

# Pathotype-specific QTL for stem rust resistance in *Lolium perenne*

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Received: 25 September 2012 / Accepted: 13 January 2013 / Published online: 30 January 2013  
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**Abstract** A genetic map populated with RAD and SSR markers was created from F1 progeny of a stem rust-susceptible and stem rust-resistant parent of perennial ryegrass (*Lolium perenne*). The map supplements a previous map of this population by having markers in common with several other *Lolium* spp. maps including EST-SSR anchor markers from a consensus map published by other researchers. A QTL analysis was conducted with disease severity and infection type data obtained by controlled inoculation of the population with each of two previously characterized pathotypes of *Puccinia graminis* subsp. *graminicola* that differ in virulence to different host plant genotypes in the F1 population. Each pathotype activated a specific QTL on one linkage group (LG): qLpPg1 on LG7 for pathotype 101, or qLpPg2 on LG1 for pathotype 106. Both pathotypes also activated a third QTL in common, qLpPg3 on LG6. Anchor markers, present on a consensus map, were located in proximity to each of the three QTL. These QTL had been detected also in previous experiments in which a genetically heterogeneous inoculum of the stem rust pathogen activated all three QTL together. The results of this and a previous study are consistent with the involvement of the pathotype-specific QTL in pathogen recognition and the pathotype-nonspecific QTL in a generalized resistance response. By aligning the markers common to other published reports, it appears that two and possibly all three of the stem rust QTL

reported here are in the same general genomic regions containing some of the *L. perenne* QTL reported to be activated in response to the crown rust pathogen (*P. coronata*).

## Introduction

An important goal for the genetic improvement of perennial ryegrass (*Lolium perenne*), a major forage and turf grass, is host resistance to rust diseases caused by species of the fungus *Puccinia*. Crown rust (caused by *P. coronata* f. sp. *lolii*) and stem rust (*P. graminis* subsp. *graminicola*) are the important rust diseases of this grass (Kimbeng 1999; Pfender 2009a; Schubiger et al. 2010). Most of the literatures on rust resistance in *Lolium* spp. concerns crown rust (Dracatos et al. 2010); stem rust has received less attention, though it is the more important disease in seed production fields in some of the world's major ryegrass seed production regions (Hampton 1986; Pfender 2009a). From the dearth of published research on genetics of stem rust resistance in *Lolium*, it follows that there is no information on relative genomic location for resistance determinants to the crown and stem rust pathogens of this host.

*Lolium perenne* is a highly heterozygous and heterogeneous crop plant, due to its genetic system for obligate outcrossing (Cornish et al. 1979). Marker-assisted selection could be a useful tool for genetic improvement in such species (Forster et al. 2004; Pauly et al. 2012; Wilkins and Humphreys 2003), and the mapping of quantitative trait loci (QTL) for traits of interest (such as rust resistance) is an important first step. Genetic linkage maps for *Lolium* spp. have been published for various mapping populations, and a succession of marker types has been used: AFLP and RFLP markers (Bert et al. 1999) combined with and eventually superseded by simple sequence repeat (SSR)

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Communicated by E. Guiderdoni.

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markers (Kubik et al. 2001; Jones et al. 2002; Faville et al. 2004; Warnke et al. 2004; Gill et al. 2006; Thorogood et al. 2002; Dumsday et al. 2003; Muyllé et al. 2005; Anhalt et al. 2008) some of which are derived from single-nucleotide polymorphisms (SNPs) in expressed sequence tags (EST) (Cogan et al. 2006), including candidate resistance and defense response (DR) genes (Dracatos et al. 2008a). More recently, some *Lolium* maps have been constructed with high-throughput markers such as restriction site-associated DNA (RAD) or diverse array tag (DArT) markers (Pfender et al. 2011; Tomaszewski et al. 2012). Although some of the mapping studies were conducted with common populations, most were done with different populations constructed by various research groups and for particular traits of interest. Therefore, it is often difficult to compare QTL locations across studies, because there may be few or no markers in common among the maps. To address this problem, consensus maps with markers common to more than one population have been created (Armstead et al. 2002; Jensen et al. 2005). The most recent and widely applicable of these, created by Studer et al. (2010), is based on eight previously reported mapping populations and uses EST-SSR markers common across several of the populations to anchor the map. In addition to advances possible through creating consensus maps within *Lolium*, synteny with the better-studied cereal grasses can be a source of genomic information; several mapping studies with markers common to *Lolium* and cereals have documented synteny (Armstead et al. 2004; Jones et al. 2002; Devos 2005; Dracatos et al. 2009a).

Most of the published researches on QTL for rust diseases in *Lolium* concern crown rust. Dracatos et al. (2010) provide a summary of crown rust resistance QTL described by more than ten research groups, and note that these QTL have been found on all seven *Lolium* linkage groups (LG), most commonly on LG2, LG7, LG1 and LG5. The diversity of QTL for crown rust resistance is likely due to a combination of diversity in host resistance genes and diversity in pathogenicity-related genotypes among pathogen populations. Variability in and among *P. coronata* f. sp. *lolii* populations for virulence to different plant populations has been noted in several studies from different regions around the world (Wilkins 1978; Potter et al. 1990; Aldaoud et al. 2004; Dracatos et al. 2008b). Genetic diversity (not specifically for pathogenicity) within and between natural populations of the pathogen has been documented using molecular genetic markers (Dracatos et al. 2009b). These observations indicate with near-certainty that there are pathotypes of the crown rust pathogen, i.e., genotypes that differ in their pathogenicity and/or virulence across different host plant genotypes. Nonetheless, the published research on QTL and resistance mechanisms for crown rust in *Lolium* has been done with

genetically heterogeneous inoculum (Dracatos et al. 2010), and, therefore, specificity of *Lolium* QTL to crown rust pathotypes has not been investigated.

*Lolium perenne* challenged with a field-collected, genetically heterogeneous inoculum of the stem rust pathogen (*P. graminis* subsp. *graminicola*) displayed QTL consistently on LG1, LG6 and LG7 (Pfender et al. 2011). The *L. perenne* F1 mapping population in that study was derived from a two-way pseudo test cross between susceptible and resistant parents chosen after inoculation with a similar heterogeneous inoculum. The QTL on LG7 was the most prominent, having LOD scores of 11–20 and explaining 26–37 % of phenotypic variance. As noted previously (Pfender et al. 2011), most plants carrying the resistance-associated allele at the marker nearest to the LG7 QTL did not display qualitative (complete) resistance in these experiments. This response could be explained as qualitative resistance to some (but not all) pathogen genotypes in the inoculum, or alternatively as quantitative (partial) resistance of varying degree to all pathogen genotypes. The other two QTL (on LG1 and LG6) each explained 6–12 % of the variance. Another previous study, with a mapping population not specifically derived with respect to stem rust reaction, detected a weak QTL for stem rust response on LG4 when challenged with a heterogeneous inoculum (Jo et al. 2008). Pathotypes for stem rust on *L. perenne* have been definitively demonstrated (Pfender 2009b) with single-pustule isolates derived from a heterogeneous inoculum similar in origin to the field-collected inoculum of the other cited studies.

The objectives of this study were to determine the pathotype specificity, if any, for QTL in stem rust of *L. perenne*, and to locate the QTL in relation to markers common to consensus or other maps of *Lolium*.

## Materials and methods

### Plant materials

This research was conducted with a previously described (Pfender et al. 2011) mapping population of perennial ryegrass (*L. perenne*) cvar Kingston. 193 F1 individuals were derived from a cross between plants susceptible (S20, female) or resistant (R2, male) as determined by inoculation with a genetically mixed, field-collected inoculum of *P. graminis* subsp. *graminicola* (Pfender 2009b). Plants were grown from seed in a greenhouse as previously described (Pfender et al. 2011) and vegetatively propagated to produce multiple ramets of each individual.

Genomic DNA was extracted from lyophilized leaf tissue using a modified rose buffer protocol (Steiner et al. 1995). The DNA samples were quantified and diluted to 20 ng/ul.

## Marker development

To facilitate the alignment of our stem rust resistance QTL with existing genetic maps of *Lolium*, we sought to add markers from other maps to our previously published (Pfender et al. 2011) linkage map. EST-SSR primers were selected from recent publications of consensus anchor markers for *L. perenne* (Studer et al. 2008, 2010), and we produced genotype scores for these markers by means of high-resolution melting (HRM) curve analysis as described by Studer et al. (2009).

PCR reactions were performed in a total volume of 11  $\mu$ L containing 20 ng DNA, 1  $\times$  PCR buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTP mix, 0.25  $\mu$ M each forward and reverse primer, 0.005 % DMSO, 0.175U BIOLASE™Taq polymerase (Bioline, USA) and 1  $\times$  LC Green<sup>R</sup> PLUS (Idaho Technology, Inc UT, USA). Oil (20  $\mu$ L) was placed over each reaction mixture to prevent evaporation during HRM scanning (Sathuvalli 2011). PCR amplification was conducted in a Dyad Peltier Thermal Cycler (BIO-RAD Inc., Hercules, CA, USA) using the following profile: 95 °C for 2 min followed by 45 cycles of 94 °C for 30 s, 56–60 °C (depending on the Ta for the primer pair) for 30 s and 72 °C for 30 s, then a final 5-min extension at 72 °C followed by a 30-s incubation at 95 °C to intercalate the fluorescent dsDNA binding dye into the double-stranded DNA amplicon. Once amplification was complete, melt analysis was performed using a 96-well format LightScanner<sup>R</sup> (Idaho Technology, Inc UT, USA) apparatus. Continuous fluorescence signal was collected between 57 and 98 °C at a rate of 0.1 °C/s. Results were analyzed using the LightScanner and Call-IT<sup>R</sup> software Version 2.0 (Idaho Technology, Inc, UT, USA). Normalized fluorescence was graphed as a function of temperature to produce melting curves. The melting curves, one for each individual plant, were examined to determine the allele configuration of the marker and to classify each individual's marker genotype. For most markers, the PCR product (9  $\mu$ L) also was run on 2 % agarose sodium borate gels (Broody and Kern 2004) at 150 V for 2 h to help discern the marker allele configuration. In some cases, amplicon lengths were estimated from gels.

## Linkage maps

Linkage maps were constructed using JoinMap4 software (Van Ooijen 2006) with CP (cross-pollinated) population type codes as described (Pfender et al. 2011), except using the settings for linkages with a recombination frequency smaller than 0.40, LOD larger than 1.00, and goodness-of-fit 5.0. The Kosambi mapping function was used to translate the recombination frequencies into map distances.

Separate female and male maps were built using the SSR/STS markers utilized for the study reported in Pfender

et al. (2011), RAD-tag SNP markers in testcross configuration (Pfender et al. 2011), and recently described EST-SSR markers (Studer et al. 2010) scored using HRM curve analysis (Mader et al. 2008; Studer et al. 2009). Candidate EST-SSRs for HRM analysis were selected based on distribution across the *Lolium* genome. Previously mapped RAD-tag SNP markers in intercross configuration were omitted to minimize the missing data. JoinMap segregation codes were assigned to the EST-SSR markers based on the pattern of HRM melting curves produced from 193 F1 individuals in comparison to the patterns from parental (heterogeneously heterozygous and homozygous) DNAs. Markers that produced two patterns in the F1s identical to the parental patterns (i.e., locus heterozygous in one parent, two alleles), were scored as nn  $\times$  np or lm  $\times$  ll; markers that produced four patterns in the F1s of which two were identical to the parents and two were unique (i.e., locus heterozygous in both parents, three alleles) were scored as ef  $\times$  eg; markers that produced four patterns in the F1s of which none was the same as parental patterns (i.e., locus heterozygous in both parents, four alleles) were scored as ab  $\times$  cd. As DNAs from grandparents of this population were not available, HRM patterns that differed from parental patterns could not immediately be assigned genotype codes (e.g., genotypes ee, fg, ac, ad, bc, and bd). For these cases, genotyping was assisted by first considering the published positions of the EST-SSR markers in the consensus linkage map for ryegrass (Studer et al. 2010) followed by finding the position/genotyping codes that produced a minimum number of cross-overs in S20  $\times$  R2 LG. This process was aided by working with graphical genotypes that revealed recombination breakpoints in the S20  $\times$  R2 F1 chromosomes. In one case, empirical genotyping was confirmed by fragment analysis of FAM-labeled PCR products produced from each parental DNA and two representatives of each of the four HRM pattern classes (G01-027, an ab  $\times$  cd marker). Three markers (G06-049, G02-047, G02-058) produced length-variant alleles that were scored on agarose gels. In addition to the 28 EST-SSR markers, a RAD marker (LpRa060) in the previous map (Pfender et al. 2011) was re-analyzed by HRMC to be scored as ef  $\times$  eg due to detection of three alleles using this technique. For the re-analysis, PCR primers for a 151 nucleotide amplicon were developed from 223 nucleotide of paired-end sequence linked to the LpRa060 locus.

## Rust reaction phenotype determination

Whereas we previously conducted a QTL analysis of plants challenged with a genetically mixed, field-collected population of *P. graminis* subsp. *graminicola* (Pfender et al. 2011), the inocula for the current study were genetically

homogenized through single-pustule isolation. Two such single-pustule isolates, previously characterized as having different pathotype specificities (Pfender 2009b), were used. Pathotype 101 (represented in this study by isolate 101) characteristically produces either a high-severity (susceptible) or low-severity (resistant) reaction on various *L. perenne* individuals exposed to a uniform inoculum level, whereas pathotype 106 (isolate 106 in this study) characteristically produces a continuous range of severities (low to high) on various *L. perenne* individuals. Urediniospores of these isolates were stored, prepared and applied as inoculum according to the previously described methods (Pfender 2009b).

Four experiments were conducted, several months apart from one another, two experiments for each pathotype. Inoculations were done under controlled conditions and plants were kept in a glasshouse during the experiment. In each experiment, 193 F1 individuals, three individually potted ramets (replicates) per individual, were inoculated with urediniospores of the pathogen suspended in oil. Incubation conditions for infection during the 24 h after inoculation were followed as previously described (Pfender et al. 2011), except that the following temperatures (°C) were recorded for night maximum, night minimum, morning (light period) maximum: Expt I 16, 12, 22; Expt II 21, 12, 22; Expt III 19, 12, 26; Expt IV 21, 14, 24. Each experiment was conducted as a randomized complete block design and plants were maintained in a glasshouse as previously described (Pfender et al. 2011). At the end of the incubation period [1.8–2.0 latent period (Pfender 2001), approximately 20 days], each ramet was examined to determine the number of eruptent pustules. The number of pustules per replicate plant was transformed to its square root to normalize the data. An additional phenotype score was assigned for the character of the pustule (termed “infection type” in rust pathology literature). Infection type was scored on a 0–4 scale, where zero represents the absence of symptoms and four represents large, abundantly sporulating lesions with no surrounding chlorosis or necrosis in the host tissue (Roelfs et al. 1992).

Heritability of rust severity in each experiment and across the two experiments for each pathotype was calculated from variance components obtained by analysis of variance for the square-root-transformed pustule number data. For each experiment, the individual values in each replicate block were adjusted by a constant factor for the overall mean block effect before calculating variances (Calenge et al. 2004). Heritability in each experiment was computed as variance among genotypes divided by the total of genotype plus residual variances. The heritability estimate for the combined two experiments with each pathotype was computed as the genotype variance divided by the sum of variances for (genotype + genotype-by-experiment + residual).

## QTL analysis

MapQTL5 (Van Ooijen 2004) was used to conduct the QTL analysis. Rust severity trait data consisted of the square-root-transformed pustule number, averaged across three replicates, for each individual. An additional data set for each experiment consisted of the infection type scores, using the predominant infection type for each plant. Male and female maps were as described in previous paragraphs. LOD thresholds for testing significance of QTL peaks were obtained using 1,000 permutations for each of the trait data sets and a genome-wide significance level of 5 %. The interval-mapping (IM) procedure of MapQTL was used to identify major QTL peaks. Automatic cofactor selection (ACS) and/or inspection of IM and Kruskal–Wallis scores were used to select a cofactor for the major peak in the genome, and additional cofactor(s) for other peak(s) were then selected after inspection of the multiple QTL-mapping routine (MQM) results. In some cases, the use of a cofactor in addition to the one for the highest peak in the genome did not effectively improve the definition of the lesser peaks; in those cases, no additional cofactor was used. No attempt was made to define more than one cofactor per LG. QTL peaks that reached the genome-wide LOD significance level, and their 2-LOD support intervals, were determined from the MQM results tables.

## Results

### Genetic mapping

Seventy-six EST-SSR markers from the consensus map of Studer et al. (2010) were screened. Of these, we successfully amplified 44, and 30 were polymorphic in our mapping population as scored by our HRM procedures. Twenty-eight of these were mapped, whereas two scorable markers (G01-047 and G04-056) could not be assigned a map location. Our revised genetic map of *L. perenne* supplements a previous map (Pfender et al. 2011) by adding the 28 SSR markers, all of which have been mapped on other published maps for this species (Table 1). Most of these additional markers are on LG1, LG6 or LG7, but all LG have at least one of these markers. Sixteen of the markers are bi-allelic (eleven in male testcross configuration, four in female testcross configuration, one heterozygous in both parents), seven markers have three alleles (ef × eg) and five markers have four alleles (ab × cd). In addition, a RAD marker (LpRa060) in the previous map (Pfender et al. 2011) was re-analyzed by HRM to be scored as ef × eg. Twenty-five of the markers were placed on the male map and 17 were placed on the female map. The revised maps are 621 and 629 cM in length for the S20 (female, rust

susceptible) and R2 (male, rust-resistant) parents, respectively (Fig. 1). The female map has 182 markers and an average inter-marker distance of 3.4 cM; the male map has 173 markers and average inter-marker distance of 3.6 cM. Largest gaps are 17.1 cM for the female (LG2) and 20.6 cM for the male (LG7). Consistent with our previous work (Pfender et al. 2011), EST-SSR markers added to LG2 exhibited strong segregation distortion in the revised map. With the inclusion of the new markers, the revised map can be compared with several other published maps of *Lolium*. Map order of these common markers was generally well conserved relative to the published consensus map (Studer et al. 2010), except for a telomeric marker (G01-040) that mapped to the opposite end of LG2 in the S20 × R2 female and male maps as compared to the consensus map. We inverted our previous orientation of LG7 to conform with the consensus map (Studer et al. 2010).

### Rust phenotyping

The distribution of disease phenotypes (square root of the average number of pustules per plant) in the mapping population exposed to pathotype 101 was markedly skewed (Fig. 2a), with a high proportion of plants having no disease. If individuals with <1 pustule per plant (averaged across three ramets) were counted as resistant, the population segregated approximately 1:1 for resistance and susceptibility. Average disease phenotype of the susceptible (female, S20) parent was 9.2 and 5.6 in experiments I and II, respectively; the resistant (male, R2) parent showed zero infection in both experiments. Heritability of disease response to pathotype 101 was high within each experiment (0.89), and repeatable across experiments (0.81) (Table 2).

Distribution of disease phenotypes in response to pathotype 106 (Fig. 2b) was more even than that for pathotype 101. Few plants were completely resistant to infection (i.e., <1 pustule per plant). The male parent, which had been selected as moderately resistant in response to a field-collected, mixed inoculum of the pathogen (Pfender 2009b; Pfender et al. 2011), had average disease phenotypes of 2.7 and 3.8 when inoculated with pathotype 106 in experiments III and IV, respectively. The female parent had disease phenotypes of 7.6 and 2.8, respectively. Heritability of disease response to pathotype 106 was 0.77 in each of the experiments, and 0.57 when calculated across the two different experiments (Table 2). There was no significant correlation ( $R^2 = 0.01$ ) between an individual plant's responses to pathotype 106 and pathotype 101 (Fig. 2c), i.e., plants resistant to pathotype 101 were equally likely to have low or high susceptibility to infection by pathotype 106. This lack of correlation between severities caused by

isolates 101 and 106 was also observed within the subset of plants susceptible to pathotype 101.

### QTL analysis

Three major QTL were detected in these experiments (Table 3). One QTL (on LG6) was associated with resistance to both stem rust pathotypes, and the other two were each associated with only one of the pathotypes (on LG1 for pathotype 106, on LG7 for pathotype 101).

The QTL on LG6, designated qLpPg3, was observed in a previous study (Pfender et al. 2011), but its significance in that analysis was somewhat diminished by the fact that the markers were lacking to extend LG6 of that previous map to what now appears to be the peak of the QTL. In the map presented in the current report, qLpPg3 was detected as a significant peak (LOD scores 4.7–8.4) on the female map in both experiments with both pathotypes. On the male map, this QTL was significant in both experiments with pathotype 106, and in experiment I with pathotype 101; it was a distinct peak on the experiment II pathotype 101 male map, but at LOD 4.7 (LOD significance threshold 6.1). QTL qLpPg3 explains 7–10 % of the phenotypic variance in response to pathotype 101, and 9–11 % in response to pathotype 106. This QTL is located between 60 and 68 cM on the female map (Table 3; Fig. 1), with a 2-LOD interval of 55–68 cM, across the four experiments. On the male map, it is located between 59 and 63 cM (2-LOD interval 53–63 M). In both maps the peak of qLpPg3 is located between markers G01-002 and LP20. Examination of linkage relations between the fully informative (4-allele) markers, G01-002 or G01-027, and the markers closest to the QTL peak (LMgSSR03-04F, LP20) shows that the alleles linked with resistance originate on one chromosome of the male parent in this cross. The markers closest to qLpPg3 have two or three alleles (nn × np for LMgSSR03-04F, ef × eg for LP20). Due to their coupling/repulsion relationships these markers are less informative than G01-002 or G01-027 in determining the parental source of the resistance.

Resistance response to pathotype 101 is associated also with a QTL on LG7, designated qLpPg1 in the previous study that used genetically mixed inoculum. In the current study qLpPg1 has LOD scores of 31–32 on male and female maps in both experiments with pathotype 101 (Table 3). It occurs at 45 cM (2-LOD interval 42–47 cM) on the female map, and at 49 cM (2-LOD interval 46–52 cM), on the male map. On both maps, qLpPg1 is located in a 7-cM interval between markers G02-048 and NFFS275. These two markers, as well as NFFA103, were scored as ef × eg; resistance is associated with the “g” allele that is in cis configuration among the three markers. It has a large phenotypic effect, explaining 50–58 % of the

phenotypic variance in response to pathotype 101. In this mapping population, progeny of a susceptible  $\times$  resistant cross, plant reaction to inoculation with isolate 101 segregates approximately 1:1 for resistance (if defined as average of  $<1$  pustule per plant) and susceptibility. Scored as a binary trait in this way, this phenotypic trait mapped to LG 7 at 49.2 cM on the male map (Fig. 1), less than 1 cM from the peak of qLpPg1 and midway between genotypic markers G02-048 and NFFS275.

Resistance response to pathotype 106 is associated with a QTL (designated qLpPg2) on LG1. It is located between 23 and 26 cM (2-LOD interval 22–28 cM) on the female

**Fig. 1** Linkage maps of parents (S20 rust-susceptible female, R2 rust-resistant male) of *Lolium perenne* F1 population used to detect QTL activated by inoculation with pathotypes of the stem rust pathogen, *Puccinia graminis* subsp. *graminicola*. QTL (2-LOD interval) are indicated by shaded sections of chromosomes. Two QTL, qLpPg1 (LG7) and qLpPg3 (LG6), were detected when plants were inoculated with pathotype 101. The QTL qLpPg2 (LG1) and qLpPg3 (LG6) were detected when plants were inoculated with pathotype 106. See Table 3 for QTL peak location and other QTL data. The star, within qLpPg1 on male LG7, indicates map location of binary phenotype (resistant vs. susceptible) for plants inoculated with pathotype 101. Markers in bold font were selected from those used on other *Lolium* maps as shown in Table 1, and were added to our previously published (Pfender et al. 2011) map for this population

**Table 1** Genetic markers genotyped by high-resolution melting curve analysis and added to previous (Pfender et al. 2011) map of *Lolium perenne*

Marker <sup>a</sup> name	LG	Genotype configuration	Original source <sup>b</sup>	Other maps <sup>b</sup> with this marker
G01-031	1	ef $\times$ eg	Studer et al. (2008)	Studer et al. (2008)
G02-047	1	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)
G02-058	1	lm $\times$ ll	Studer et al. (2008)	Studer et al. (2008)
G06-049	1	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)
LpRa060	1	ef $\times$ eg	Pfender et al. (2011)	Pfender et al. (2011)
LpSSR085	1	lm $\times$ ll	Jensen et al. (2005)	Jensen et al. (2005), VanDaele et al. (2008), Studer et al. (2008)
PR37	1	ab $\times$ cd	Kubik et al. (2001)	Jensen et al. (2005), Studer et al. (2008), Pauly et al. (2012)
G01-040	2	ef $\times$ eg	Studer et al. (2008)	Studer et al. (2008)
G02-025	2	ab $\times$ cd	Studer et al. (2008)	Studer et al. (2008), Pauly et al. (2012)
G03-054	2	ef $\times$ eg	Studer et al. (2008)	Studer et al. (2008)
G04-054	3	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)
G05-088	3	ef $\times$ eg	Studer et al. (2008)	Studer et al. (2008)
G01-037	4	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)
G06-078	4	lm $\times$ ll	Studer et al. (2008)	Studer et al. (2008)
G05-094	5	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)
B1-A8	6	im $\times$ ll	Lauvergeat et al. (2005)	Studer et al. (2008)
G01-002	6	ab $\times$ cd	Studer et al. (2008)	Studer et al. (2008)
G01-027	6	ab $\times$ cd	Studer et al. (2008)	Studer et al. (2008)
G03-015	6	ef $\times$ eg	Studer et al. (2008)	Studer et al. (2008)
LmgSSR03-04F	6	nn $\times$ np	Hirata et al. (2006)	Studer et al. (2008)
LP20	6	ef $\times$ eg	Kubik et al. (2001)	Studer et al. (2008), Pauly et al. (2012)
LpSSR058	6	nn $\times$ np	Jensen et al. (2005)	Studer et al. (2008)
xpps0210 <sup>c</sup>	6	nn $\times$ np	Faville et al. (2004)	Faville et al. (2004), Dracatos et al. (2009a), Pauly et al. (2012)
xpps0299 <sup>c</sup>	6	ab $\times$ cd	Faville et al. (2004)	Faville et al. (2004), Dracatos et al. (2009a), Pauly et al. (2012)
xpps0374 <sup>c</sup>	6	hk $\times$ hk	Faville et al. (2004)	Faville et al. (2004), Dracatos et al. (2009a)
G01-046	7	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)
G02-048	7	ef $\times$ eg	Studer et al. (2008)	Studer et al. (2008)
G03-045	7	lm $\times$ ll	Studer et al. (2008)	Studer et al. (2008)
G03-092	7	nn $\times$ np	Studer et al. (2008)	Studer et al. (2008)

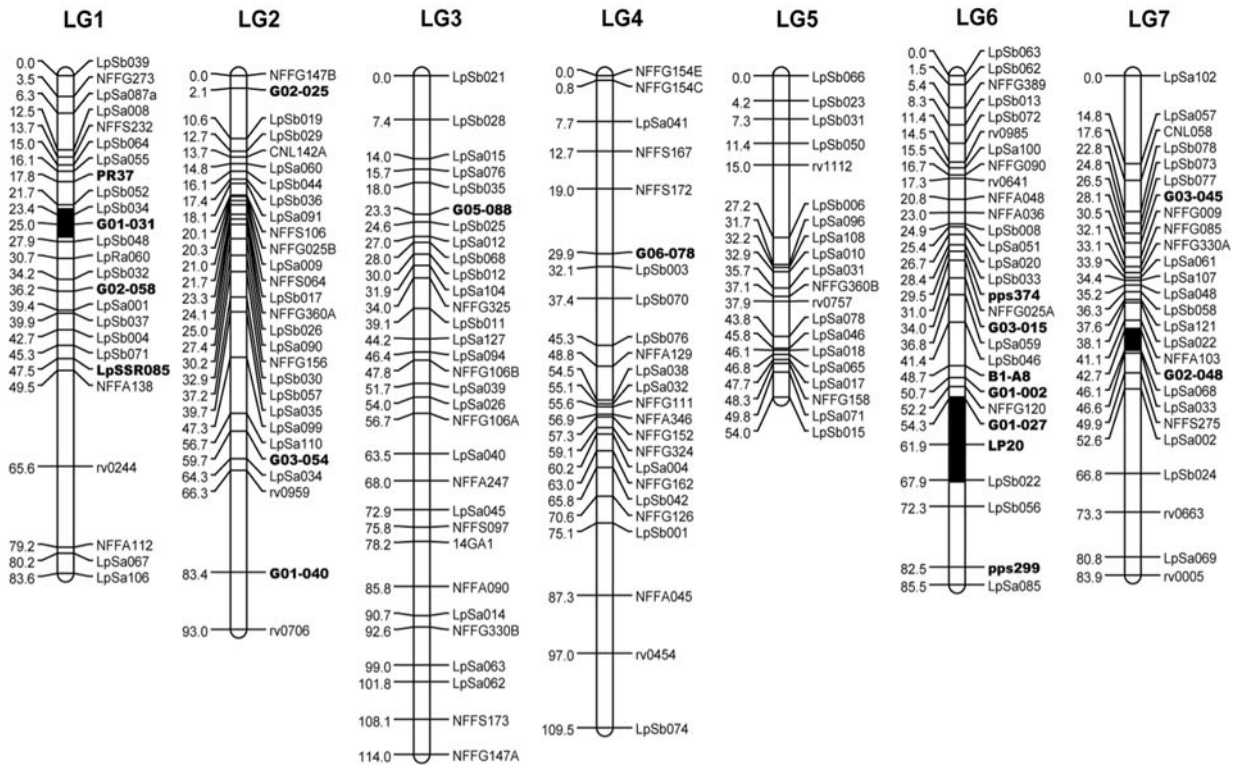
LG linkage group

<sup>a</sup> All markers are EST-SSR except LpRa060, which is a RAD marker

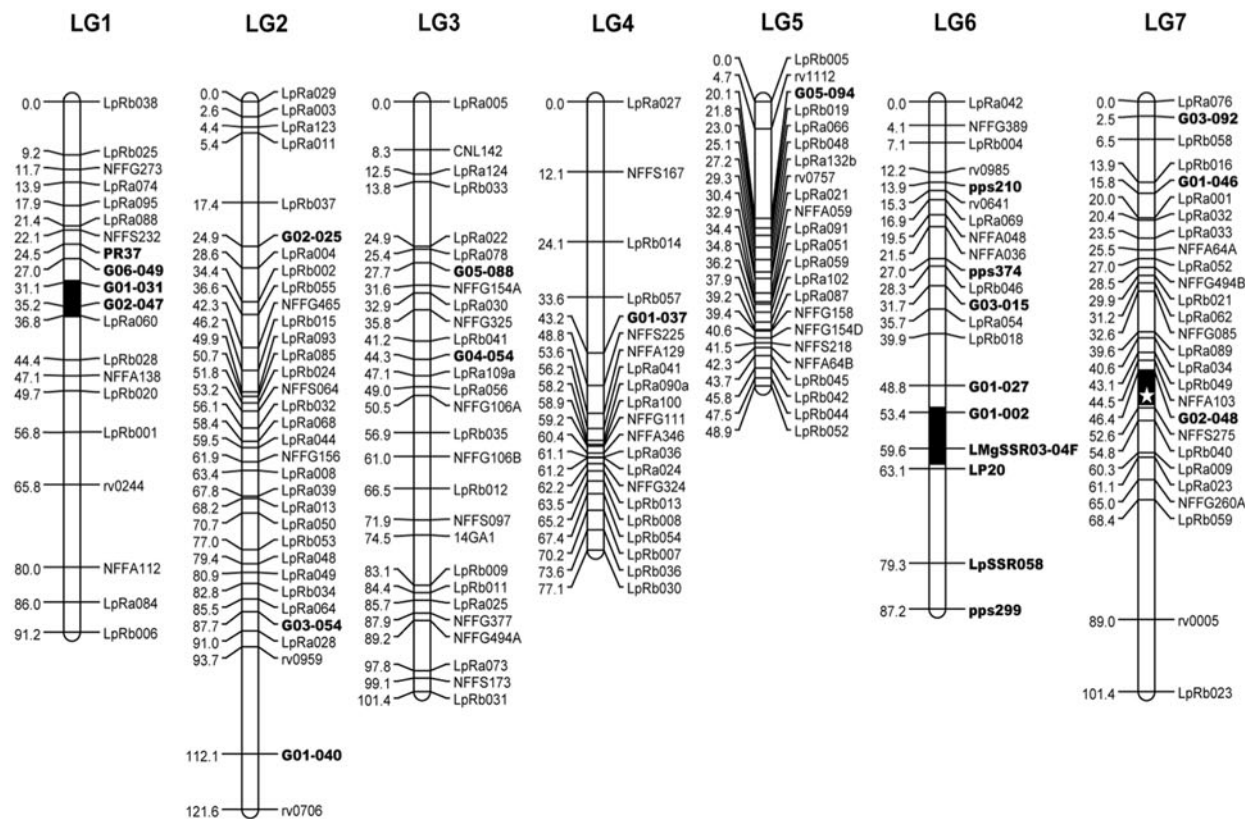
<sup>b</sup> References for marker sources and maps

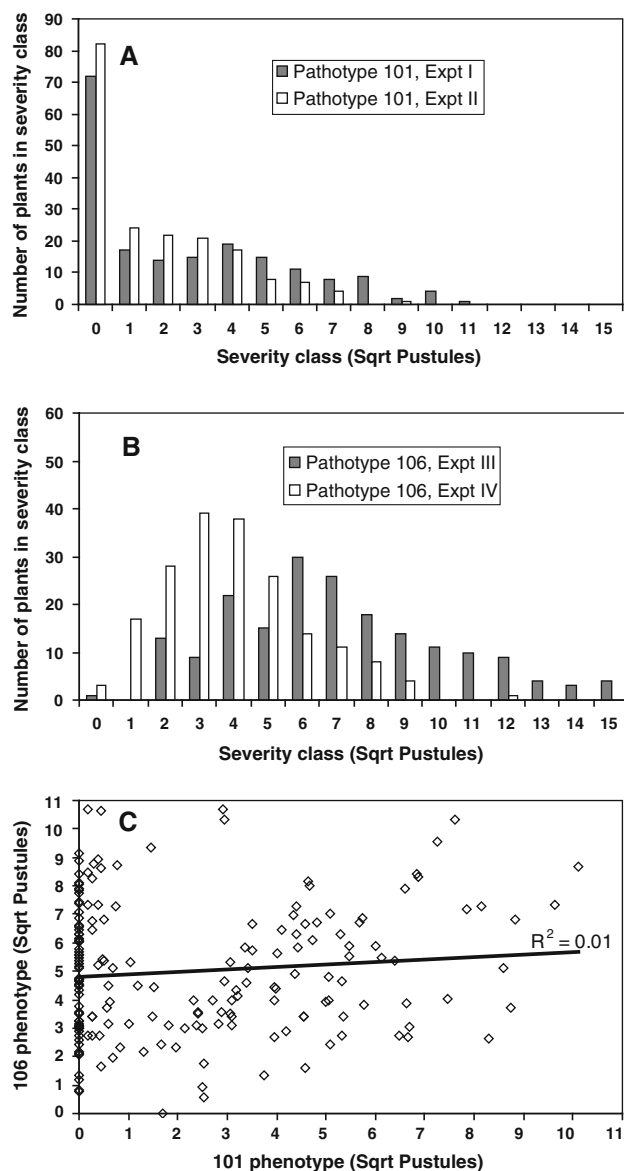
<sup>c</sup> Labels for these markers are shortened on our map (Fig. 1) to pps210, pps229 and pps374

**Female (S20)**



**Male (R2)**





**Fig. 2** Phenotypes of *L. perenne* individuals in F1 mapping population inoculated with pathotypes of *P. graminis* subsp. *graminicola* under controlled conditions. **a** Frequency distribution of disease severities induced by pathotype 101 in two independent experiments (average of square-root-transformed number of pustules per individual, averaged across three replicate ramets per individual). **b** Frequency distribution of disease severities for plants inoculated with pathotype 106. **c** Data points and regression line for severities of each plant inoculated (in separate experiments) with pathotype 101 or 106. Each data point represents one plant in the mapping population

map, and between 34 and 36 cM (2-LOD interval 31–37 cM) on the male map, in the two experiments with pathotype 106. The LOD scores for qLpPg2 ranged from 8 to 15, and this QTL explained 17–30 % of the phenotypic variance in these experiments. It is located between markers G01-031 and LpRa060 on the female and male maps. Marker LpRa060, developed from a testcross RAD marker (Pfender et al. 2011), was scored as ef × eg in the

data obtained by HRM curve analysis. The “g” allele, carried by the male parent, is associated with resistance; this allele is in coupling with the differentiating allele for the testcross marker Rb028, and with the non-differentiating allele for the testcross marker G02-047 (both nn × np).

QTL qLpPg3 and qLpPg1 together explained 60–65 % of the phenotypic variance in response to pathotype 101, whereas qLpPg2 was not detected in response to this pathotype. qLpPg3 and qLpPg2 together explained 30–39 % of the phenotypic variance in response to pathotype 106; qLpPg1 was not detected in response to pathotype 106.

QTL analysis for infection type data (size and development characteristics of pustules) showed that the qLpPg1 is associated with infection type for pathotype 101 (LOD 50, peak at 49 cM on LG7 of the male map). Infection type for pathotype 106 is associated with qLpPg2 (LOD 6–15, peak at 36.8 cM on LG1 of the male map). There was no association of infection type with qLpPg3 (LG6) for either pathotype.

## Discussion

In this research, we determined and documented specificity of stem rust resistance QTL in *L. perenne* for pathotypes of *P. graminis* subsp. *graminicola* obtained through sequential single-pustule culture. Plant response to challenge with pathotype 101 included activation of QTL qLpPg1 on LG7, whereas challenge with pathotype 106 activated qLpPg2 on LG1. Neither of these QTL was activated by challenge with the opposite pathotype, i.e., there was no evidence for qLpPg1 in plants inoculated with pathotype 106, and no evidence for qLpPg2 in plants inoculated with pathotype 101. The third QTL, qLpPg3 on LG6, was activated by either pathotype. The QTL on LG7, specific for pathotype 101, has a major effect on susceptibility to this pathotype, explaining 50–58 % of the phenotypic variance. The response associated with the qLpPg1/pathotype 101 interaction is essentially qualitative, in that 92 % of plants carrying the resistance-associated allele at the marker closest to the QTL are resistant (<3 pustules total among the three replicate ramets of an individual), whereas 5 % without the “g” allele are resistant (data not shown). When this response was scored as a binary trait (<1 pustule per plant vs. >1 pustule per plant) it mapped to nearly the same location as qLpPg1 (Fig. 1). Thus, this locus behaves genetically like a single dominant gene. The QTL on LG1, qLpPg2, is associated with a more quantitative response. In previous research using a genetically heterogeneous, field-collected inoculum of *P. graminis* subsp. *graminicola*, all three of these QTL were activated (Pfender et al. 2011).



**Table 2** Analysis of variance<sup>a</sup> and heritability<sup>b</sup> calculations for reaction to stem rust pathotypes in perennial ryegrass cvar

Pathotype	Experiment	Factor	df	SS	MS	p	H <sup>b</sup>
101	I	Genotype	187	4,110	22.0	<0.001	0.89
		Residual	376	1,042	2.8		
		Total	563	5,152			
	II	Genotype	187	1,856	9.9	<0.001	0.89
		Residual	376	463	1.2		
		Total	563	2,319			
	Combined	Genotype	187	5,147	27.5	<0.001	0.81
		Expt	1	261	261.0	<0.001	
		Genotype × Expt	187	819	4.4	<0.001	
Residual		752	1,519	2.0			
Total		1,127	7,746				
106	IV	Genotype	188	5,305	28.2	<0.001	0.77
		Residual	378	3,243	8.6		
		Total	566	8,548			
	V	Genotype	188	865	4.6	<0.001	0.77
		Residual	378	521	1.4		
		Total	566	1,386			
	Combined	Genotype	188	3,980	21.2	<0.001	0.57
		Expt	1	6,813	6813.4		
		Genotype × Expt	188	2,146	11.4		
		Residual	756	3,564	4.7		
		Total	1,133	16,503			

Kingston F1 mapping population scored for severity (pustules per plant)

<sup>a</sup> Data (square-root of number of pustules per plant) were first adjusted for block effect (three randomized complete blocks, one ramet of each F1 plant per block), then subjected to ANOVA to obtain variances for heritability calculation. Mapping populations was inoculated with pathotype 101 or 106 of *Puccinia graminis* subsp. *graminicola* under greenhouse conditions

<sup>b</sup> For individual experiments, heritability is genotype variance as a proportion of genotype + residual variances. For combined experiments, it is genotype variance as a proportion of (genotype + genotype × experiment + residual) variances

From the results presented here, it appears that the multiple-QTL response to mixed inoculum was due to independent activation of different QTL by specific pathotypes, as well as their activation of a common QTL.

We have no data on the mechanism of interaction among these three QTL. One plausible but untested explanation is that the pathotype-specific QTL (on LG1 or LG7) are involved in initial pathogen recognition event(s), whereas the qLpPg3 is involved in the ensuing metabolic response triggered by recognition. We note that, with the limited number of pathogen genotypes tested, we cannot presume that qLpPg3 is non-specific; clearly it could be specific to some group of pathotypes that includes types 101 and 106 but not other genotypes. However, in the proposed scenario of specificity and general response, qLpPg1 on LG7 could mediate a marked, and perhaps rapid, recognition and implementation of qLpPg3-mediated metabolism, whereas qLpPg2 on LG1 could mediate a slower and/or less complete recognition that is, nonetheless, followed by qLpPg3-mediated activity. The pathotype-specific QTL could contribute to post-recognition

events in addition to the recognition. This hypothesis is consistent with our observation that the qLpPg1 and qLpPg2, but not qLpPg3, are detected as QTL in our analysis of infection type, a measure which reflects the character of the recognition response according to the well-accepted gene-for-gene model of plant/pathogen interaction (Flor 1956). In addition, consistent with this hypothesis is our observation that qLpPg1 and qLpPg2 each had a greater phenotypic effect in the single-pathotype challenge than in the previously observed mixed-inoculum challenge (Pfender et al. 2011), whereas no such difference was observed for qLpPg3. Specifically, the proportion of phenotypic variance explained by qLpPg1 was  $54 \pm 4\%$  in the single-pathotype challenge (Table 3) versus  $31 \pm 5\%$  in the mixed-inoculum challenge (Pfender et al. 2011); the respective values for qLpPg2 are  $23 \pm 7\%$  versus  $9 \pm 3\%$ . In contrast, the phenotypic effect of qLpPg3 was similar in the two challenges:  $9 \pm 3\%$  versus  $8 \pm 2\%$ . The existence and genomic location of candidate genes for pathogen recognition (NBS-LRR) and DR in *Lolium*, as determined by Dracatos et al. (2009a), are relevant to this

**Table 3** Quantitative trait locus (QTL) analysis for stem rust resistance in perennial ryegrass inoculated with pathotypes of *Puccinia graminis* subsp. *graminicola* under greenhouse conditions

QTL	Linkage group	Pathotype	Parent	Experiment <sup>a</sup>	LOD threshold <sup>b</sup>	Peak (cM)	LOD	2-LOD window (cM)	Nearest marker <sup>d</sup>		Allele configuration	
									Marker	K-W <i>p</i> value		Variance (%)
qLpPg3	6	101	Female	I	4.3	67.9	5.6	55–67.9	LpSb022	0.1	6.8	lm × ll
				II	4.4	60.3	8.4	54–68	LP20	0.05	10.2	ef × eg
		106	Female	III	3.8	61.9	4.7	54–68	LP20	0.05	8.6	ef × eg
				IV	3.9	62.9	5.2	54–68	LP20	0.005	10.9	ef × eg
qLpPg1	7	101	Male	I	6.1	59.6	4.7	53–63	LMgSSR03-04F	0.01	5.6	nn × np
				II	6.3	57.4	7.6	53–63	LMgSSR03-04F	0.001	9.9	nn × np
		106	Male	III	4.1	59.4	5.2	54–63	LMgSSR03-04F	0.005	9.1	nn × np
				IV	4.1	62.6	5.3	54–63	LMgSSR03-04F	0.0005	10.5	nn × np
qLpPg2	1	101	Female	I	4.3	44.8	31.8	43–46.5	LpSa068	ns	58.3	lm × ll
				II	4.4	45.7	30.7	42–46.5	LpSa068	0.1	50.2	lm × ll
		106	Female	III	3.8	26.4	14.8	22.5–27.5	LpRa060	0.0001	30.2	ef × eg
				IV	3.9	22.9	8.6	22.5–27.5	LpSb034	ns	19.0	lm × ll
qLpPg2	1	106	Male	III	4.1	36.2	14.0	31–37	G02-047	0.0001	26.9	nn × np
				IV	4.1	34.1	8.0	31–36.5	G02-047	0.0001	16.9	nn × np

<sup>a</sup> QTL analyses were done with each dataset from two independent experiments for each pathotype

<sup>b</sup> Threshold of LOD values for significance at *p* 0.05, determined from 1,000 permutations of the experimental data

<sup>c</sup> ns: QTL peak is lower than threshold value; but included to indicate size of local peak near the QTL site

<sup>d</sup> The marker located closest to the position of the peak of LOD scores for the QTL. Values are given for the Kruskal–Wallis test, proportion of phenotypic variance explained by the genotype at the QTL peak, and the allele configuration as coded in JoinMap

scenario for the function of these QTL. Although there are relatively few markers in common between our map and that of Dracatos et al. (2009a), it is plausible that our qLpPg3 on LG6 could be co-located with their DR candidate gene *xlprg-1435ct*. This locus maps in a 60-cM region between markers *xpps0374* and *xpps0229* on LG6 in the Dracatos et al. (2009a) map, and qLpPg3 maps within a 60-cM region between these same two markers on our map (labeled *pps374* and *pps229* in Fig. 1). On LG1 in Dracatos et al. (2009a), *xlprg30-707ag* (NBS-LRR candidate) and *xlpthbna-317ag* (DR candidate) are both within a 40 cM distance of *xlpsrk15h05*; on our map qLpPg2 on LG1 is near G01-031, which maps within about 25 cM of *xlpsrk15h05* on the map of Studer et al. (2010). Our qLpPg1 maps to the central portion of LG7, the same general region as the NBS-LRR candidate *xlprg60-216gt* of Dracatos et al. (2009a). We are currently attempting to map the disease-response-related markers reported by Dracatos et al. (2009a) in our population, which would help clarify whether these genomic relationships are likely, rather than merely plausible. To further test the above-proposed hypothesis of specificity in function of these stem rust QTL would require research on the time course of metabolic and morphological response to challenge with the different pathotypes.

In elucidating the genomic organization of disease resistance in the grasses, an interesting question is the co-location of resistance determinants for crown rust and stem rust. The *Lolium* stem rust QTL qLpPg3 appears to be in the same general region of LG6 as some QTL detected for crown rust reaction. On our map, qLpPg3 is distal to markers *rv0641* and *pps374*, proximal to *pps299*, and near LP20 as well as G01-001 and G01-027. Dracatos et al. (2008a) detected a crown rust QTL on LG6 distal to *pps374* and proximal to *pps299*. Pauly et al. (2012) detected a crown rust QTL near LP20, between *rv064* and *pps299*. The genetic distance between common markers among these maps is fairly large (40–60 cM), so it is difficult to ascertain how similar the location of these QTL for stem rust and crown rust might be. Our analysis shows G01-001 and G01-027, anchor markers of Studer et al.'s (2010) consensus map, to be highly associated with the stem rust QTL on LG6; if these markers are mappable in populations used by Dracatos et al. (2008a) or Pauly et al. (2012) their association with the crown rust phenotype could shed more light on the commonality of the stem rust and crown rust QTL.

Stem rust QTL qLpPg2, on LG1, also may be near a previously reported crown rust QTL. Schejbel et al. (2007) detected a QTL for crown rust slightly distal to marker PR37; our qLpPg2 covers an interval starting from approximately 8 cM distal to PR37 (Fig. 1). This QTL apparently is not, however, near a crown rust QTL on LG1

of *L. multiflorum* detected by Studer et al. (2007). qLpPg2 on our map is 10 cM proximal to the marker G02-058, which in turn is proximal to NFFA032 on the consensus map (Studer et al. 2010), whereas the *L. multiflorum* LG1 QTL is 30 cM further distal to NFFA032 on its map (Studer et al. 2007). Stem rust QTL qLpPg1 is in the central third of LG7 on our map, and crown rust QTL also have been found in the central third of this LG in analyses by Sim et al. (2007) and Dracatos et al. (2008a), although the maps have no common markers to confirm these QTL's proximity to one another. Muylle et al. (2005) detected two QTL for crown rust reaction in *Lolium* that map to the lower end of their LG1. Again, lack of common markers prevents us from determining whether these QTL for crown rust are located near our stem rust QTL on this LG.

Research is in progress to further test and select the genetic markers that co-segregate with the stem rust resistance QTL, in crosses of plants from this mapping population with other plants. Such markers could be useful in marker-assisted selection (MAS) strategy for genetic improvement of *L. perenne*, particularly after more information is gained about the nature of the genes at these loci. If qLpPg1 and qLpPg2 are, in fact, co-located with genes that function in recognition of particular pathotypes, MAS could be used to pyramid these and other signaling genes to improve resistance durability. Because perennial ryegrass varieties are released as synthetics, MAS to place various pathotype-specific resistance loci in the various female parents offers the possibility of resistance-gene mixtures in the crop (such as are obtained in inbreeding cereal crops by mechanical mixture of isolines). When the genetic and physiological nature of the resistance at qLpPg3 is better understood, MAS with this locus could be implemented with the goal of providing either back-stopping non-specific resistance and/or improved functioning of resistance triggered by recognition events due to other loci. Deployment strategies for genetic resistance could, thus, be designed, given detailed comparative knowledge of resistance function at these loci. However, even without that level of knowledge, MAS should be useful as an empirical approach to collect these loci into breeding stock.

Our observations add to current information about genomic organization of disease resistance in grasses. As others have noted (Armstead et al. 2004, 2006; Devos 2005; Dracatos et al. 2010; Jones et al. 2002), there is a degree of synteny for disease resistance loci among *Lolium*, other related grasses such as *Festuca* (Roderick et al. 2003), and economically important grasses such as cereal grains. Perennial ryegrass LG1 has a locus (LrK10) orthologous to a crown rust resistance locus that is syntenous on wheat, barley and rye LG (Dracatos et al. 2009a). Although this locus does not appear to map near our qLpPg2 on LG1, its synteny with a cereal grass resistance

locus demonstrates that rust resistance in *Lolium* and cereals offers opportunities for synergy in research.

**Acknowledgments** We thank Sheila Seguin and Lori Evans-Marks (USDA-ARS) for excellent technical assistance essential to this research.

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