Title:

QTL mapping reveals complex genetic architecture of quantitative virulence in the wheat pathogen *Zymoseptoria tritici*

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Summary

We conducted a comprehensive analysis of virulence in the fungal wheat pathogen *Zymoseptoria tritici* using QTL mapping. High throughput phenotyping based on automated image analysis allowed measurement of pathogen virulence on a scale and with a precision that was not previously possible. Across two mapping populations encompassing more than 520 progeny, 540,710 pycnidia were counted and their sizes and grey values were measured, yielding over 1.6 million phenotypes associated with pathogen reproduction. Large pycnidia were shown to produce more numerous and larger spores than small pycnidia. Precise measures of percent leaf area covered by lesions provided a quantitative measure of host damage. Combining these large and accurate phenotype datasets with a dense panel of RADseq genetic markers enabled us to genetically dissect pathogen virulence into components related to host damage and components related to pathogen reproduction. We show that different components of virulence can be under separate genetic control. Large- and small-effect QTLs were identified for all traits, with some QTLs specific to mapping populations, cultivars and traits and other QTLs shared among traits within the same mapping population. We associated the presence or absence of accessory chromosomes with several virulence traits, providing the first evidence for an important function associated with accessory chromosomes in this organism. A large-effect QTL involved in host specialization was identified on chromosome 7, leading to identification of candidate genes having a large effect on virulence.

Introduction

Much plant pathology research has been oriented around understanding the classic gene-for-gene (GFG) interaction (Flor, 1955) between the pathogen and its host, largely because early plant breeding programs focused on the introgression of major, qualitative resistance genes (R-genes) to control plant pathogens. Because most pathogens evolved quickly to defeat major R-genes, and quantitative resistance appeared more durable, the genetic basis of quantitative resistance became an area of intensive investigation, leading eventually to identification of host genes encoding quantitative resistance (Krattinger et al, 2009, St. Clair, 2010). The term aggressiveness is typically used by plant pathologists to describe the quantitative degree of damage caused by a pathogen strain. In the broader literature, virulence is defined as the quantitative degree of damage caused by a pathogen to its host. We will use the latter definition to be consistent with the wider field of life sciences. In contrast to quantitative resistance in the host, quantitative virulence in pathogens is much less studied and remains poorly understood (Lannou, 2012, Pariaud et al, 2009), though quantitative virulence appears to be common in plant pathogens (Pariaud et al, 2009).

Given the broad spectrum in observed virulence phenotypes, the genetic architecture of virulence may be mediated both by single genes of large effect that follow the GFG model (Flor, 1955) and by many genes that make smaller contributions to quantitative virulence. Quantitative traits often exhibit a continuous distribution in a population and are typically governed by independent assortment of multiple alleles with small individual effects, with different combinations of alleles responsible for the range of observed phenotypes in recombining populations (Mackay 2001). Numerous confounding factors such as dominance, pleiotropy and additive allele effects also contribute to the genetic architecture of a trait (Hansen 2006). In order to disentangle

72 individual loci contributing to overall virulence phenotypes, we utilized quantitative
73 trait locus (QTL) mapping, an established technique for determining the genetic
74 architecture of quantitative traits. QTL mapping was successfully used to identify
75 candidate genes underlying traits of interest in plants (Alonso-Blanco and Méndez-Vigo,
76 2014), animals (Solberg Woods, 2014) and humans (Almasy and Blangero, 2009). It has
77 also been employed in fungi (Larraya et al, 2003), though its use in fungi remains rare
78 compared to other eukaryotes (Foulongne-Oriol, 2012).
79
80 Virulence in the wheat pathogen *Zymoseptoria tritici* (formerly *Mycosphaerella*
81 *graminicola*), causal agent of Septoria tritici blotch (STB), is a predominantly
82 quantitative trait (Stewart and McDonald 2014, Zhan et al, 2007). *Z. tritici* is the most
83 damaging wheat pathogen in Europe (Jorgensen et al, 2014; O'Driscoll et al, 2014) and
84 is considered an important fungal pathogen worldwide (Dean et al, 2012). Under
85 optimal conditions, yield losses can reach 50% (Eyal 1987). Even with the use of
86 resistant cultivars and regular fungicide treatments, yield losses of 5–10% can be
87 expected (Fones and Gurr, 2015). Ten to fourteen days after infection chlorotic areas
88 begin to appear that later become necrotic lesions. Pycnidia containing asexual
89 pycnidiospores develop mostly within these necrotic lesions in the sub-stomatal
90 cavities. Pycnidiospores are exuded from the pycnidia in a gelatinous cirrhus during
91 periods of high humidity and are spread throughout the plant canopy by rain splash.
92 Numerous asexual infection cycles occurring during a growing season are the main
93 cause of STB epidemics in the field.
94
95 Despite its agricultural importance, the mechanisms responsible for virulence in *Z.*
96 *tritici* and their underlying genetics remain poorly understood. Twenty genes affecting

97 virulence in *Z. tritici* have been described and functionally characterized (Figure S1).
98 Most of these genes have developmental regulatory functions or affect inherent fitness
99 without having a specific role in virulence. Host resistance is also quantitative, with 21
100 genes and 89 genomic regions implicated in resistance to *Z. tritici* (Brown et al, 2015).
101 The quantitative nature of virulence in *Z. tritici* makes QTL mapping an ideal technique
102 to elucidate the genetic determinants of quantitative virulence, as already validated
103 using several other quantitative traits in *Z. tritici* (Lendenmann et al, 2014, Lendenmann
104 et al, 2015, Lendenmann et al, 2016).
105
106 Epidemic development is strongly affected by the ability of a pathogen to reproduce and
107 cause subsequent infections (Parlevliet, 1979), hence the reproductive output of a
108 pathogen is an important determinant of the total damage it can cause during an
109 epidemic. Damage caused by plant pathogens is frequently assessed by quantifying the
110 area covered by disease lesions on the host. However, a pathogen's ability to induce
111 lesions does not necessarily reflect its ability to reproduce. Indeed, several studies have
112 shown that pathogen damage, as indicated by the plant area covered by disease lesions,
113 can be independent of pathogen reproduction, as indicated by the number of spores
114 produced during an infection (Burns et al, 2014, Habgood, 1977, Halama et al, 1999,
115 Pariaud et al, 2009, Twizeyimana et al, 2014), suggesting that these traits may be under
116 separate genetic control. The contribution of each virulence component combines to
117 produce quantitative disease phenotypes.
118
119 The success of a QTL mapping project depends on the availability of genetic markers
120 and accurate phenotypic data. With current availability of genomic tools that can
121 generate very large numbers of genetic markers comparatively easily and cheaply, the

122 limiting factor in most studies is accurate phenotyping (Furbank and Tester, 2011).
123 Using image analysis to phenotype *Z. tritici* infection allows for measurement of both
124 leaf damage, based on the percentage of the leaf covered by necrotic lesions, and
125 pathogen reproduction, based on the number and size of the pycnidia formed on the
126 infected leaves (Stewart and McDonald 2014, Stewart et al, 2016).
127
128 The aim of this study was to investigate the genetic architecture of virulence traits in *Z.*
129 *tritici*. We conducted a QTL mapping study using two mapping populations derived
130 from parental isolates exhibiting varying degrees of virulence. Combining digital image
131 analysis with a large number of RADseq SNP markers in a large number of offspring
132 allowed us to genetically separate traits related to host damage and pathogen
133 reproduction. We also identified novel candidate genes involved in host specialization
134 and virulence.
135
136
137 **Results**
138
139 Phenotyping
140 A phenotyping method based on digital image analysis (Stewart and McDonald, 2014)
141 was used to measure % leaf area covered by lesions (PLACL), pycnidia density (# of
142 pycnidia per cm^2 leaf), pycnidia size and pycnidia melanization in two *Z. tritici* mapping
143 populations (3D1x3D7 and 1A5x1E4) that have also been used to study melanization
144 (Lendenmann et al, 2014), fungicide sensitivity (Lendenmann et al, 2015) and
145 temperature sensitivity (Lendenmann et al, 2016). The pairs of parental isolates used to
146 make the crosses were chosen based on differing levels of virulence observed in

147 previous work (Zhan et al, 2005). To investigate the effect of host on virulence, we used
148 cultivars Runal (moderately susceptible to *Z. tritici*) and Titlis (moderately resistant)
149 (Courvoisier et al, 2015). All phenotypes showed a continuous distribution in both
150 crosses. Transgressive segregation was evident for all phenotypes, with some progeny
151 exhibiting more extreme phenotypes than the parent isolates (Figure 1). In cross
152 3D1x3D7 all phenotypes were significantly higher on Runal than Titlis, including mean
153 pycnidia size ($F=3.93$, $p=0.048$), pycnidia melanization ($F=22.7$, $p<0.001$), PLACL
154 ($F=21.4$, $p<0.001$), and pycnidia density ($F=19.2$, $p<0.001$). In cross 1A5x1E4, PLACL
155 ($F=22.7$, $p<0.001$) and pycnidia melanization ($F=17.7$, $p<0.001$) were significantly
156 higher in Runal whereas pycnidia size ($F=11.5$, $p<0.001$) and pycnidia density ($F=73.6$,
157 $p<0.001$) were significantly higher in Titlis. Across both crosses, a total of 540,710
158 pycnidia were counted and their sizes and melanization measured, yielding over 1.6
159 million phenotypic data points associated with fungal fruiting bodies. In order to
160 evaluate whether counting and measuring pycnidia provides an accurate estimate of
161 spore number, the spore output of a subset of isolates was measured. The mean number
162 of spores per pycnidium was 2598. The mean spore size, as measured by length, was
163 17.6 µm. There were weak but significant positive correlations between pycnidia size
164 and spore size ($r^2=0.111$, $p=0.001$) and between pycnidia size and the number of spores
165 per pycnidium ($r^2= 0.112$, $p=0.001$). These findings indicate that pycnidia size is
166 correlated with reproductive output of *Z. tritici*.
167
168 <u>Quantitative Trait Locus (QTL) mapping</u>
169 We used QTL mapping to elucidate the genetic architecture of the quantitative virulence
170 phenotypes. QTLs were identified for all phenotypes. All QTLs in cross 3D1x3D7 were
171 found only on Runal and mapped to a single large effect QTL on chromosome 7 (Figure

172 2a). In 1A5x1E4 the same QTL for pycnidia density was found on chromosome 5 on
173 both cultivars. A second QTL for pycnidia density was found on chromosome 9 in Titlis
174 and on chromosome 3 in Runal. PLACL in Titlis and pycnidia grey value in Runal both
175 mapped to the same QTL on chromosome 5. Pycnidia size also mapped to the same QTL
176 on chromosome 3 in cv Runal (Figure 2b). No QTLs were shared between the two
177 crosses. The 95% confidence interval for a QTL ranged from 91.3 kb for the
178 chromosome 5 QTL in 1A5x1E4 to 717.7 kb for the chromosome 7 QTL for grey value
179 on Runal in 3D1x3D7.
180
181 The single, large effect QTL on chromosome 7 in cross 3D1x3D7 explained 54% of the
182 phenotypic variance for PLACL, 57% of the variance for pycnidia density and 18% of the
183 variance for pycnidia grey value. PLACL and pycnidia density shared the same
184 confidence interval containing 35 genes. The wider confidence interval for pycnidia
185 grey value contained 227 genes (Table 1A). The allele from the 3D7 parent was
186 responsible for higher PLACL and pycnidia density and decreased pycnidia
187 melanization.
188
189 In cross 1A5x1E4 the variance explained by each QTL was smaller and ranged from
190 6.1% for pycnidia density on Titlis to 8.7% for pycnidia size on Runal. The number of
191 genes within each confidence interval was generally higher than in cross 3D1x3D7,
192 ranging from 89 for grey value in Runal to 280 in the QTL on chromosome 5 for
193 pycnidia density in Runal and PLACL in Titlis (Table 1B). The 1A5 allele was responsible
194 for the higher phenotype values in the QTL on chromosome 5 whereas the 1E4 allele
195 was responsible for the higher phenotype values in the chromosome 3 and 9 QTLs.
196

197 Additive effects were observed for the two QTLs found for pycnidia density in 1A5x1E4.
198 On Runal, isolates with the 1A5 allele at the chromosome 5 QTL peak and the 1E4 allele
199 at the chromosome 3 QTL peak had significantly higher pycnidia density than isolates
200 with the reverse alleles ($F=11.2$, $p<0.001$) (Table 2A). On Titlis, isolates with the 1E4
201 allele at the chromosome 9 QTL peak and the 1A5 allele at the chromosome 5 QTL peak
202 had significantly higher phenotypes than isolates with the opposite allele combinations
203 ($F=19.1$, $p<0.001$) (Table 2B). On both cultivars, isolates with the same parental allele
204 at both QTL peaks were not different from each other and were intermediate to the
205 isolates with the alleles coming from different parents.
206
207 Effect of Accessory Chromosomes on Virulence
208 Among the parent isolates used to make the crosses, all accessory chromosomes were
209 present in 3D1 whereas 3D7 was missing chromosomes 14, 15, 18 and 21. In the
210 1A5x1E4 cross chromosome 17 was absent in 1E4. QTLs could only be mapped on
211 chromosomes present in both parent isolates. No QTLs were found on the accessory
212 chromosomes in either cross.
213
214 The presence or absence of accessory chromosomes was established for each of the
215 progeny in cross 3D1x3D7. Chromosome 14 was absent in 67 (26%) progeny,
216 chromosome 15 in 68 (26%) progeny, chromosome 18 in 77 (30%) progeny and
217 chromosome 21 in 73 (28%) progeny. All accessory chromosomes were present at a
218 significantly higher frequency than the expected 1:1 Mendelian inheritance ratio
219 (chromosome 14: $\chi^2 =61.1$, $p<0.001$, chromosome 15: $\chi^2 = 59.1$, $p<0.001$, chromosome
220 18: $\chi^2 = 43.1$, $p <0.001$, chromosome 21: $\chi^2 = 49.9$, $p<0.001$). This is consistent with

221 previous work that reported skewed inheritance of accessory chromosomes in a subset
222 of 48 isolates from the same population (Croll et al, 2013).
223
224 We studied the effect of accessory chromosome presence-absence on virulence. On
225 Runal, isolates with chromosome 21 had significantly larger pycnidia (F=6.2, p=0.014)
226 than isolates without chromosome 21, but the effect size was small (η^2=0.023). On Titlis,
227 isolates with chromosome 18 had significantly larger pycnidia (F=5.0, p=0.027) and
228 darker pycnidia (F=5.7, p=0.018) than isolates without chromosome 18. The effect sizes
229 for pycnidia size (η^2=0.021) and pycnidia melanisation (η^2=0.022) were small. A
230 significant interaction between chromosomes 15 and 18 was also observed for pycnidia
231 size (F=7.0, p= 0.009), with isolates having both accessory chromosomes showing larger
232 pycnidia, but the effect size of the interaction was small (η^2=0.028).
233
234 Candidate genes for pathogen virulence within QTLs
235 In total, 918 candidate genes were identified in the 95% confidence intervals of all
236 QTLs, including 227 from cross 3D1x3D7 and 691 from cross 1A5x1E4. No candidate
237 genes were shared between the two crosses. In the literature to date, 20 genes have
238 been implicated in virulence in *Z. tritici* and functionally characterized (Figure S1). None
239 of the genes previously shown to play a role in virulence were found within the QTLs.
240
241 In depth investigation of the chromosome 7 QTL
242 The QTL for PLACL and pycnidia density in cross 3D1x3D7 on Runal had a high LOD
243 score, a low number of candidate genes within the 95% confidence interval, and
244 explained approximately half of the observed phenotypic variance. Existing gene
245 models were manually checked for accuracy, sequence polymorphisms between the two

246 parent isolates were analyzed and gene expression during the infection cycle was
247 investigated. Population genomic approaches were used to investigate the genomic
248 features of the region in a natural population.
249
250 Re-annotation of the genes in the chromosome 7 QTL
251 Within the 95% confidence interval, 25 of the 35 gene models from the reference
252 annotation were not convincingly supported by RNAseq reads. The annotations of 20
253 genes were replaced with those of Grandaubert et al. (2015). Four genes not present in
254 any previous annotation were discovered and supported by our RNAseq data. The
255 annotations of four additional genes were altered slightly from those of Grandaubert et
256 al. (2015) or the JGI. After re-annotation, 38 genes were found in the 95% confidence
257 interval (Table 3). Among the newly annotated genes, GO terms could be assigned to 17
258 genes while the remaining 21 genes had no known function. Six genes within the QTL
259 encode proteins with a predicted signal peptide but lack a trans-membrane domain and
260 are therefore predicted to be secreted. Among these six genes, two meet the criteria for
261 being small secreted effector-like proteins (<300 amino acids, secreted and cysteine
262 rich).
263
264 Identification of high priority candidate genes within the chromosome 7 QTL
265 Sequence variation within gene coding regions of the chromosome 7 QTL was
266 investigated in the parental genomes. 82 sequence variants were identified, comprising
267 51 synonymous and 31 non-synonymous mutations as well as 4 insertions/deletions
268 (indels). 17 genes were found to have at least one mutation affecting amino acid
269 sequence. 19 genes showed differential expression *in planta* between the two parental
270 isolates for at least one time point during the infection process. Several high-priority

271 candidate genes were identified based on putative function, sequence variation and
272 expression levels during infection. Candidate gene *Zt_QTL7_5* is not present in any
273 existing annotation and BLAST searches showed no similarities to other proteins. With
274 a length of 65 aa, six cysteine residues and a signal peptide domain, it fulfills the criteria
275 of a typical small secreted effector protein. Its peak expression is between 12 and 14
276 dpi, during the transition from symptomless growth to the onset of chlorosis (Figure 4).
277 It is the second most expressed gene in the QTL confidence interval, but shows
278 significantly lower expression in 3D7 (the more virulent parent) at 14 dpi.
279 Candidate gene 00558 is also a small secreted protein containing an *Alternaria*
280 *alternata* allergen domain. This domain is unique to the Dothideomycete and
281 Sordariomycete classes of fungi (Chruszcz et al, 2012) and can induce major allergic
282 reactions in the human respiratory systems (Bush at al, 2001). This gene shows peak
283 expression during the symptomless phase and lower expression in 3D7 (Figure 4).
284 Candidate gene 105313 is a fungal-specific transporter from the Major Facilitator
285 Superfamily (MFS). It contains a trichothecene efflux domain that has been shown to
286 play a role in toxin secretion in Fusarium (Alexander et al, 1999). The parental alleles
287 differed by two non-synonymous SNPs. Candidate gene 00579 is a fungal-specific
288 membrane protein containing 7 trans-membrane domains, a CFEM domain and a signal
289 peptide. Expression peaks at 7 dpi (Figure 4).
290
291 <u>Population genomics of genes in the chromosome 7 QTL</u>
292 As QTL mapping is highly cross specific, genetic variation in the chromosome 7 QTL was
293 investigated in a natural population of 28 re-sequenced *Z. tritici* isolates from
294 Switzerland. The 163 kb confidence interval contained an alternation of gene-rich and
295 gene-poor regions. A large gene-poor region (found between ca. 1.79 - 1.84 Mb in the

296 reference genome assembly) contained only two genes (Figure 3E). This gene-poor
297 region contained all transposable elements (TEs) found within the confidence interval
298 (Figure 3D). Three classes of TEs were found in this region, including Copia, Gypsy and
299 Tad1 elements. The gene-rich regions contained a higher density of SNPs than the
300 average on the chromosome, reaching up to 120 SNPs per kb in some regions (Figure
301 3C). The genotyping rate was high in the gene-rich region. The gene-poor and TE-rich
302 regions contained few SNPs and had a low genotyping rate (Figure 3C) and generally
303 had a lower GC content than the gene-rich regions (Figure 3B).
304
305 Genes within the confidence interval were highly variable for the number of SNPs
306 within the natural population (Figure 3F). Seven genes contained fewer than 10 SNPs
307 per kb whereas 9 genes contained more then 50 SNPs per kb. On average, the genes
308 within the confidence interval contained 33 SNPs per kb. The Tajima's D statistic for 27
309 genes having 10 or more SNPs showed an alternation of clusters of genes with positive
310 D values and genes with negative D values (Figure 3G). Genes with negative D values are
311 more likely to be under purifying selection while genes with positive D values are likely
312 to be under balancing selection. This suggests variability in the selection processes
313 acting on these genes, including a possibility of hitchhiking effects.
314
315
316 **Discussion**
317
318 Here we report the most comprehensive QTL mapping analysis of fungal virulence to
319 date, utilizing different mapping populations and different host cultivars as well as
320 measuring multiple traits associated with virulence. We were able to genetically dissect

321 pathogen virulence into separate components, showing that different components of
322 virulence were under separate genetic control. QTLs were identified for all traits,
323 including QTLs that were specific to mapping populations, cultivars and traits as well as
324 QTLs that were shared among traits within the same mapping population. We identified
325 genome regions associated with single large effects as well as regions of smaller
326 additive effects.
327
328 The genetic architecture of virulence in *Z. tritici* is complex
329 Our results point to a complex genetic architecture involving multiple factors that
330 combine to produce quantitative virulence. Strong evidence was found for transgressive
331 segregation, with many progeny showing more extreme phenotypes than the parent
332 isolates in both crosses. QTLs were specific to each cross and in some cases specific to a
333 phenotype or cultivar. In all but one case, multiple traits mapped to the same QTL, an
334 observation also made for other traits in *Z. tritici* (Lendenmann et al. 2014; 2015; 2016).
335
336 Both quantitative (Zhan et al, 2005) and large-effect interactions consistent with the
337 gene-for-gene (GFG) hypothesis have been reported in *Z. tritici* (Brading et al, 2002,
338 Kema et al, 2000). GFG interactions are frequently reported as conferring
339 compatible/incompatible interactions (Flor, 1955). The large effect size of the 3D1x3D7
340 chromosome 7 QTL on one of the cultivars shows similarity to a GFG interaction
341 between a major resistance gene in Runal and a corresponding avirulence effector gene
342 in the 3D1 strain of the pathogen. In our case the interaction explains ~50% of the
343 overall variance for the virulence traits which also show a continuous distribution in the
344 mapping population. These observations suggest that numerous additional genes of

345 small effect may also be involved, in addition to the genotype-by-environment
346 interactions typically observed in quantitative phenotypes.
347
348 In contrast to the single large effect QTL in 3D1x3D7, numerous QTLs distributed across
349 several chromosomes were found for the different phenotypes in cross 1A5x1E4. These
350 QTLs had relatively small effects on phenotype and contained a high number of
351 candidate genes. This suggests that the genetic architecture of virulence in 1A5x1E4 is
352 more complex than that of 3D1x3D7 and demonstrates the contribution of both large
353 and small effects on virulence.
354
355 Additive effects of QTL loci on virulence
356 The multiple factor hypothesis (Morgan et al 1915) based on additive effects of many
357 genes is the key principle underlying quantitative traits. Recombination generates
358 progeny with different combinations of genes than the parents and can contribute to
359 novel virulence phenotypes (Joseph et al, 2011, Kanvil et al, 2015). Strong additive
360 effects were observed in cross 1A5x1E4 for the QTL for pycnidia density. For both
361 cultivars, alternative alleles from the two parents combined to produce a higher
362 phenotype. Isolates with the same parental allele at both QTLs showed lower virulence
363 than isolates with the different parental alleles at the two QTLs. Transgressive
364 segregation was observed for all phenotypes in both crosses. These findings illustrate
365 the power of recombination to generate progeny phenotypes that are significantly
366 different from their parents, an observation shared with other plant pathogenic fungi
367 (Sommerhalder et al, 2010; Stefansson et al. 2014) and show how a range of
368 quantitative virulence phenotypes can be generated through sexual reproduction in
369 natural field populations.

370

371 The contribution of accessory chromosomes to virulence

372 By using the novel approach of associating virulence phenotypes with the presence or
373 absence of specific accessory chromosomes, we discovered a significant correlation for
374 some phenotypes, with higher virulence observed in offspring carrying certain
375 accessory chromosomes. Accessory chromosomes were shown to play an important
376 role in virulence in other fungal pathogens (Akagi et al, 2009, Ma et al, 2010, Miao, et al,
377 1991) but until now their role in *Z. tritici* was elusive (Stukenbrock et al, 2010). The
378 increase in virulence was small, around 2-3%, but significant. If this correlation also
379 exists under natural field conditions, this increase in virulence and the associated
380 increase in reproduction would represent a significant fitness advantage that could
381 explain why accessory chromosomes are retained in natural field populations.

382

383 Cultivar specificity

384 Cultivar specificity is well documented in *Z. tritici* (Ahmed et al, 1995, Cowger and
385 Mundt 2002, Zhan et al, 2002) and is commonly reported as qualitative
386 compatible/incompatible interactions (e.g. Brading et al, 2002) or as generally
387 quantitative with isolates being more virulent or less virulent on a particular host (e.g.
388 Zhan et al, 2005). In cross 3D1x3D7, all phenotypes were higher on the more
389 susceptible cultivar as expected. However, in cross 1A5x1E4, PLACL was higher in
390 Runal whereas pycnidia size, density and melanization were higher in Titlis, the more
391 resistant cultivar. Additionally, in 1A5x1E4 the same chromosome 5 QTL was found for
392 pycnidia density in both cultivars. However, a second QTL was found on chromosome 3
393 in Runal and on chromosome 9 in Titlis, suggesting that these QTL loci play a role in
394 quantitative specialization to these two cultivars. This is in contrast to the findings of

395 Mirzadi Gohari et al, (2015) who found a QTL at a similar position on chromosome 5
396 that was implicated in host specificity in a different set of cultivars. The results from the
397 accessory chromosome presence/absence analysis also point towards cultivar
398 specificity, with different accessory chromosomes affecting the same phenotype on
399 different cultivars.
400
401 Contribution of pycnidia number and size to epidemic potential
402 The basic reproductive number (R_0) is the theoretical number of infections arising from
403 a single infection event and can be useful in predicting the potential development of
404 plant disease epidemics (van den Bosch et al, 2008). Pycnidia represent the asexual
405 reproductive output of a *Z. tritici* strain, with larger pycnidia correlated with a greater
406 number of conidia in earlier work (Gough, 1978). It was previously postulated that the
407 number and size of pycnidia could be important measures of virulence in *Z. tritici*
408 (Stewart and McDonald 2014, Suffert et al, 2013). Our results show a significant
409 correlation indicating that larger pycnidia bear both larger and more numerous spores.
410
411 The number and size of pycnidia influence the R_0 and therefore provide a prediction of
412 the epidemic potential associated with a pathogen strain under field conditions. In
413 contrast, measuring the leaf area covered by lesions (PLACL) gives an indication of the
414 amount of damage caused by a pathogen strain on the host plant. The ability to directly
415 measure both the epidemic potential of an isolate and the amount of damage it causes to
416 its host represents a powerful improvement in the overall measurement of pathogen
417 virulence that can also be used to improve measurements of resistance in the host
418 (Stewart et al. 2016). Furthermore, the identification of a QTL for pycnidia size shows
419 that this important trait can be under separate genetic control. A separate experiment

420 oriented around measuring differences in host resistance and conducted using naturally
421 infected plants under field conditions showed that host genotype could affect pycnidia
422 formation independently of damage caused by leaf lesions (Stewart et al 2016).
423 Theoretically, a pathogen strain which causes high levels of leaf necrosis but has a low
424 R_0 (i.e. producing fewer pycnidia per infected leaf) may appear more damaging at a
425 small spatial scale but an isolate which has a higher R_0 (i.e. producing more pycnidia per
426 infected leaf) has a greater potential to cause damage over larger spatial scales. From an
427 epidemiology perspective, directly measuring pathogen reproduction on a given host
428 could inform disease management decisions oriented around inhibiting pathogen
429 reproduction (e.g. through seeking resistance genes that lower pathogen reproduction
430 in addition to resistance genes that lower host damage), as well as providing a novel
431 way of selecting for quantitative host resistance. The results from cross 1A5x1E4
432 illustrate the potential usefulness of this approach with different levels of pycnidia and
433 PLACL found on the two different cultivars.
434
435 Candidate genes affecting pathogen virulence
436 By combining several disciplines, we were able to narrow the candidate gene list for the
437 chromosome 7 QTL affecting PLACL, pycnidia density and melanization, but it remains
438 possible that different genes located within this QTL interact to affect all three traits. It
439 also is possible that one gene in this QTL is primarily responsible for only one
440 phenotype, which influences the other two without any genetic or regulatory
441 connection, a situation referred to as spurious, vertical, relational or reactive pleiotropy
442 (Paaby and Rockman, 2013). For example, it is plausible that a gene encoding higher
443 PLACL would lead to greater pycnidia density that could in turn affect pycnidia
444 melanization. The results from cross 1A5x1E4 offer support for the latter hypothesis,

445 with multiple traits mapping to the same QTL on chromosome 5, though independent
446 QTLs were found on different chromosomes for the same traits. In previous work, genes
447 up-regulated in the biotrophic phase of the infection cycle showed reduced pycnidia
448 production in knockout mutants (Poppe et al, 2015), providing evidence that pycnidia
449 development is somewhat dependent on prior processes.
450
451 Among the candidate genes within the chromosome 7 QTL, the one we consider most
452 likely to explain the observed virulence is *Zt_QTL7_5*, a previously un-described small
453 secreted protein (SSP) that is highly expressed during the switch to necrotrophic
454 growth. SSPs are common virulence factors in fungi that facilitate infection or elicit a
455 response in the host (Lo Presti, 2015). SSPs often conform to the GFG paradigm by
456 interacting with a major R gene in the host. A GFG interaction would explain the large
457 effect of the chromosome 7 QTL on only one host. Interestingly, no significant sequence
458 differences exist for this gene between the parent isolates, but the expression levels are
459 significantly lower in 3D7, the more virulent parent. This is consistent with the theory
460 of effector triggered immunity (ETI), where recognition of a pathogen effector triggers a
461 defense response in the host. Another SSP, candidate gene 00558, contains a conserved
462 domain known to elicit allergic response in humans (Bush et al, 2001). It has
463 additionally been implicated in virulence in *Alternaria brasicicola* on Arabidopsis
464 (Cramer and Lawrence, 2004) and inhibits plant antimicrobial proteins (Gomez-Casado
465 et al, 2014). Given the existing knowledge of this protein, it appears that it could play a
466 role in *Z. tritici* virulence. An MFS transporter implicated in toxin secretion was also
467 located in the QTL. MFS transporters contribute to virulence by secretion of fungal
468 toxins and secondary metabolites as well as efflux of plant-derived antimicrobial
469 compounds (Coleman and Mylonakis, 2009). Candidate gene 00579 contains a CFEM

470 domain that is unique to fungi and found at significantly higher frequency in pathogenic
471 than non-pathogenic fungi (Zhang et al, 2015). Putative functions of the CFEM domain
472 include cell surface receptors, signal transduction or adhesion of molecules in plant-
473 pathogen interactions (Kulkarni et al, 2003). None of the genes previously described as
474 having a role in virulence in *Z. tritici* were identified in this study, highlighting the
475 usefulness of forward genetic approaches such as QTL mapping to identify novel
476 virulence genes.
477
478 Population genomics of the chromosome 7 QTL
479 Analysis of the large effect QTL on chromosome 7 in a Swiss field population revealed
480 several interesting genomic features. The region was highly variable, with genes
481 exhibiting variable numbers of SNPs as well as evidence for both purifying and
482 balancing selection. Genes involved in virulence are often under positive selection in
483 plant pathogens (Lo Presti et al, 2015; Stukenbrock and McDonald, 2009), including in
484 *Z. tritici* (Poppe et al, 2015). An island of transposable elements (TEs) was found within
485 a gene-poor region of the QTL. The *Z. tritici* genome contains 16.7% repetitive elements
486 (Dhillon et al, 2014). Effector genes show a tendency to cluster in gene-poor regions of
487 repetitive DNA that are TE-rich in numerous filamentous fungal plant pathogens (Dong
488 et al, 2015). TEs can influence the expression of effector genes by insertion into
489 promoter regions (Ali et al 2014) or through epigenetic gene silencing (Shaaban et el,
490 2010). We hypothesize that these processes may explain the differences in expression
491 observed in *Zt_QTL7_5.*
492
493 Conclusions

494 This study highlights the complex nature of virulence in the wheat-*Z. tritici*
495 pathosystem, illustrating that many factors contribute to quantitative phenotypes. We
496 showed that virulence is comprised of different traits, some affecting host damage and
497 others affecting pathogen reproduction, that can be under independent genetic control.
498 In light of this, researchers and breeders should reconsider the best way to measure
499 virulence in this pathosystem. We propose that more attention should be focused on
500 resistance that reduces pathogen reproduction in order to decrease R_0 during
501 epidemics.
502
503 **Experimental Procedures**
504
505 Two *Z. tritici* mapping populations (described in Lendenmann et al, 2014) were
506 phenotyped in a greenhouse-based seedling assay as described in Stewart and
507 McDonald (2014). The Swiss winter wheat cultivars 'Runal' and 'Titlis' (DSP Ltd, Delly,
508 Switzerland) were inoculated using each offspring from both crosses along with the
509 parental isolates. Two plants of each cultivar were inoculated with each *Z. tritici* isolate
510 to give two technical replicates. Each technical replicate was placed in a separate
511 greenhouse compartment. This process was repeated three times over three
512 consecutive weeks to generate three biological replicates, resulting in six replicates
513 total for each isolate-cultivar combination.
514
515 At 23 days post inoculation (dpi) the second leaf from each plant was excised,
516 photographed and phenotyped using automated image analysis as described previously
517 (Stewart and McDonald 2014). The method was modified slightly to include a measure
518 of pycnidia melanization. RGB images were converted to 8-bit greyscale and the mean

519 grey value for the pixels making up each pycnidium was calculated. The grey scale runs
520 from 0 (black) to 255 (white). Grey values can be used as a proxy for the degree of
521 melanisation (Lendenmann et al, 2015). The phenotypes percent leaf area covered by
522 lesions (PLACL), pycnidia density (pycnidia per cm^2 leaf area), pycnidia size (in mm^2)
523 and pycnidia melanization were used as phenotypes for QTL mapping (see below).
524
525 For the 3D1x3D7 progeny, the mean pycnidia size was calculated for each isolate on
526 Runal. The 10 isolates showing the largest mean pycnidia sizes and 10 showing the
527 smallest mean pycnidia sizes were selected for further analysis. The leaves from the six
528 replicates for each isolate were retrieved from storage. Each leaf was cut into ~30 mm
529 long sections. All sections from each leaf were placed into a 1.2 ml collection microtube
530 (QIAGEN) along with a 5 mm x 25 mm strip of filter paper. 100 µl sterile water was
531 added to each tube to moisten the filter paper. Tubes were capped and placed at 25 °C
532 for 24 h to provide the humid environment needed to exude the cirrhi containing spores
533 from pycnidia. 800 µl of water containing 0.001% TWEEN 20 was added to each tube.
534 Tubes were vortexed for 20 seconds to suspend the released spores. 100 µl of the spore
535 solution was placed into a black glass-bottomed 96 well plate (Greiner bio one, µClear).
536 Spores were imaged with an Olympus IX 81 inverted microscope coupled with a
537 Hamamatsu ORCA-ER camera using transmission illumination. Four images were made
538 from each well at 20x magnification with a 10% overlap between images. Images from
539 each well were stitched together using ImageJ (Rasband, 1997–2015). Each spore was
540 counted and its length was measured using the line selection tool and the measure
541 command in ImageJ (Figure S2). The mean spore length was used as a proxy for spore
542 size. The total number of spores per leaf was calculated (Figure S2) and divided by the
543 number of pycnidia per leaf to derive the mean number of spores per pycnidium.

544

545 All analyses were performed in base R (R core team, 2012) unless specified otherwise.
546 To normalize environmental variation between technical replicates and biological
547 replicates and account for genotype-by-environment (GxE) interactions, phenotypes
548 were mean centered (Schielzeth, 2010). For each phenotype, the mean value for each
549 greenhouse chamber and time point was calculated. This value was subtracted from all
550 individual values from the corresponding greenhouse chamber and time point, resulting
551 in a mean value of ~0 for each greenhouse chamber time point. The mean of these
552 transformed values was calculated for each progeny and used for subsequent analyses.
553 PLACL and pycnidia density values were log transformed prior to mean centering. Mean
554 centered values were used for analysis but untransformed values are reported in the
555 text and figures for clarity.
556
557 Phenotype differences between wheat cultivars were calculated using ANOVA. Effects
558 on phenotypes of accessory chromosome presence or absence were calculated using
559 ANOVA with the presence or absence of each chromosome as factors. Non-significant
560 factors were removed from the models. Effect sizes (η^2) were calculated using the lsr
561 package (Navarro, 2015). Fisher's LSD test was used to calculate the differences
562 between isolates with different parental alleles at QTL peaks using the Agricolae
563 package (de Mendiburu, 2014). Correlations between pycnidia size and spore size and
564 between pycnidia size and number of spores per pycnidia were made with Pearson's
565 correlation coefficient.
566
567 Progeny from both crosses were genotyped using restriction site associated DNA
568 sequencing (RADseq) based on a method adapted from Etter et al (2011) as described

569 previously (Lendenmann et al, 2014). The genetic map of Lendenmann et al (2014) was
570 used to perform QTL mapping. QTL mapping was performed using the r/QTL package
571 (Arends et al, 2010) following the methods described in Lendenmann et al (2014).
572
573 To establish the presence or absence of accessory chromosomes in 3D1x3D7, sequence
574 reads from the RADseq genotyping were aligned to the IPO323 reference genome
575 (Goodwin et al, 2011). For each isolate, the mean read depth was calculated for each
576 chromosome and divided by the mean sequence depth over all chromosomes. Thus,
577 chromosomes with read depth similar to the genome average have a value of ~1 and
578 were deemed present whereas chromosomes with low or no read depth have a value of
579 ~0 and were deemed absent. Deviations from Mendelian inheritance among the
580 accessory chromosomes exhibiting presence/absence polymorphisms in the parents
581 were tested using a Chi square (χ^2) test.
582
583 Parent genome sequences (Croll et al, 2013) were aligned to the IPO323 reference
584 genome. Sequence variants were identified using the unified genotyper within GATK
585 (McKenna et al, 2010) and effects of variants found within genes were predicted using
586 snpEFF (Cingolani et al, 2012a) and snpSIFT (Cingolani et al, 2012b) following the
587 methods outlined in Lendenmann et al (2014).
588
589 The genes within the 95% confidence interval containing a QTL with large effect on
590 chromosome 7 were manually re-annotated using existing annotations from the IPO323
591 reference annotation (Goodwin et al, 2011) and Grandaubert et al. (2015) along with
592 RNAseq data from IPO323 (Rudd et al, 2015) and from the 3D7 parent isolate (Palma-
593 Guerrero et al, 2016). Genes were annotated using blast2go (Conesa et al. 2005). Signal

594 peptides were identified using signalP 4.1 (Petersen et al, 2011) and trans-membrane
595 domains were identified with TMHMM 2.0 (Krogh et al, 2001). The re-annotated genes
596 were used for all subsequent analyses of this region.
597
598 An *in planta* infection assay was performed with the parent isolates 3D1 and 3D7
599 (Javier Palma-Guerrero, unpublished). RNA was sequenced from leaf samples collected
600 at 7, 12, 14 and 28 days post inoculation (dpi) as described earlier (Palma-Guerrero et
601 al. 2016).
602
603 The population genomics of the genes in the 95% confidence interval of the
604 chromosome 7 QTL was characterized using 28 re-sequenced *Z. tritici* isolates sampled
605 from a single field in Switzerland. Sequences were aligned to the reference genome
606 using Bowtie 2 version 2.2.3 (Langmead and Salzberg, 2012). SNPs among the isolates
607 were identified using GATK tools (De Pristo et al. 2011). GC content was calculated in
608 the reference genome using 1000 bp overlapping windows, with 100 bp overlaps. The
609 locations of transposable elements within the reference genome were identified using
610 RepeatMasker (Smit et al. 2013-2015). The repeat library used for annotation was
611 version 20150807 downloaded from Repbase in September 2015 (Jurka et al. 2005).
612 The SNP genotyping rate in the 28 re-sequenced isolates was calculated in windows of
613 1000 bp. The SNP density was calculated in windows of 500 bp for SNPs that were
614 genotyped in >50% of the re-sequenced isolates. The SNP density per coding sequence
615 was calculated by dividing the number of SNPs with a genotyping rate > 90% by the
616 total coding sequence length. Tajima's D statistic (Tajima, 1989) was calculated per
617 gene using Popgenome in R (Pfeifer, et al, 2014). Only SNPs with a genotyping rate >

618 90% were included. Tajima's D values for genes with less than 10 SNPs were not
619 calculated.
620
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631
632

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Tables

Table 1. Significant QTLs for *in planta* virulence traits from *Z. tritici* mapping populations 3D1x3D7 (A) and 1A5x1E4 (B) on wheat cultivars Runal and Titlis.

A

Cultivar	Phenotype	Chr	LOD Score	Variance Explained (%)	LOD Threshold	p value	Confidence Interval (Mb)	Number of Genes	Mean Phenotype (3D1 allele)	Mean Phenotype (3D7 allele)
Runal	PLACL	7	43.7	53.9	3.34	<0.001	1.75-1.91	35	38.98	78.19
Runal	Pycnidia per cm^2	7	48.2	57.3	3.30	<0.001	1.75-1.91	35	11.26	34.23
Runal	Grey Value†	7	11.4	18.1	3.29	<0.001	1.18-1.91	227	71.72	78.44

B

Cultivar	Phenotype	Chr	LOD Score	Variance Explained (%)	LOD Threshold	p value	Confidence Interval (Mb)	Number of Genes	Mean Phenotype (1A5 allele)	Mean Phenotype (1E4 allele)
Runal	Pycnidia per cm^2	3	4.9	8.6	3.38	0.003	1.27-1.87	209	24.79	32.13
Runal	Pycnidia per cm^2	5	3.8	7.0	3.38	0.018	0.14-0.23	280	31.02	26.60
Runal	Pycnidia Size ($mm^2 \times 10^{-3}$)	3	5.1	8.7	3.33	0.001	1.27-1.98	143	4.773	4.998
Runal	Grey Value†	5	4.7	8.1	3.31	0.001	0.14-0.15	89	88.06	84.74
Titlis	Pycnidia per cm^2	5	4.15	7.2	3.38	0.006	1.36-2.27	280	28.03	22.62
Titlis	Pycnidia per cm^2	9	3.51	6.1	3.38	0.045	3.20-9.19	204	22.37	28.01
Titlis	PLACL	5	4.56	8.0	3.17	0.015	1.36-2.27	280	95.49	90.40

†The grey scale ranges from 0 (black) to 255 (white). Lower values indicate darker pycnidia.

Table 2. Additive allele effects for pycnidia density in cross 1A5x1E4 for cultivars Runal (A) and Titlis (B). Chromosome alleles represent the parental allele of the marker at the QTL peak. Phenotypes are the mean pycnidia density of all isolates with the corresponding allele combination. Superscript letters denote significant differences. n represents the number of isolates with each allele combination.

A

Cultivar	Chr 3 allele	Chr 5 allele	Mean Phenotype	n
Runal	1E4	1A5	39.28[a]	43
Runal	1A5	1A5	26.10[b]	32
Runal	1E4	1E4	26.96[b]	44
Runal	1A5	1E4	23.09[c]	46

B

Cultivar	Chr 5 allele	Chr 9 allele	Mean Phenotype	n
Titlis	1A5	1E4	33.38[a]	37
Titlis	1A5	1A5	25.00[b]	41
Titlis	1E4	1E4	25.15[b]	54
Titlis	1E4	1A5	20.11[c]	36

Table 3. Re-annotated candidate genes located in a QTL for pycnidia density and PLACL on chromosome 7 in *Z. tritici*. Superscripts denote the source of the gene annotation: [a] new genes not present in previous annotations, [b] annotations modified from existing annotations, [c] annotation from the JGI reference annotation, [d] annotation from Grandaubert et al. (2015).

Gene Name	Description	Protein Length	Signal Peptide	TM Domain
Zt_QTL7_1[a]	NA	504	N	N
Zt_QTL7_2[a]	NA	228	Y	N
Zt_QTL7_3[a]	NA	357	N	N
Zt_QTL7_4[b]	Hypothetical protein	471	N	Y
Zt_QTL7_5[a]	NA	198	Y	N
Zt_QTL7_6[b]	AMP-binding enzyme family	921	N	Y
Zt_QTL7_7[b]	Serine carboxypeptidase	966	N	N
Zt_QTL7_8[b]	Hypothetical protein	879	N	N
Mycgr3T100839[c]	Fe-containing alcohol dehydrogenase,	1251	N	N
Mycgr3T100840[c]	Vacuolar membrane pq loop repeat	972	N	Y
Mycgr3T105313[c]	Trichothecene efflux pump	1788	N	Y
Mycgr3T44611[c]	Radical s-adenosyl methionine domain-containing 2	1074	N	Y
Mycgr3T44946[c]	Hypothetical protein	666	N	N
Mycgr3T45542[c]	Lipoate- ligase B	720	N	N
Mycgr3T74280[c]	Prolyl 4-hydroxylase	1503	N	N
Mycgr3T94634[c]	Hypothetical protein	525	N	N
Mycgr3T94648[c]	Hypothetical protein	1143	N	N
Mycgr3T94659[c]	Hypothetical protein	972	N	N
Zt09_model_7_00553[d]	Ferric-chelate reductase (Fre2)	2085	N	Y
Zt09_model_7_00555[d]	Hypothetical protein	255	N	N
Zt09_model_7_00556[d]	Hypothetical protein	1038	N	N
Zt09_model_7_00557[d]	Hypothetical protein	378	N	N
Zt09_model_7_00558[d]	Major allergen alt a1, partial	540	Y	N
Zt09_model_7_00559[d]	Hypothetical protein	789	N	Y
Zt09_model_7_00561[d]	Hypothetical protein	1302	N	N
Zt09_model_7_00562[d]	Hypothetical protein	3663	Y	N
Zt09_model_7_00563[d]	Hypothetical protein	1851	N	N
Zt09_model_7_00564[d]	Hypothetical protein	1761	N	Y
Zt09_model_7_00567[d]	Hypothetical protein	651	N	N
Zt09_model_7_00569[d]	Acid phosphatase	456	N	Y
Zt09_model_7_00571[d]	Adenylate kinase	696	N	N
Zt09_model_7_00573[d]	NA	420	N	N
Zt09_model_7_00574[d]	Glycoside hydrolase	933	N	N
Zt09_model_7_00575[d]	Glycoside hydrolase family 36	2238	Y	N
Zt09_model_7_00577[d]	Ca2+-modulated nonselective cation channel polycystin	1917	Y	N
Zt09_model_7_00578[d]	Urease accessory	780	N	N
Zt09_model_7_00579[d]	CFEM domain-containing	1230	Y	Y
Zt09_model_7_00581[d]	Hypothetical protein	294	N	N

Figure Legends

Figure 1. Frequency histograms of virulence phenotypes from the *Z. tritici* mapping populations 3D1x3D7 (upper panels) and 1A5x1E4 (lower panels) on the wheat cultivars Runal (white bars) and Titlis (grey bars). Vertical lines represent phenotype values of the parent isolates, dotted line represents 3D1 and 1A5, dashed line represents 3D7 and 1E4.

Figure 2. LOD (logarithm of the odds) plots from QTL mapping of virulence traits in the *Z. tritici* mapping populations 3D1x 3D7 (A) and 1A5x1E4 (B) for the 13 core chromosomes (x axis). The dashed horizontal line represents the 0.05 significance threshold calculated with 1000 permutations.

Figure 3. Population genomic characterization of the QTL located on chromosome 7 (region 1746 kb to 1909 kb) in the reference isolate IPO323 and a population of 28 *Zymoseptoria tritici* isolates drawn from a single field in Switzerland. (A). GC content in the IPO323 reference genome. (B). SNPs density (bottom) and genotyping rate (top) in the 28 field isolates. Black rectangles represent the frequency of missing SNP genotypes. The horizontal line represents the mean SNP density across chromosome 7 in the 28 isolates. (C). Transposable element locations in the IPO323 reference genome. (D). *Bottom:* Location of coding sequences in the IPO323 reference genome. *Top:* Frequency of the allele carried by 3D7 in the 28 field isolates for the SNPs shared by the 3D1 and 3D7 parents in the gene coding sequences. (E). SNP density per kb in the coding sequences of the genes. Only SNPs with a genotyping rate ≥ 90% were

included. (F). Tajima's D statistic per gene coding sequence. Tajima's D values for genes with less than 10 SNPs are not shown.

Figure 4. Gene expression profiles of *Zymoseptoria tritici* candidate genes at 7, 12, 14 and 28 days post infection (DPI). Solid red lines represent parent isolate 3D1, dashed blue lines represent parent isolate 3D7.

Supplementary Figures

Figure S1. Genes previously implicated in virulence for *Z. tritici*. Adapted from Orton et al, 2011.

Figure S2. Typical microscope images of *Z. tritici* pycnidiospores showing many long, overlapping spores (top left) and short, less dense spores (top right). Yellow lines represent spores counted and measured via a semi-automated ImageJ macro (bottom panels).

Figure S3. GFF file containing details of the re-annotated genes in the chromosome 7 QTL.

Figure S4. blast2go results for the chromosome 7 QTL.