuthbert, R. Molecular Mapping of Septoria tritici Blotch Resistance in Hexaploid Wheat (*Triticum aestivum* L.). Ph.D. Thesis, University of Manitoba, Manitoba, MB, Canada, 2011.
33. Yu, H.; Wu, J.; Xu, N.; Peng, M. Roles of f-box proteins in plant hormone responses. *Acta Biochim. Biophys. Sin.* **2007**, *39*, 915–922. [CrossRef]
34. Bari, R.; Jones, J.D.G. Role of plant hormones in plant defence responses. *Plant Mol. Biol.* **2009**, *69*, 473–488. [CrossRef] [PubMed]
35. Glazebrook, J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Ann. Rev. Phytopathol.* **2005**, *43*, 205–227. [CrossRef] [PubMed]
36. Zamani, E.; Mohammadi-goltapeh, E.; Safaie, N. Effects of salicylic acid on the growth and pathogenicity of *Zymoseptoria tritici*. *Biol. J. Microorg.* **2019**, *7*.
37. Dangl, J.L.; Jones, J.D. Plant pathogens and integrated defence responses to infection. *Nature* **2001**, *411*, 826. [CrossRef] [PubMed]
38. Initiative, A.G. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **2000**, *408*, 796. [CrossRef] [PubMed]

39. Monosi, B.; Wisser, R.; Pennill, L.; Hulbert, S. Full-genome analysis of resistance gene homologues in rice. *Appl. Genet.* **2004**, *109*, 1434–1447. [CrossRef]
40. Ji, H.-M.; Zhao, M.; Gao, Y.; Cao, X.-X.; Mao, H.-Y.; Zhou, Y.; Fan, W.-Y.; Borkovich, K.A.; Ouyang, S.-Q.; Liu, P. Frg3, a target of slmir482e-3p, provides resistance against the fungal pathogen *Fusarium oxysporum* in tomato. *Front. Plant Sci.* **2018**, *9*, 26. [CrossRef]
41. Sun, J.; Li, L.; Zhao, J.; Huang, J.; Yan, Q.; Xing, H.; Guo, N. Genetic analysis and fine mapping of rpsjs, a novel resistance gene to *Phytophthora sojae* in soybean [*Glycine max* (l.) merr.]. *Appl. Genet.* **2014**, *127*, 913–919. [CrossRef]
42. Jianyuan, L.; Xiaodong, W.; Lirong, Z.; Qingfang, M.; Na, Z.; Wenxiang, Y.; Daqun, L. A wheat nbs-lrr gene targa19 participates in lr19-mediated resistance to *Puccinia triticina*. *Plant Physiol. Biochem.* **2017**, *119*, 1–8. [CrossRef]
43. Bittner-Eddy, P.D.; Crute, I.R.; Holub, E.B.; Beynon, J.L. Rpp13 is a simple locus in *Arabidopsis thaliana* for alleles that specify downy mildew resistance to different avirulence determinants in *Peronospora parasitica*. *Plant J.* **2000**, *21*, 177–188. [CrossRef]
44. Shiu, S.-H.; Bleecker, A.B. Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10763–10768. [CrossRef] [PubMed]
45. Greeff, C.; Roux, M.; Mundy, J.; Petersen, M. Receptor-like kinase complexes in plant innate immunity. *Front. Plant Sci.* **2012**, *3*, 209. [CrossRef]
46. Saintenac, C.; Lee, W.-S.; Cambon, F.; Rudd, J.J.; King, R.C.; Marande, W.; Powers, S.J.; Bergès, H.; Phillips, A.L.; Uauy, C. Wheat receptor-kinase-like protein stb6 controls gene-for-gene resistance to fungal pathogen *Zymoseptoria tritici*. *Nat. Genet.* **2018**, *50*, 368. [CrossRef] [PubMed]
47. Ma, X.; Keller, B.; McDonald, B.A.; Palma-Guerrero, J.; Wicker, T. Comparative transcriptomics reveals how wheat responds to infection by *Zymoseptoria tritici*. *Mol. Plant. Microbe Interact.* **2018**, *31*, 420–431. [CrossRef] [PubMed]
48. Odilbekov, F.; Armonienė, R.; Henriksson, T.; Chawade, A.C. Proximal phenotyping and machine learning methods to identify Septoria tritici blotch disease symptoms in wheat. *Front. Plant. Sci* **2018**, *9*, 685. [CrossRef] [PubMed]
49. Van Ooijen, J. Joinmap® 4, software for the calculation of genetic linkage maps in experimental populations. *Kyazma Bvwagening* **2006**, *33*, 1371.
50. Wang, S.; Wong, D.; Forrest, K.; Allen, A.; Chao, S.; Huang, B.E.; Maccaferri, M.; Salvi, S.; Milner, S.G.; Cattivelli, L. Characterization of polyploid wheat genomic diversity using a high-density 90,000 single nucleotide polymorphism array. *Plant. Biotechnol. J.* **2014**, *12*, 787–796. [CrossRef]
51. Van Ooijen, J.; Kyazma, B. *Mapqtl 6. Software for the Mapping of Quantitative Trait Loci in Experimental Populations of Diploid Species*; Kyazma BV: Wageningen, The Netherlands, 2009.
52. Voorrips, R. Mapchart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered* **2002**, *93*, 77–78. [CrossRef]
53. Andrews, S. Fastqc: A Quality Control Tool for High Throughput Sequence Data. 2010. Available online: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (accessed on 21 March 2018).
54. Kopylova, E.; Noé, L.; Touzet, H. Sortmerna: Fast and accurate filtering of ribosomal rnas in metatranscriptomic data. *Bioinformatics* **2012**, *28*, 3211–3217. [CrossRef]
55. Bolger, A.M.; Lohse, M.; Usadel, B. Trimmomatic: A flexible trimmer for illumina sequence data. *Bioinformatics* **2014**, *30*, 2114–2120. [CrossRef]
56. Ewels, P.; Magnusson, M.; Lundin, S.; Käller, M. Multiqc: Summarize analysis results for multiple tools and samples in a single report. *Bioinformatics* **2016**, *32*, 3047–3048. [CrossRef] [PubMed]
57. Alaux, M.; Rogers, J.; Letellier, T.; Flores, R.; Alfama, F.; Pommier, C.; Mohellibi, N.; Durand, S.; Kimmel, E.; Michotey, C. Linking the international wheat genome sequencing consortium bread wheat reference genome sequence to wheat genetic and phenomic data. *Genome Biol.* **2018**, *19*, 111. [CrossRef] [PubMed]
58. Dobin, A.; Davis, C.A.; Schlesinger, F.; Drenkow, J.; Zaleski, C.; Jha, S.; Batut, P.; Chaisson, M.; Gingeras, T.R. Star: Ultrafast universal rna-seq aligner. *Bioinformatics* **2013**, *29*, 15–21. [CrossRef] [PubMed]
59. Li, H.; Handsaker, B.; Wysoker, A.; Fennell, T.; Ruan, J.; Homer, N.; Marth, G.; Abecasis, G.; Durbin, R. The sequence alignment/map format and samtools. *Bioinformatics* **2009**, *25*, 2078–2079. [CrossRef] [PubMed]
60. Roberts, A.; Pachter, L. Streaming fragment assignment for real-time analysis of sequencing experiments. *Nat. Methods* **2013**, *10*, 71. [CrossRef] [PubMed]

61. Robinson, M.D.; McCarthy, D.J.; Smyth, G.K. Edger: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **2010**, *26*, 139–140. [CrossRef]
62. Du, Z.; Zhou, X.; Ling, Y.; Zhang, Z.; Su, Z. Agrigo: A go analysis toolkit for the agricultural community. *Nucleic Acids Res.* **2010**, *38*, W64–W70. [CrossRef] [PubMed]