Identification of genomic loci associated with crown rust resistance in perennial ryegrass (Lolium perenne L.) divergently selected populations

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Disciplines
Agricultural Science | Agronomy and Crop Sciences | Plant Breeding and Genetics

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Identification of genomic loci associated with crown rust resistance in perennial ryegrass (*Lolium perenne* L.) divergently selected populations

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Abstract

The inheritance of crown rust resistance in perennial ryegrass is complex with both major and minor quantitative trait loci (QTL) being detected on all seven linkage groups. However, QTL mapping populations have only few segregating alleles, limiting the transferability of results to other materials. In this study, a synthetic population was developed from four crown rust resistant and susceptible parents as starting material for a divergent selection experiment of crown rust resistance to be closer to practice in plant breeding programs, and to identify genome regions relevant across a broader range of genotypes. Following three cycles of directional selection, perennial ryegrass populations were produced with a two-fold difference in average rust resistance. Divergently selected populations were genotyped at 7 resistance gene analog-derived expressed sequence tag (RGA-derived EST) as well as 15 simple sequence repeat (SSR) loci. A test for selective neutrality (Waples test), which tests the hypothesis of genetic drift versus selection, identified significant differences in allele frequencies for 7 loci (32 %). The selection effect was bidirectional with the same loci showing significant response in both positively and negatively selected populations. A region under selection represented by markers LpSSR006 and EST13 on linkage group (LG) 4 was further confirmed by colocation with two separate QTL for crown rust resistance in a VrnA, a two-way pseudo-testcross mapping population. This suggests suitability of alleles identified for introgression into perennial ryegrass germplasm, where quantitative resistance to crown rust is desired.

Keywords: perennial ryegrass, crown rust resistance, single nucleotide polymorphism,
quantitative trait locus
Introduction

Perennial ryegrass (*Lolium perenne* L.) is widely grown in temperate climate zones in the world for feeding ruminant livestock and/or as amenity grass in sport courses and residential areas. Crown rust (*Puccinia coronata* Corda) is an important pathogen of ryegrass, which leads to significant reductions in green and dry matter yield and quality of forage-type perennial ryegrass [1]. Chemical control is often limited because the time for pesticide spraying is difficult to determine, the cost for forage production is increasing and pesticide residues pollute the environment. Therefore, breeding resistant ryegrass cultivars is the preferable strategy to control crown rust.

Germplasm has been screened for cultivars highly resistant to crown rust. Resistance to crown rust has been shown to be both qualitatively and quantitatively inherited [2, 3], indicating a complex genetic basis of crown rust resistance. Resistance to crown rust in perennial ryegrass involves both major and minor resistance genes [4]. Genetic analyses of crown rust resistance using molecular markers have been conducted in the past decade. Several major and minor QTL were detected for crown rust resistance in different mapping populations on all linkage groups (LGs) [5, 6, 7, 8, 9, 10]. However, high genetic heterogeneity determined by the obligate outbreeding in perennial ryegrass has hindered genetic analysis of the rust pathogen-host grass interaction and it remains unclear whether multiple minor genes mediate resistance to a single dominant pathogen race, or a few major genes for resistance to different pathogen races are present.

As compared to major crop species, such as rice (*Oryza sativa* L.) and maize (*Zea mays* L.), genomic information and tools for perennial ryegrass are limited so far. Thus, first
generation DNA markers such as random amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP) [11], amplified fragment length polymorphism (AFLP) [12], and simple sequence repeat (SSR) markers [13] have been used to construct genetic linkage maps and for QTL mapping in perennial ryegrass. Single nucleotide polymorphism (SNP) and diversity arrays technology (DArT) markers, DNA markers with potential for high-throughput genotyping, have only recently been employed for QTL mapping in ryegrass [14, 15, 16, 17, 18].

Most major resistance genes isolated so far, code for nucleotide binding site-leucine rich repeat (NBS-LRR) proteins, which mediate recognition of pathogens by host plants [19, 20]. NBS-LRR proteins contain conserved amino acid motifs [21]. In perennial ryegrass, resistance gene analogs (RGA) have been isolated using degenerated primers and further used to compare their locations in the genetic map with resistance genes of several plant species [22, 23, 24, 25]. SNP markers derived from candidate genes for pathogen defense response [26] and cleaved amplified polymorphic sequence (CAPS) markers derived from expressed disease resistance genes [23] have been assigned to linkage maps and evaluated with regard to their association with pathogen resistance.

Due to self-incompatibility, a genetic mechanism promoting cross pollination in ryegrass, bi-allelic mapping populations are difficult to develop but can be easily maintained by vegetative cloning. In most cases, segregating F₁ or F₂ (pseudotestcross F₂) populations derived from two heterozygous genotypes are used for genetic linkage map development and QTL analysis [9, 13]. The limitation of all QTL mapping populations is that they have only few segregating alleles, limiting the transferability of results to other materials. Therefore,
we decided to perform an experiment with four founder genotypes to be closer to the practice in plant breeding programs, and to identify genome regions relevant across a broader range of genotypes. In this study, a synthetic population was developed as starting material for a divergent selection experiment for crown rust resistance over three cycles of selection. Divergently selected populations were genotyped with RGA-derived SNPs as well as SSR markers. Our objectives were to compare the allele frequencies between (1) the initial synthetic population and the divergently selected populations, and (2) the resistant and susceptible selected populations. Finally, we evaluated, if any of the RGA-derived SNP markers are significantly associated with crown rust resistance.

**Materials and methods**

Plant materials

Four of the 20 perennial ryegrass genotypes (Lolium Test Set, LTS) studied in the EU project GRASP [27], namely, LTS01, LTS05, LTS09 and LTS18, were crossed in all possible pairwise combinations including reciprocal crosses during winter 2003/2004. Equal amounts of seed from each combination were sown to form the synthetic SYN1 population (n = 200), which was harvested in bulk (Fig. 1). The C0 population (n = 200) was subjected to divergent phenotypic selection for susceptibility to crown rust. A selection intensity of 15 % was used with the 30 most resistant plants in replicated trials taken to produce the resistant C1 subpopulation (C1R). All 30 plants were open pollinated in an isolation greenhouse during winter 2004/2005. Accordingly, the 30 most susceptible genotypes were selected to produce the susceptible C1 population (C1S) during winter 2004/2005. Following the same procedure
for selection as in the C₀ population, 200 C₁R and C₁S genotypes, respectively, were employed in crown rust infection experiments. Within both the C₁R and C₁S subpopulations, the 30 most resistant genotypes within C₁R and the 30 most susceptible within C₁S were identified for generation of the respective C₂ populations (C₂R and C₂S, respectively). Thirty random C₀ and 30 selected genotypes from each, C₂R and C₂S, were used for DNA extraction (Fig. 1). In order to synchronize flowering, all plants were treated at 6 °C with 8-hour light per day for 100 days for vernalization. Vernalized plants flowered within 1 week.

Crown rust infection and scoring

A leaf segment test (LST) was used for our crown rust infection experiments [8, 28]. Two weeks before leaf segments were harvested, plants were cut back in order to have leaves at the same developmental stage. A leaf segment of 2–3 cm from the middle part of the youngest fully developed leaf was laid with the abaxial side facing up on a 0.54 % water agar containing 3 p.p.m. benzimidazol (5.49 grams of agarose gel and 7.5 ml 4 % benzimidazol in 1000 ml) in a transparent rectangular tray of a size of 22 × 30 cm. For each generation, two infection experiments separated by 20 days were conducted, each with 3 replicates. In each experiment, the susceptible control cultivar Aurora was included with five leaf-fragments evenly distributed in each tray. The replicates were arranged in randomized complete block design. Urediniospores of crown rust (P. coronata f. sp. lolii) for artificial inoculation were collected in Les Alleuds (France) in August 2003. 20 μg of rust spores were dispersed to the leaf fragments in a closed box by air pressure. The first 24 hours after
infection, the leaf fragments were placed in the dark at 17°C. After 24 h, the trays were subsequently kept under constant light at 17°C in a Termaks KBP 6395 cooling incubator (Bergen, Norway). Fourteen days after inoculation, the leaf segments were scored on a 1–6 scale [8], where 1 = no symptoms, 2 = chlorotic/necrotic spots, 3 = small pustules and chlorotic/necrotic spots, 4 = medium size pustules and chlorotic/necrotic spots, 5 = large pustules and chlorotic/necrotic spots, and 6 = large pustules without chlorotic/necrotic spots.

DNA extraction

Young leaves from 30 C_0 plants, 30 C_2R and 30 C_2S plants were collected for DNA extraction followed by the CTAB method [29].

SNP genotyping

Eight expressed candidate disease resistance genes were selected from EST sequences generated within the Danish genome project DAFGRI (http://www.dafgri.dk), which included homologs of NBS-LRR, pathogenesis response (PR), mitogen activated protein kinase (MAPK), enhanced disease resistance (EDR), and plastid pyruvate kinase A (PKpA) protein coding genes. Details on primer design and SNP detection can be found in the study of Xing et al. [22]. The minimum number of SNP markers to discriminate all candidate resistance gene haplotypes was determined by PolyMin [30]. Ninety plants (30 plants in each C_0, C_2R and C_2S) were amplified for the eight genes with their corresponding primer pairs. SNP genotyping was performed using Sequenom MassARRAY (Maldi-TOF mass spectrometry) system at the Centre for Integrative Genomics (CIGENE, Aas, Norway).
SSR genotyping

Fifteen SSR markers distributed across all seven LGs were used to investigate allele frequencies in the 30 C₀ plants, 30 C₂R and 30 C₂S plants. Genotyping of SSR markers was done on a MegaBACE 1000 capillary sequencer preceded by PCR with fluorescent labeled primers as described earlier [13].

QTL mapping

Previously published QTL for resistance to crown rust in the VrnA mapping population [8] were recalculated including the markers shown in Table 5. The map positions of these markers were taken from a transcriptome-based genetic linkage map of the VrnA population [17] or inferred by a comparative genomics approach using the perennial ryegrass GenomeZipper [31]. QTL analysis was performed with MapQTL version 5.0 [32] using multiple QTL mapping (MQM). Automatic cofactor selection (backward elimination, P < 0.02) was used for the detection of significantly associated markers as cofactors. LOD significance threshold levels were determined using 1,000 permutations. Parental genotypes from the selection experiment and the VrnA mapping population were unrelated.

Data analysis

The mean of the scoring data was calculated for each population (C₀, C₁R, C₁S, C₂R, C₂S). The 30 plants showing extreme responses to crown rust infection in two directions were used to produce the next generations. Selection response (R) was calculated as R = Y - µ, where Y
was the averaged infection score value of progeny from selected parents, \( \mu \) was the averaged infection score value from the parental generation. Selection differential (D) calculated as \( D = y - \mu \), where \( y \) was the average infection score value of selected plants. Lattice analysis using the PLABSTAT software [33] was performed in order to estimate quantitative parameters, such as standard deviation, error and repeatability, summarised in Table 2.

SNP genotyping results were used to calculate haplotype frequencies in each of the four samples, LTS, C0, C2R and C2S. A test of selective neutrality (Waples test) was applied. It tests, whether the observed variation in allele frequency between two samples taken at different times can be explained as a sample drawn from a population of size \( N_e \) (effective population size) that has undergone \( t \) generations of genetic drift. The test statistic is distributed as a chi-square [34] and is calculated as \( \chi^2 = (x-y)^2/\text{var}(x-y) \), where \( x \) equals the estimated allele frequency in an initial sample, \( y \) equals the estimated allele frequency in a subsequent sample and \( \text{var}(x-y) \) equals the variance of this difference. The derivation of the variance in \( (x-y) \) is explained by Waples [34] in detail. We assumed that the effective population size is equal to the number of founder genotypes \( (N = 4) \) in a population of allogamous diploid genotypes, where migration was controlled by isolation and mutation could be ignored. Bonferroni correction was used to account for multiple testing.

**Results**

A synthetic population was generated from crown rust resistant and susceptible parents and submitted to two rounds of recombination and three rounds of selection on the basis of
resistance to crown rust (Fig. 1) in order to identify loci associated with resistance to crown rust in perennial ryegrass.

Effect of selection on response to crown rust

The control genotype ‘Aurora’ was more susceptible to crown rust (score = 5.7) across all infections, than the four founder genotypes. Genotype LTS05 had a score of 1.8 across experiments, and was thus the most crown rust resistant genotype among the four founder genotypes. LTS01 expressed intermediate resistance (score = 2.8). LTS09 and LTS18 were highly susceptible with scores above 5.0 (Table 1).

The distribution of scoring values in the C0 population was normal with a mean value of 3.7 (Fig. 2). As expected, the distribution in the C1 and C2 populations was skewed towards low (C1+, C2+) or high (C1-, C2-) scores. Both C1+ and C2- showed a steady increase towards higher mean scoring values, while C1+ and C2+ mean scoring values were of the same magnitude, and were significantly lower as compared to C0. Moreover, in the C2- population, the majority of plants showed extreme susceptibility to crown rust, a few plants were highly resistant. From C0 to C1, selection responses were -0.5 and 1.6 for positive and negative selection, respectively (Table 2). Both values were highly significant (p < 0.001). However, selection response of only 0.4 (p < 0.001) was observed for the negative selection from C1- to C2- and no selection response for respective positive selection. The highest selection differentials were obtained in C0- and C0+ with values of 2.2 and -1.9, respectively (Table 2).

Effect of selection on haplotype frequency
Genotyping with 17 SNPs and 15 SSRs was performed on a random sample of 30 genotypes (C₀), 30 selected genotypes in each of the two C₂ populations and four parental genotypes (Fig. 1). The number of SNPs for each of the ESTs was selected to represent all of the haplotypes present in the parental genotypes and ranged from 1 to 3 SNPs per EST. In total 25 SNP haplotypes and 64 SSR alleles were found in the studied material. Surprisingly, 4 SSR alleles (B1C9-219, LpSSR021-296, LpSSR021-312 and PR25-110) detected in C₀ were absent in the parental genotypes. These ‘novel’ alleles most likely indicate some mis-scoring of multiallelic SSRs. Alien pollen contamination could also be the cause of ‘novel’ allele appearance. However, those were detected at the C₀ stage only and no further contamination was found in the C₂ populations. Furthermore, novel alleles were detected for three loci only and higher rates would be expected in case of alien pollen contamination. In order to comply with Waples neutrality test model for genetic drift assumption of no migration we eliminated those three SSR loci (B1C9, LpSSR021 and PR25) from further analysis. Furthermore, 5 EST haplotypes (one haplotype in EST13 and two haplotypes in each EST24 and EST31) were absent in the C₀ population and subsequently were not transferred to either C₂⁺ or C₂⁻. This indicates an effect of genetic drift caused by the sample size in C₀, which was equal to the sizes of selected samples of 30 genotypes.

After two cycles of recombination and three cycles of selection, two alleles (2.7%) in C₂⁺ and eight alleles (11.0%) in C₂⁻ were eliminated from the populations. One of the eliminated alleles, allele DLF020-360, was lost in both C₂⁺ and C₂⁻. Most of the eliminated alleles had low (< 0.15) frequencies in C₀, however, two alleles of LpSSR058 of sizes 310 bp and 318 bp and with a cumulative initial frequency of 0.30 in C₀ were both completely
eliminated in C₂⁻, but not in C₂⁺. No fixed alleles were detected in our data set. The highest frequency of 0.96 reached allele LpSSR076-252 in C₂⁺ while the most frequent allele in C₂⁻ was EST28-hap1 with a frequency of 0.93.

Waples test [34] was applied to each locus to detect if any of the observed changes in allele (haplotype) frequency between C₀ and C₂ within populations were greater than those expected by drift alone. Loci were tested for statistically significant changes in allele frequency assuming an effective population size of Nₑ = N = 4, where N is a number of founder genotypes (N = 4). The Waples test indicated selection for seven loci of the total 22 in this study (32 %) (Table 5). As expected for a divergent selection scheme, all of the loci under selection showed response to both positive and negative selection. Out of the seven selection-responsive loci, four were ESTs and three SSRs, of which one was designated as EST-based SSR.

QTL analysis

Phenotypic data for resistance to crown rust from a previous study [8] were used to recalculate QTL in the VrnA mapping population with enhanced resolution. Our QTL analysis confirmed four out of six QTL on LGs 1, 4, and 5 with similar minor phenotypic variance (Vₚ) ranging from 8.4 to 15.5% (Table 6). Based on the data from the leaf segment tests, one QTL on LG5 and two closely located QTL on LG4 were detected, while no QTL was observed on LG1. Both QTL on LG1 and LG4 for individual field trials in 2004 and 2005, respectively, were confirmed in our analysis. QTL analysis on combined field data revealed three additional QTL on LGs 1, 3, and 4, with the one QTL on LG4 co-locating
with a QTL for the combined leaf segment data. Furthermore, this region represented by markers LpSSR006 and EST13 was identified as a region under selection in the Waples test.

Discussion

Various methods for the evaluation of resistance to crown rust, including both natural and artificial infection, are used in perennial ryegrass breeding programs. Leaf segment test based on field-collected inoculum was applied in this study to standardize infection scoring over different generations. High correlation among LST, glasshouse and field disease scoring was previously shown and confirmed LST as a suitable method for resistance evaluation both on phenotypic and on QTL level [35].

Following recombination and directional selection, perennial ryegrass populations with distinct crown rust resistance values were produced (Fig. 2). The change in average resistance clearly indicates the effect of selection, especially for the first round of selection from C0 to C1. However, high selection intensity in the first round and a limited number of resistance alleles in the parental genotypes (only one of the four LTS genotypes being resistant) have resulted in low selection response in the second round of selection. Nevertheless, two C2 populations were produced with a difference in average rust resistance score of almost two-fold.

Standard statistical tests (e.g. \( \chi^2 \), G tests) for assessing significant changes in allele frequencies neglect the effects of random genetic drift and are, therefore, not appropriate for the analysis of changes in allele frequencies in recurrently selected populations of a finite size. In contrast, Waples test takes into account the increased variance in allele frequencies
between generations caused by random genetic drift and distinguishes it from the effects of selection, migration, and mutation. As migration was controlled by isolation of the populations and mutation rate could be ignored in the current experimental design, a significant Waples test statistic would imply that selection for crown rust resistance was a driving force for a change in allele frequencies as opposed to genetic drift.

Significant differences in allele frequencies were found for seven loci. Waples test revealed that selection was bidirectional with the same loci showing significant response in both positively and negatively selected populations. Unfortunately, the test is not capable to identify causal alleles, as the loci under selection were multiallelic (3 to 5 alleles) and had a complex interaction between allele frequency shifts. Nevertheless, several alleles were identified as putatively causal resistance alleles being enriched after positive but not after negative selection. For example EST13-Hap2 frequency in C2+ has increased nine-fold compared to C0R while it stayed at the same level in C2− (Table 4). Similarly, LpSSR006-257 allele was enriched in C2+ but completely eliminated from C2−. Both these alleles were introduced from resistant or partially resistant parent genotypes, LpSSR006-257 coming from LTS05 and EST13-Hap2 transmitted from both LTS05 and LTS01. This is a feature of the recurrent selection process which is aimed at the enrichment of rare alleles and was observed earlier in a complex maize population selected for quantitative disease resistance for four cycles [36] as well as in a similar selection scheme for axillary tiller development in perennial ryegrass [37, 38].

The inheritance of crown rust resistance in perennial ryegrass is complex with both major and minor QTL being detected on all seven LGs (reviewed in [39]). Most of the
studies so far have used field collected inoculum and anonymous marker systems in order to detect QTL, however, direct comparison of QTL was hindered by the lack of common markers. A previous study based on both artificial (detached leaf assay) and natural infection of the VrnA population [8] identified six QTL on LGs 1, 4, and 5, accounting for 6.8 to 16.4% of phenotypic variance. The VrnA mapping population at that time was mapped with 33 SSR and 59 AFLP markers. As the resolution of the VrnA map has increased substantially with more than 700 markers being mapped [17] we have decided to reanalyze the QTL data in order to verify the markers under selection in our present study. QTL analysis confirmed four out of six QTL on LGs 1, 4, and 5 with similar minor phenotypic variance of Vp ranging from 8.4 to 15.5% but with substantially higher resolution, especially on LG1 and LG4. Waples test identified a region under selection on LG4 represented by markers LpSSR006 and EST13 which showed colocation with two separate QTL detected in the same region on LG4 in VrnA. The role of LG4 as well as LG1 for crown rust resistance was noted earlier [8] as QTL for both the leaf segment test and the field experiments were identified on these LGs. Four major QTL for crown rust resistance were identified on LG1 and LG2 [5, 6].

With this complex QTL distribution, recurrent selection is the most appropriate breeding method for improving perennial ryegrass resistance to crown rust. We were able to identify loci by Waples test and confirm at least partially their role in disease resistance in a separate QTL analysis. The trait-locus association on LG4 was detected in a different genetic background from the population that underwent selection. This suggests that the alleles identified would be stable under a range of conditions and suitable for introgression into
perennial ryegrass germplasm where quantitative resistance to crown rust is desired.

Acknowledgement

This study was funded by the EU framework V project GRASP (QLRT-2001-00862) and partly supported by the Research Council of Lithuania (grant No. MIP-032/2012 FUMAG)

References


Table 1. Response to crown rust in four ryegrass founder genotypes and control (Aurora)

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Type</th>
<th>Score level</th>
<th>Rust response</th>
</tr>
</thead>
<tbody>
<tr>
<td>LTS01</td>
<td>G00612</td>
<td>Forage</td>
<td>2.8 ± 0.83</td>
<td>Partially resistant</td>
</tr>
<tr>
<td>LTS05</td>
<td>DLF5</td>
<td>Turf</td>
<td>1.8 ± 0.75</td>
<td>Resistant</td>
</tr>
<tr>
<td>LTS09</td>
<td>RASP17-03</td>
<td>Forage</td>
<td>5.3 ± 0.94</td>
<td>Susceptible</td>
</tr>
<tr>
<td>LTS18</td>
<td>WSC 23/9</td>
<td>Forage</td>
<td>5.0 ± 0.71</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Control</td>
<td>Aurora</td>
<td>Forage</td>
<td>5.7 ± 0.23</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

Table 2. Key values of the phenotypic characterization of resistance to crown rust based on 200 perennial ryegrass genotypes in C0 and each of the selected populations

<table>
<thead>
<tr>
<th>Population</th>
<th>Mean ± SDa</th>
<th>Range</th>
<th>SEb</th>
<th>Rc</th>
</tr>
</thead>
<tbody>
<tr>
<td>C0</td>
<td>3.3 ± 1.38</td>
<td>1.0 – 6.0</td>
<td>0.53</td>
<td>65.5</td>
</tr>
<tr>
<td>C1-</td>
<td>4.9 ± 0.87</td>
<td>2.0 – 6.0</td>
<td>0.40</td>
<td>59.7</td>
</tr>
<tr>
<td>C1+</td>
<td>2.8 ± 1.29</td>
<td>1.0 – 6.0</td>
<td>0.36</td>
<td>81.1</td>
</tr>
<tr>
<td>C2-</td>
<td>5.3 ± 0.86</td>
<td>1.5 – 6.0</td>
<td>0.28</td>
<td>74.6</td>
</tr>
<tr>
<td>C2+</td>
<td>2.8 ± 1.59</td>
<td>1.0 – 6.0</td>
<td>0.37</td>
<td>87.8</td>
</tr>
</tbody>
</table>

a standard deviation
b standard error
c repeatability

Table 3. Selection efficiency across three generations

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C0+</th>
<th>C0-</th>
<th>C1+</th>
<th>C1-</th>
<th>C2+</th>
<th>C2-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Selection response</td>
<td>-0.5***</td>
<td>1.6***</td>
<td>0ns</td>
<td>0.4***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selection differential</td>
<td>-1.9</td>
<td>2.2</td>
<td>-1.5</td>
<td>0.5</td>
<td>-1.5</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*** P < 0.001
ns not significant

Table 4. Haplotype and allele frequencies for ESTs and SSRs, respectively, in parental genotypes (LTS), C0+, C2+ and C2-.
<table>
<thead>
<tr>
<th>Locus</th>
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<td>6 (53.5)</td>
<td>4</td>
<td>30</td>
<td>29</td>
<td>28</td>
<td>84.34***</td>
<td>97.61***</td>
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<tr>
<td>B1A8</td>
<td>6 (67.6)</td>
<td>3</td>
<td>29</td>
<td>29</td>
<td>25</td>
<td>8.36</td>
<td>10.51</td>
<td>2</td>
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<tr>
<td>EST24</td>
<td>7 (38.6)</td>
<td>4</td>
<td>29</td>
<td>30</td>
<td>30</td>
<td>5.28</td>
<td>5.93</td>
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<tr>
<td>DLF020</td>
<td>7 (61.0)</td>
<td>4</td>
<td>30</td>
<td>30</td>
<td>29</td>
<td>6.42</td>
<td>2.92</td>
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<tr>
<td>EST40</td>
<td>7 (63.4)^a</td>
<td>3</td>
<td>26</td>
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<td>25.73***</td>
<td>25.94***</td>
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<td>7 (110.0)</td>
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<td>28</td>
<td>28</td>
<td>30</td>
<td>0.14</td>
<td>3.68</td>
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*P < 0.05, **P < 0.01, ***P < 0.001

^aMap position identified using the perennial ryegrass GenomeZipper [31].
<table>
<thead>
<tr>
<th>Trait</th>
<th>LG</th>
<th>QTL position, cM</th>
<th>Two LOD support interval (cM)</th>
<th>LOD score</th>
<th>% expl Vp</th>
<th>Closest marker (distance to the QTL maximum in cM)</th>
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<tbody>
<tr>
<td>L1-3</td>
<td>4</td>
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<td>50.5-56.5</td>
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<td>60.7</td>
<td>59.9-60.7</td>
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<td>9.9</td>
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<td>11.0-19.5</td>
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<tr>
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<td>33.0-34.8</td>
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<td>58.3-59.9</td>
<td>4.7</td>
<td>10.8</td>
<td>P16M47_162 (0.0)</td>
</tr>
</tbody>
</table>

L1-3, combined leaf segment tests; F04-F05, individual field trials; F04+05, combined field trials.
Figure 1. Generation of synthetic perennial ryegrass populations undergoing divergent selection for the crown rust resistance and susceptibility (PC pairwise crosses, SYN1 synthetic population 1, C0 starting population (n=200), C1 generation 1 (n=200), C2 generation 2 (n=200), S selection (n=30), R random (n=30), plus sign low infection, negative sign high infection.

Figure 2. Rust infection scores in starting population C0 and populations after one (C1) or two (C2) cycles of divergent selection for the crown rust resistance and susceptibility in perennial ryegrass plus sign low infection, negative sign high infection.