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ORIGINAL PAPER

Mapping with RAD (restriction-site associated DNA) markers to rapidly identify QTL for stem rust resistance in *Lolium perenne*

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Abstract A mapping population was created to detect quantitative trait loci (QTL) for resistance to stem rust caused by *Puccinia graminis* subsp. *graminicola* in *Lolium perenne*. A susceptible and a resistant plant were crossed to produce a pseudo-testcross population of 193 F_1 individuals. Markers were produced by the restriction-site associated DNA (RAD) process, which uses massively parallel and multiplexed sequencing of reduced-representation libraries. Additional simple sequence repeat (SSR) and sequence-tagged site (STS) markers were combined with the RAD markers to produce maps for the female (738 cM) and male (721 cM) parents. Stem rust phenotypes (number of pustules per plant) were determined in replicated greenhouse trials by inoculation with a field-collected, genetically heterogeneous population of urediniospores. The F_1 progeny displayed continuous distribution of phenotypes and transgressive segregation. We detected three resistance QTL. The most prominent QTL (qLpPg1) is located near 41 cM on linkage group (LG) 7 with a 2-LOD interval of 8 cM, and accounts for 30–38% of the stem rust phenotypic variance. QTL were detected also on LG1 (qLpPg2) and LG6 (qLpPg3), each accounting for approximately 10% of phenotypic variance. Alleles of loci closely linked to these QTL originated from the resistant parent for qLpPg1 and from both parents for qLpPg2 and qLpPg3. Observed quantitative nature of the resistance may be due to partial-resistance effects against all pathogen genotypes, or qualitative effects completely preventing infection by only some genotypes in the genetically mixed inoculum. RAD markers facilitated rapid construction of new genetic maps in this outcrossing species and will enable development of sequence-based markers linked to stem rust resistance in *L. perenne*.

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Introduction

Perennial ryegrass (*Lolium perenne*) is among the most important cool-season grasses worldwide, being used for forage production and as turfgrass, and there is ongoing interest in development of improved varieties. One important target for genetic improvement is disease resistance. Several diseases, including stem rust and crown rust, can cause serious losses to seed or vegetative production. Genetic resistance, if available, is a preferred method of disease management.

Genetic improvement in *Lolium* is challenging due to its obligate outcrossing mating system that results in high levels of individual heterozygosity and population heterogeneity. The pseudo-testcross approach to mapping (Grattapaglia and Sederoff 1994) is useful for such populations, and there are numerous reports of QTL studies that identify genomic regions and markers associated with disease resistance genotypes in outcrossing plant species (Fischer et al. 2004; Mehlenbacher et al. 2006; Muylle et al. 2005; Wilcox et al. 1996).

For genetic studies of disease resistance in *Lolium* spp., the major focus of QTL discovery has been on resistance to crown rust, caused by *Puccinia coronata*. Because plant disease phenotype is determined by pathogen genotype(s) and can be strongly influenced by environmental conditions, it is to be expected that different QTL will be identified in experiments conducted by various methods and across a range of geographic regions. Studies conducted in the field and under controlled conditions in Europe, North America and Australasia have identified QTL for crown rust reaction on all linkage groups (Dumsday et al. 2003; Muylle et al. 2005; Schejbel et al. 2007; Sim et al. 2007; Studer et al. 2007), typically on 2–4 linkage groups in any particular study. The LGs most commonly found to contain QTL for crown rust resistance are LG1 and LG2 (Dumsday et al. 2003; Muylle et al. 2005; Schejbel et al. 2007; Studer et al. 2007).

Genetic research on stem rust caused by *Puccinia graminis* subsp. *graminicola* has thus far received relatively little attention, although it is the major disease problem in seed production areas in parts of Australasia (Hampton 1986) and North America where seed losses as great as 98% can occur if the disease is not controlled (Pfender 2009a). Stem rust affects the leaves and stems of perennial ryegrass equally and is a problem primarily in seed production, rather than in turf or forage use. Most commercial cultivars and populations of *Lolium* spp. lack sufficient genetic resistance to avoid fungicide use for stem rust, and there is little information on inheritance of resistance to the disease in this species (Rose-Fricker et al. 1986). However, the existence of pathotypes in *Lolium* stem rust was demonstrated recently (Pfender 2009b). Also, a comparative analysis of disease resistance QTL (Jo et al. 2008) in a *Lolium* population developed for a different purpose (Warnke et al. 2004) detected a QTL for stem rust resistance on LG4, although resistance levels in the mapping population were not high.

To quickly develop moderately dense maps suitable for QTL analysis of stem rust resistance in a new *Lolium* population, we combined conventional SSR genotyping with a recently developed method in which massively parallel and multiplexed sequencing of reduced-representation restriction-site associated DNA (RAD) libraries facilitates population-specific marker discovery and progeny genotyping (Baird et al. 2008; Miller et al. 2007; Van Tassell et al. 2008). The RAD process produces two types of markers. Single nucleotide polymorphisms (SNPs) within the restriction enzyme recognition site lead to dominant RAD tags, whereas biallelic SNPs in sequences adjacent to the enzyme site are discovered in parents and scored co-dominantly in progeny. SNPs are stable, heritable, and abundant DNA features that are preferred markers for linkage mapping and genome-wide association studies that require large numbers of markers. SNP resources for *Lolium* are presently limited (Cogan et al. 2006), and development of a RAD-SNP database will increase the availability of these important tools for molecular breeding and genetic studies. SSR markers on the other hand are advantageous for linkage mapping and breeding applications due to ease of scoring, reproducibility, high levels of polymorphism, and co-dominant mode of inheritance. Framework and consensus linkage maps populated with publicly available SSR markers have been developed for *Lolium* in recent years (Jensen et al. 2005; Gill et al. 2006; Studer et al. 2010).

The objectives of this research were to: (1) determine utility of RAD markers for map construction in *Lolium*, (2) construct genetic linkage maps of a pseudo-testcross ryegrass population with a wide range of stem rust resistance, (3) detect QTL conferring resistance to a mixed population of *P. graminis* sub sp. *graminicola*, and (4) identify molecular markers associated with stem rust resistance for potential use in marker-assisted breeding.

Materials and methods

Plant materials

A mapping population for stem rust resistance was constructed by crossing two parental plants selected from the New Zealand perennial ryegrass cultivar Kingston (PGG Wrightson Seeds, Christchurch NZ). The resistant and susceptible parents, designated R2 (male) and S20 (female), respectively, were selected from a group of plants challenged with a genetically mixed inoculum of *P. graminis* subsp. *graminicola* collected in 2005, as previously described (Pfender 2009b). The mapping population consisted of 193 F_1 individuals from this cross. Plants were grown from seed in a glasshouse, and vegetatively propagated to produce multiple ramets of each plant. Ramets were grown in cylindrical pots (3.8 cm diameter × 24 cm tall) of soil in a glasshouse, fertilized at 2-week intervals with pH-adjusted NPK solution, and trimmed periodically to a height of 8 cm. Leaf tissue was collected from each of the 193 plants for DNA extraction, and plants were subjected to inoculation with *P. graminis* subsp. *graminicola* for phenotype determination.

DNA isolation

Lolium perenne genomic DNA was initially purified from pulverized freeze-dried leaf tissue using a sodium lauryl sarkosyl buffer as described by Steiner et al. (1995). Due to impurities in some samples DNA was subsequently re-purified for Illumina sequencing using DNeasy kits (Qiagen, Valencia, CA) following manufacturer's specifications.

RAD marker development

Population-specific *Lolium* RAD markers were developed from S20 and R2 parental DNA and genotyped on F_1 progeny by Floragenex, Inc. (Eugene, OR). *Lolium* genomic DNA was digested with the restriction endonuclease *Sbf*I, which recognizes an 8-nucleotide (nt) sequence (CCTGCAGG), and separately processed into RAD tag libraries similarly to the method of Baird et al. (2008). Briefly, genomic DNA (~300 ng) from parental genotypes and F_1 progeny was digested for 60 min at 37°C in a 50 μl reaction with 20 units (U) of *Sbf*I [New England Biolabs (NEB), Ipswich, MA] and heat-inactivated for 20 min at 65°C. 2.0 μl of 100 nM P1 adapter, a modified Solexa adapter (Illumina, all rights reserved), were added to each sample along with 1 μl of 10 mM rATP (Promega, Madison, WI), 1 μl 10× NEB Buffer 4, 1.0 μl (1,000 U) T4 DNA Ligase (high concentration, NEB), 5 μl H_2O, and incubated at room temperature for 20 min. P1 adapters contained a forward amplification primer site, an Illumina sequencing primer site, and a unique sequence index (barcode) to be read as the first five nucleotides of the Illumina sequence read. Heat-inactivated samples were pooled and randomly sheared by sonication to an average size of 500 bp. DNA fragments from 300 to 700 bp were separated on a 1.5% agarose 0.5× TBE gel and isolated using a MinElute Gel Extraction Kit (Qiagen). Klenow exo$^-$ (Enzymatics, Beverly, MA) was used to add 3′ adenine overhangs after ends were enzymatically blunted (Enzymatics). After purification (MinElute column, Qiagen) 1 μl of 10 μM P2 adapter, a divergent modified Solexa adapter (Illumina, all rights reserved), was ligated to the obtained DNA fragments. The re-purified eluate was quantified using a fluorimeter and 20 ng was used in a PCR amplification containing 50 μl Phusion Master Mix (NEB), 5 μl of 10 μM modified Solexa amplification primer mix (Illumina, all rights reserved), and H_2O to 100 μl. Phusion PCR settings followed product guidelines for a total of 16–18 cycles. Samples were gel purified, excising DNA 300–650 bp, and diluted to 1 nM.

Lolium libraries were sequenced on an Illumina Genome Analyzer II at the University of Oregon High Throughput Sequencing Facility. Illumina/Solexa protocols were followed for single read (1 × 36 bp) sequencing chemistry.

RAD sequence analysis, SNP discovery and genotyping

Solexa sequences were segregated by the barcode assigned to each sample. Floragenex proprietary sequence tools and custom programs were used to process raw data. Reads were trimmed to 29 nts of genomic sequence and those with greater than five low-quality bases (Q score < 20) were scrubbed from the dataset. In the marker discovery phase of the study, sequence reads from each parent were first grouped into clusters of identical sequences (RAD tags) and clusters with <7 or >200 sequences were discarded. RAD tags were compared to each other and nearly-identical tags with one or two mismatches (SNPs or 1–2 nt indels) were considered as putative alleles that were heterozygous in the parental DNA. Identification of markers that were polymorphic between the S20 and R2 parents was accomplished by pairwise grouping of sequence data from each genotype with a maximum of two mismatches allowed per 29-nt alignment. To minimize errors in assigning markers to segregation classes, sequence clusters with <8× coverage in both parental libraries were excluded from consideration.

F_1 genotypes were scored based on comparison of progeny RAD tags to the parental marker panel. Scoring parameters were set to produce robust genotyping calls while retaining as many markers as possible. Each marker was required to have an allele present in at least 90 F_1 individuals and each allele had to be present in at least 20 F_1 individuals. We expected to observe both homozygous and heterozygous genotypes for RAD marker loci in testcross and intercross configurations. With an overall average of ~14 reads per locus in each F_1 plant many genotyping calls were unambiguous. However, for cases with limited reads, we trialed requiring a minimum of three versus four tag sequences of one SNP allele combined with zero instances of the alternate allele to make a homozygous call. The less stringent protocol (i.e., at least three reads of one allele) resulted in twice the number of markers reaching the minimum threshold as resulted from the more stringent protocol (i.e., at least four reads of one allele). Because both marker sets integrated well with SSR/STS markers and produced maps of similar lengths, we adopted the less stringent protocol. Marker genotypes not meeting minimum thresholds were scored as missing data (19% overall).

SSR genotyping

Six hundred and eighty-seven microsatellite and STS primers developed from EST and genomic libraries from tall fescue (Saha et al. 2004, 2005), cereals (Kantety et al. 2002), and *Lolium* × *Festuca* F_1 hybrids (Lauvergeat et al. 2005) were screened against S20 and R2 parental DNA. SSR and STS markers were assayed at the Samuel Roberts

Noble Foundation (Ardmore, OK). Forward primers were modified by M13 tail at the 5′ end (Schuelke 2000). The final reaction volume of the PCR was 10 µl, which contained 15 ng of template DNA, 0.1 µl of 10 µM reverse primer, 0.25 µl of 10 µM forward primer, 3.0 mM $MgCl_2$, 2.5 mM of each dNTPs, 1 µM M13 fluorescent dye (Applied Biosystems, Foster City, CA) and 0.45 units of *Taq* DNA polymerase with 1× PCR Buffer, (GeneScript Corp. Piscataway, NJ). PCR reactions were done using an Applied Biosystems (Foster City, CA) Geneamp 9700 thermocycler. Touch-down PCR program was used for all primer sets: 95°C for 3 min followed by 6 cycles of 94°C for 45 s, 68°C for 5 min, and 72°C for 1 min with the annealing temperature reduced by 2°C per cycle, then 8 cycles of 94°C for 45 s, 58°C for 2 min and 72°C for 1 min with the annealing temperature reduced by 1°C per cycle, followed by 25 cycles of 94°C for 45 s, 50°C for 2 min and 72°C for 1 min with a final extension of 72°C for 7 min. PCR products were separated in an ABI 3730 genetic analyzer using the LIZ®™ 500 size standard. The Genescan tracefiles were imported and analyzed by GeneMapper 3.0 software (Applied Biosytems). Primer pairs that produced clean PCR products (142) were re-tested against parental DNA and a panel of six randomly selected F_1 progeny. Markers with segregating alleles (98) were genotyped on F_1 progeny and scored co-dominantly. To identify and orient linkage groups with respect to published genetic maps we genotyped 28 markers from perennial ryegrass, tall fescue, cereal EST superclusters, and wheat that were previously mapped in *Lolium* (Gill et al. 2006; Sim et al. 2009; Warnke et al. 2004).

Linkage maps

Linkage maps were assembled using JoinMap4 software with CP population type codes (cross-pollinated progeny from a cross between two heterogeneously heterozygous and homozygous diploid parents with no prior knowledge of linkage phases) (Van Ooijen 2006). The input datasets were constructed from 192 F_1s genotyped for SSR/STS alleles and 177 F_1s genotyped for RAD markers including one F_1 not genotyped for SSR/STS alleles, bringing the mapping population to 193 individuals. Separate maps for the female parent (S20) and the male parent (R2) were built using RAD markers alone, SSR/STS markers alone, and with selected RAD markers and SSR/STSs combined. Markers were assigned to seven linkage groups using a test for independence LOD score of 6.0, and ordered using the regression mapping algorithm with a recombination frequency threshold of 0.5, a LOD threshold of 0.5, and a jump threshold value of 5.0. A ripple was performed after addition of each locus. JoinMap4 automatically determined the linkage phases of the loci during the estimation of recombination frequencies. The Kosambi mapping function was used to translate recombination frequencies into map distances. Diagnostic tools in JoinMap4 were used to identify markers exhibiting segregation distortion. The maximal distortion permitted was 2.5:1 for a marker with expected Mendelian segregation of 1:1 (e.g., Chi-square value <40 for markers with $df = 1$). Additionally, markers identified by JoinMap4 as having poor fit were discarded. The map3 output was accepted as the final result after removal of excessively distorted and poorly performing markers.

Pathogen inoculum

Urediniospores of *P. graminis* subsp. *graminicola* were collected in 2008 from a field of perennial ryegrass near Corvallis, OR (44°38′N, 123°12′W). The spores were dried overnight at 30% relative humidity, stored at −60°C until needed, and heat-shocked immediately before use, as previously described (Pfender 2003). Viability of inoculum was tested by applying a sample of spores to plates of water agar and determining percentage germination by observation with a microscope after 4 h of incubation in the dark.

Stem rust phenotype determination

Reaction of each of the 193 F_1 individuals to the stem rust pathogen was quantified in two separate experiments 3 months apart by exposing plants to a uniform inoculum of *P. graminis* subsp. *graminicola* urediniospores. In each experiment, three cloned ramets (replicates) of each individual F_1 were inoculated, and the experiment was conducted as a randomized complete block. Additional pots with plants of the susceptible cultivar 'Jet' were interspersed at random among the pots of F_1 ramets, as an additional check for uniformity of the test. The inoculum concentration was 6 mg urediniospores per milliliter of Soltrol oil, prepared as described previously (Pfender 2003), with each ramet receiving 30 µl of spore suspension. After the Soltrol oil had dried plants were placed in an environmental enclosure and incubated under conditions favorable for infection (Pfender 2003), i.e., plant leaves were kept moist in the dark overnight and during 4 h of natural light the following morning. In the first experiment the temperatures were 22°C maximum and 16°C minimum during the moist dark period, rising to 25°C in the moist light period. In the second experiment the temperatures were 17°C maximum and 11°C minimum during the moist dark period, rising to 20°C in the moist light period. After exposure in the moist environment, plants were removed from the enclosure and incubated for the duration of the experiment on a glasshouse bench under natural light. Pots were watered from below to avoid water on the leaves which could permit additional infections to occur. Duration of the

post-inoculation incubation on the glasshouse bench was 15 days in the first experiment and 21 days in the second experiment; these durations were equivalent in heat units (degree days), constituting 1.8 latent periods for stem rust development (Pfender 2001) in each experiment. The average daily minimum and maximum temperatures during this incubation period were 15 and 24°C in Experiment I, and 10 and19°C in Experiment II. At the end of the post-inoculation incubation period the disease severity of each plant was measured as the number of rust pustules. Disease severity values were transformed to $\log_{10}(0.5 + \text{number of pustules})$ to achieve normally distributed data. The log-transformed values of the three ramets of each F_1 individual were averaged to produce the severity value of an individual for QTL analysis. Three data sets were prepared, one for each experiment and an additional data set pooled across the two experiments. The pooled data set was constructed by first normalizing the two data sets to the same overall mean severity to avoid biasing the values toward the higher-severity experiment, then averaging the two experiments' values for each F_1 individual.

Heritability was calculated from variance components obtained by analysis of variance for each experiment, with disease severity (log-transformed number of pustules per ramet) as the dependent variable. As described by Calenge et al. (2004) individual values in each block were adjusted by a constant factor for the overall mean block effect before calculating variances, and heritability in each of the two experiments was then computed as variance among genotypes divided by the total of genotype plus residual variances. Similarly, the two datasets (Experiments I and II) were used together for an estimate of heritability that includes the genotype × environment interaction.

QTL analysis

Quantitative trait locus analysis was conducted using the software MapQTL5, build 9apr08.7apr08 (Van Ooijen 2004). Trait data (log-transformed disease severity values) and map data (male and female maps) were as described in previous paragraphs. LOD thresholds for testing significance of QTL peaks were calculated using 1,000 permutations for each of the trait data sets and a genome-wide significance level of 5%. The interval mapping (IM) procedure was used to identify major QTL peaks, then the automatic cofactor selection routine (ACS) was used to select among the markers in the region of the highest significant IM peak, starting with the linkage group having the highest peak. This cofactor was retained while the ACS routine was applied to the linkage group showing the next highest significant peak, and this procedure was repeated until cofactors were identified for all linkage groups with significant peaks. No attempt was made to designate more than one cofactor for any linkage group. The multiple QTL mapping routine (MQM) was then applied using the selected cofactors. In some cases use of cofactor(s) beyond those for the highest peak(s) did not substantially improve definition of the additional peaks, and these additional cofactors were omitted. QTL were designated for peaks that reached the genome-wide LOD significance level of 5%, and 2-LOD support levels were determined from the MQM results tables.

Results

RAD tag sequencing and marker discovery in *Lolium*

Massively parallel Solexa sequencing of reduced-representation libraries generated ~1 million reads from each *Lolium* parent. Reads were aggregated into ~17,000 raw RAD clusters per parent. After removal of low abundance (<7, 9%) and high abundance (>200, 13%) reads, ~14,000 RAD clusters were obtained, of which 94% had depths of >8 reads/cluster. Proportion of clusters that had read depths of >10, >15, >25, >35 and >50 were 90, 83, 70, 50 and 40%, respectively.

Clusters from the S20 and R2 libraries were compared to identify useful markers for the S20 × R2 cross (Table 1). The cluster analysis revealed 1,733 potentially scoreable RAD markers with alleles distinguished by 1 or 2 biallelic SNPs or small indels. RAD tag clusters that were homozygous in one parent but entirely absent from the other (1,988 from S20 and 2,775 from R2) were not utilized for mapping in this study because the depth of sequencing required for accurate detection of the null progeny class was beyond the scope of the project. Two-thirds of the potential markers were in testcross configuration and were expected to segregate 1:1 in the F_1 mapping population. RAD markers in testcross configuration (homozygous in one parent and heterozygous in the other) were of two types. One type, designated by the prefix LpSa or LpRa in the S20 or R2 parent, respectively, had the null (missing) sequence in the homozygous parent (presumably because the sequence of the restriction site was altered). The other type, designated by LpSb or LpRb, had a detectable homozygous sequence in the homozygous parent. The markers present in intercross configuration (designated by the prefix LpIC) were expected to segregate 1:2:1 and were included in both S20 and R2 datasets. Representative markers from the three RAD marker classes were scored on a panel of 12 F_1 progeny and the predicted segregation patterns were confirmed (results not shown).

Genetic maps for the S20 × R2 cross

A total of 47.5 million Solexa reads were obtained from 188 F_1 progeny libraries. The average read number was 205,380, but the number of reads per F_1 varied from 14,000

Table 1 RAD marker discovery in S20 and R2 parents and utility in S20 × R2 F_1 mapping population

RAD marker prefix	Allelic configuration in S20 × R2	JoinMap4 segregation type codes	Markers identified in parents	Markers scored in F_1 mapping population	Markers mapped in F_1 mapping population
LpSa[a,d]	Testcross	lm × ll	445	221	219
LpRa[a,e]	Testcross	nn × np			
LpSb[b,d]	Testcross	lm × ll	661	129	119
LpRb[b,e]	Testcross	nn × np			
LpIC[c]	Intercross	hk × hk	627	142	120

[a] Cluster heterozygous in S20 or R2, null in other parent

[b] Cluster heterozygous in S20 or R2, homozygous in other parent

[c] Cluster heterozygous in both S20 and R2

[d] S denotes markers heterozygous in S20 parent

[e] R denotes markers heterozygous in R2 parent

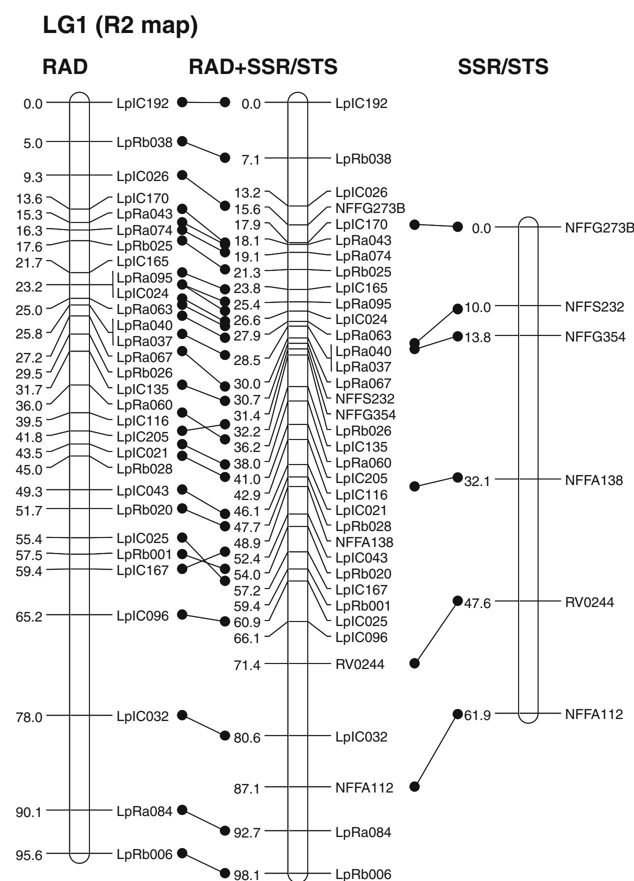

Fig. 1 Three LG1 maps for rust resistant male parent (R2) of the S20 × R2 *Lolium perenne* mapping population. Maps were constructed with RAD tags, RAD tags plus SSR/STS markers, and SSR/STS markers alone. *Lines* connect homologous markers

to 935,000. Eleven progeny with <50,000 reads were excluded from the mapping dataset, leaving 177 progeny for RAD tag genotyping. We identified 1,156 potential RAD markers for the female (S20) map, of which 329 yielded F_1 genotyping scores that met the criteria outlined in "Materials and methods". For the male (R2) map, 1,216 potential markers were identified and 305 markers produced acceptable F_1 genotyping scores. At a minimum LOD of 6.0, the regression mapping algorithm grouped more than 90% of the input markers into 7 LGs, the same as the haploid chromosome number for *Lolium perenne* (Supplementary Fig. 1). The total lengths of the RAD tag maps were 707 and 710 cM for S20 and R2 maps, respectively. The average distance between markers was 2.3 cM (S20) and 2.6 cM (R2), and the largest gaps were 18.1 cM on LG2 (S20) and 17.5 cM on LG5 (R2). In both maps a tight cluster of ~25 markers occupied approximately 3 cM in the middle of LG4. These clusters consisted exclusively of markers in the LpSa (S20 map) or LpRa (R2 map) configuration, characterized by null alleles in one parent. LG6 was sparsely populated with markers in both maps, relative to other LGs.

SSR/STS maps were developed using primer pairs derived from several sources (Table 2). The S20 map was built using 56 primer pairs (64 loci) and the R2 map was built using 51 primer pairs (56 loci). Two-thirds of the SSR/STS markers on each map were co-dominant, and more than half were heterozygous in both parents with three or four alleles per locus. The maps were 470 and 471 cM in length (S20 and R2 map, respectively), with an average distance between loci of 7.3 and 8.4 cM (Supplementary Fig. 2). *Lolium* SSR markers in common with the framework map of Gill et al. (2006) enabled us to orient the LGs and assign LG names to correspond to previously published *L. perenne* maps.

For both marker types (RADs and SSRs) we observed marked segregation distortion on LG2, where 85% of the markers deviated significantly from Mendelian segregation ratios. Fully informative markers (i.e., ab × cd and ef × eg segregation type codes) revealed that deficient alleles were inherited from one grandparent (i.e., same phase) on the

Table 2 The utility of simple sequence repeat (SSR) and sequence-tagged site (STS) markers from various sources in the S20 × R2 F_1 mapping population

Marker prefix	Type	Origin	Number prescreened on S20 and R2[a]	Number screened on S20, R2, and 6 F_1s	Number genotyped	Number mapped
General mapping primers						
NFFA[b]	EST-SSR	Tall fescue	91	36	23	14
NFFG[c]	Genomic-SSR	Tall fescue	221	64	50	37 (41)
NFFS[d]	STS	Tall fescue	321	35	23	17
CNL[e]	EST-SSR	Cereal superclusters	26	4	1	1 (2)
B#-X#[f]	Genomic-SSR	*Lolium* × fescue	28	3	1	0
LG-specific primers						
RV[g]	Genomic-SSR	Ryegrass		19	13	13
NFFA[b]	EST-SSR	Tall fescue		14	10	3
CNL[e]	EST-SSR	Cereal superclusters		11	2	1 (2)
KSUM[h]	EST-SSR	Wheat		21	3	0

[a] Table entries are number of primers (number of loci mapped)

[b] EST-SSRs from 11 tall fescue cDNA libraries (http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=f_arundinacea)

[c] Genomic-SSRs from enriched tall fescue genomic library (http://www.ncbi.nlm.nih.gov/nucgss/57551941?report=gss)

[d] Tall fescue sequence-tagged sites (STS); primer sequences available from Dr. M. Saha, Noble Foundation

[e] Conserved grass EST-SSRs; primer sequences available from Dr. M. Sorrells, Cornell University

[f] Hybrid *Lolium* × fescue genomic-SSRs, primer sequences available from Dr. M. Ghesquiere, Lusignan, INRA, France (http://www.ncbi.nlm.nih.gov/nuccore/59668411?report=genbank)

[g] Enriched genomic and methyl-filtered GeneThresher libraries from perennial ryegrass; primer sequences available in Gill et al. (2006)

[h] Wheat EST-SSRs; primer sequences available from Dr. M. Sorrells, Cornell University

S20 side of the pedigree. Maximum distortion was observed near the middle of LG2 (X^2 test p values <0.00001 for markers with $df = 1$–3) and tapered toward each telomere. In this region, markers expected to segregate 1:1 instead exhibited as much as a 2.5:1 ratio. Markers at ~95–125 cM on LG 3 showed moderate segregation distortion (X^2 test p values <0.001) and mild distortion was observed in the middle of LG5 (X^2 test p values <0.01 at $df = 3$). Remaining LGs were undistorted in the S20 × R2 cross.

The SSR/STS dataset was added to the RAD dataset and the maps were recalculated. Combining data from both marker types had no effect on the groupings and caused only minor changes in marker orders and inter-SSR or inter-RAD distances. RAD tag loci served to increase marker density and extend linkage groups by ~30% in both S20 and R2 maps, as compared to the SSR/STS maps, as shown in Fig. 1 which compares RAD-tag, combined, and SSR/STS maps for LG1 from the R2 map. For QTL analysis of stem rust resistance, we decreased marker density in the maps to approximately one marker per 6 cM by reducing RAD tag loci to 118 (S20) and 126 (R2), while retaining most SSR/STS markers (Fig. 2). Preference was given to RAD tag markers with the fewest missing genotypes. The resulting maps were 738 cM (S20) and 721 cM (R2) in length.

Rust reaction phenotyping

The F_1 plants displayed a range of rust severity reactions (Fig. 3). Number of pustules per plant (averaged across the 3 ramets of each F_1) ranged from 0.3 to 300 in Experiment I, and from 0.3 to 235 in Experiment II. The corresponding log-transformed averages were 0.1–2.5 (Experiment I) and −0.3 to 2.4 (Experiment II). The experiment-wide average severity (log-transformed) was 1.37 and 0.91, and the average variance among the three replicate ramets per F_1 was 0.23 and 0.34, in Experiments I and II, respectively. The log-transformed disease severity in the susceptible parent (S20) was 1.9 and 1.5, and in the resistant parent (R2) 0.7 and 1.2, for Experiments I and II, respectively. There was transgressive segregation toward susceptibility and resistance in both experiments. Ramets of the susceptible cultivar 'Jet' that were placed randomly among the F_1s had average disease severities of 2.4 and 2.1, and variances of 0.03 and 0.07, in Experiments I and II, respectively.

Heritability estimates for stem rust reaction, expressed as genotype variance divided by the total variance that had been adjusted for block effects (Calenge et al. 2004), were 0.78 and 0.75 in Experiments I and II, respectively (Table 3). The two experiments differed in favorability of the environment for disease, so that there was a large main

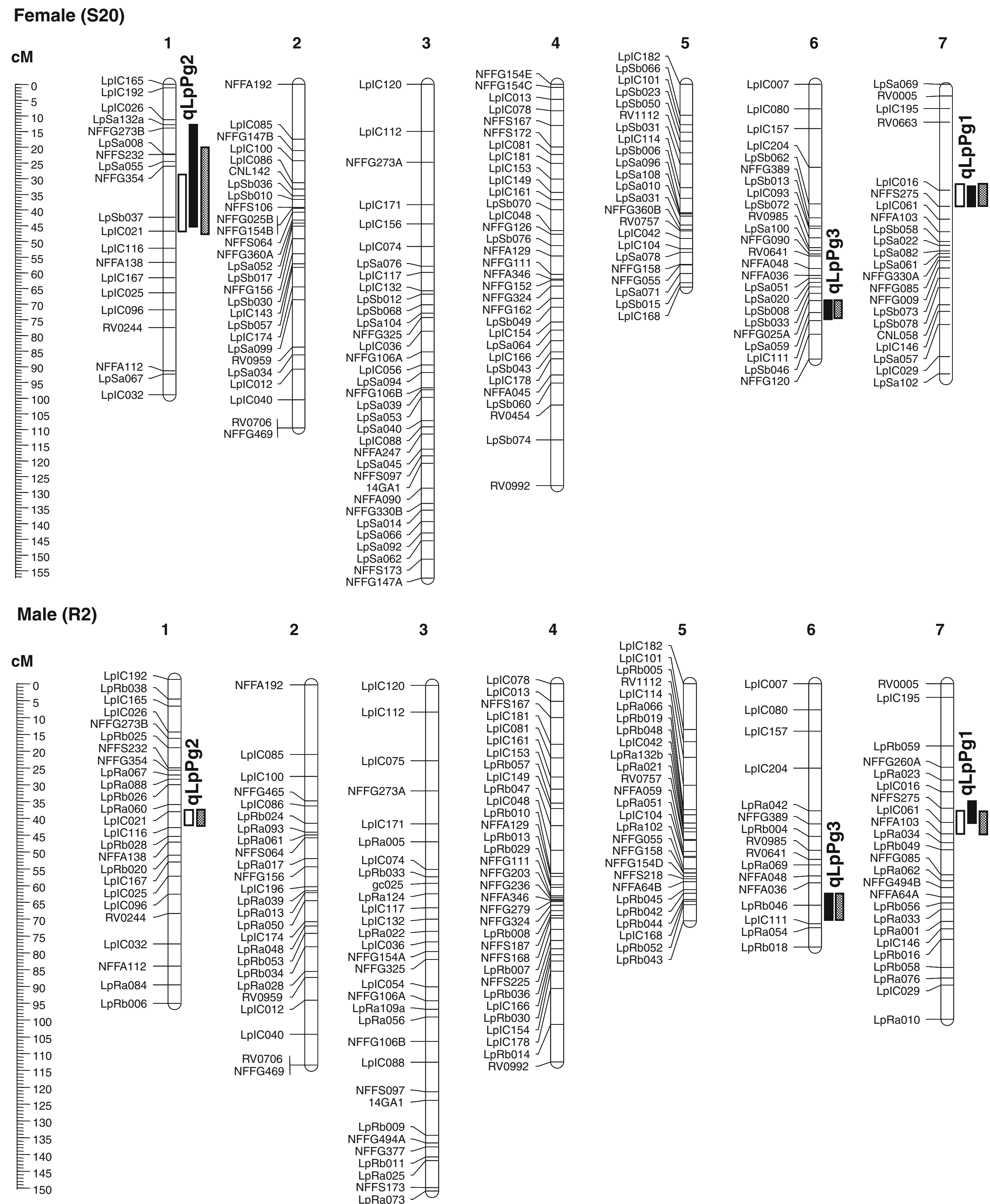

Fig. 2 Linkage maps of the rust susceptible female parent (S20) and rust resistant male parent (R2) used in QTL mapping for stem rust resistance in the S20 × R2 'Kingston' perennial ryegrass population. SSR markers (prefixed NFFA, NFFG, RV, and CNL), STS markers (prefixed NFFS) and RAD tag markers (prefixed LpSa/Sb, LpRa/Rb, and LpIC) were used in map construction. Linkage group numbering conforms to standard *Lolium* maps based on common SSR markers (Gill et al. 2006). Map distance scales in cM are placed at left margin. Location of QTL (qLpPg_) for resistance to a mixed inoculum of *P. graminis* subsp. *graminicola* is indicated for Experiment I (*white bar*), Experiment II (*black bar*) and their pooled values (*gray bar*)

effect of environment on the level of disease response across all genotypes (Table 3). The difference in relative expression of resistance among genotypes across environments is expressed as the genotype × environment factor. Heritability across experiments, expressed as the genotype variance divided by the sum of genotype, genotype × environment and residual variances, was 0.66 (Table 3).

QTL analysis

Three QTL for resistance to the stem rust pathogen *Puccinia graminis* subsp. *graminicol*a were identified consistently in this mapping population (Fig. 2; Table 4). The level of variability in the association of phenotype values with genetic loci was moderately high but consistent across experiments and parental maps, as indicated by LOD thresholds of 4.1–4.3 among the analyses. The most prominent QTL, which we designate qLpPg1, was located on LG7. qLpPg1 is well-defined (LOD scores 14.5–20.5, 2-LOD intervals <8 cM) on the male and female maps. Proportion of the phenotypic variance explained by qLpPg1 ranged from 26 to 37%. A QTL designated qLpPg2, on LG1, had significant LOD scores of 4.2–5.6 in 5 of the 6 analyses across datasets and parents. The male map for Experiment II had a local peak at this location with a non-significant LOD score of 3.6. The 2-LOD intervals for qLpPg2 among the three data sets (Experiment I, II and pooled) overlapped within each parental map, but the location of the peak differed substantially between Experiments I and II. The pooled data suggest that Experiment I indicates the more likely location for qLpPg2. A third QTL, (qLpPg3), was located on LG6 in Experiment II and the pooled data. This QTL had a consistent location in the two parental maps. There was a non-significant (LOD 2.4–3.1), but distinct local peak in LOD scores at this location in Experiment I for both the female and male maps, and the pooled phenotype data strongly supported the existence of qLpPg3. The total proportion of phenotypic variance explained by all QTL with significant peaks was 40–46% in Experiments I and II, and 54–55% in the pooled data. The ranges in proportion of phenotypic variances explained by significant peaks of the individual QTLs qLpPg1, qLpPg2 and qLpPg3 were 26–37, 7–12 and 7–11%, respectively.

Several markers showed consistent association with QTL peaks, as well as significant Kruskal–Wallis (K-W) scores indicating association of genotype with phenotype.

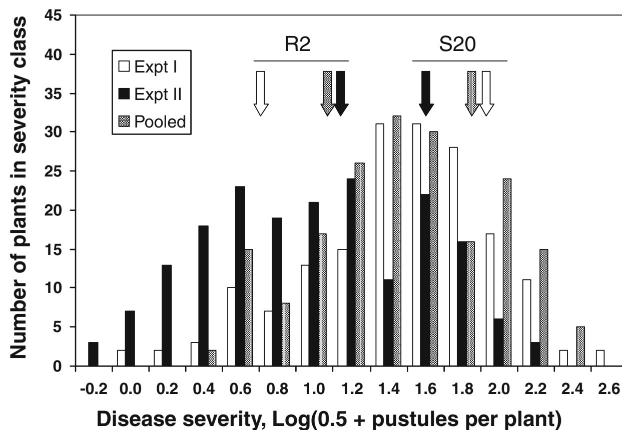

Fig. 3 Frequency distribution of rust severity (log-transformed number of pustules per plant, averaged over 3 ramets) in the *L. perenne* mapping population inoculated with field-collected urediniospores of *P. graminis* subsp. *graminicola* under greenhouse conditions. *Arrows* indicate disease severity phenotypes of male (R2) and female (S20) parents in each dataset

Table 3 Analysis of variance and heritability calculations for stem rust reaction in perennial ryegrass cvar Kingston

Experiment	Factor	*df*	SS	MS	*p*	H[a]
I	Genotype	186	114.0	0.61	<0.001	0.78
	Residual	359	63.7	0.18		
	Total	545	177.8			
II	Genotype	188	183.6	0.98	<0.001	0.75
	Residual	378	120.7	0.32		
	Total	566	304.3			
Pooled	Genotype	170	213.7	1.26	<0.001	0.66
	Expt	1	67.2	67.17	<0.001	
	Genotype × Expt	170	67.0	0.39	<0.001	
	Residual	675	166.2	0.25		
	Total	1,016	516.4	0.51		

Data were first adjusted for block effect (3 randomized complete blocks, 1 ramet of each F_1 plant per block), then subjected to ANOVA to obtain variances for heritability calculation. Kingston F_1 mapping population was inoculated with *Puccinia graminis* subsp. *graminicola* under greenhouse conditions

[a] Heritability is genotype variance as a proportion of genotype + residual variances (Experiments I and II) or as proportion of genotype + genotype × experiment + residual variances (pooled data)

Table 4 QTL (quantitative trait locus) analysis for stem rust resistance in perennial ryegrass F_1 mapping population inoculated with *Puccinia graminis* subsp. *graminicola* under greenhouse conditions

QTL	Linkage group	Parent	Experiment[a]	LOD threshold[b]	Peak (cM)	LOD[c]	2-LOD window (cM)	Nearest marker[d]				
								Marker	K-W *p* value	% variance	Allele configuration	Resistance allele source
qLpPg1	7	Female	I	4.1	40.9	15.6	36–43	NFFS275	0.0001	35.2	ef × eg	Male
			II	4.1	40.9	11.5	35–43	NFFS275	0.0001	26.4	ef × eg	Male
			Pooled	4.1	40.9	17.9	36–43	NFFS275	0.0001	35.8	ef × eg	Male
		Male	I	4.1	39.0	16.5	37–44	NFFS275	0.0001	33.9	ef × eg	Male
			II	4.1	39.0	14.5	34–41	NFFS275	0.0001	31.1	ef × eg	Male
			Pooled	4.3	40.0	20.5	37–45	LpIC061	0.0005	36.9	hk × hk	Male + female
qLpPg2	1	Female	I	4.1	46.3	5.6	31–50	LpIC021	0.05	9.2	hk × hk	Male + female
			II	4.1	24.6	4.2	15–48	LpSa055	0.05	7.1	ll × lm	Female
			Pooled	4.1	46.3	5.1	23–51	LpIC021	ns	8.6	hk × hk	Male + female
		Male	I	4.1	39.3	6.5	37–42	LpIC021	0.05	12.0	hk × hk	Male + female
			II	4.1	26.3	3.6 ns	26–28	NFFS232	0.05	6.3	nn × np	Male
			Pooled	4.3	38.2	5.4	37–43	LpRa060	0.05	9.6	nn × np	Male
qLpPg3	6	Female	I	4.1	74.8	3.1 ns	65–78	LpIC111	ns	6.1	hk × hk	Male + female
			II	4.1	74.8	4.1	72–79	LpIC111	0.01	10.9	hk × hk	Male + female
			Pooled	4.1	74.8	4.4	72–78	LpIC111	0.01	10.7	hk × hk	Male + female
		Male	I	4.1	71.4	2.4 ns	53–73	LpIC111	ns	4.4	hk × hk	Male + female
			II	4.1	70.9	4.5	65–73	LpIC111	0.01	9.2	hk × hk	Male + female
			Pooled	4.3	71.4	4.7	65–73	LpIC111	0.01	7.5	hk × hk	Male + female

Disease severity scored as number of pustules per plant, averaged over 3 ramets per F_1 plant per experiment

[a] QTL analyses were done with each dataset from two independent experiments and with a dataset of results pooled over the two experiments

[b] Threshold of LOD values for significance at p 0.05, determined from 1,000 permutations of the experimental data

[c] ns: QTL peak is lower than threshold value, but included in table to indicate size of local peak near the QTL site

[d] The marker located closest to the position of the peak of LOD scores for the QTL. NFFS275 and NFFS232 are SSR markers, the others are RAD markers. Values are given for the Kruskal–Wallis test, proportion of phenotypic variance explained by the genotype at this marker, the configuration as coded in JoinMap, and the parental source of the allele associated with the greatest average rust resistance response

qLpPg1 was consistently associated with marker NFFS275, (Table 4) which had K-W significance levels of 0.0001. Rust resistance at qLpPg1 was associated with inheritance of the allele from the more resistant parent (R2, male), and with homozygous inheritance of the C…G allele at the intercross marker LpIC061 (C/A…G/A two-SNP marker). The stem rust reaction of the heterozygote for LpIC061 was intermediate between those of the two homozygotes. For qLpPg2, the nearest marker in several analyses was the RAD intercross marker LpIC021. Maximum rust resistance at qLpPg2 was associated with homozygosity of the C allele of a C/G SNP, and the heterozygote of LpIC021 was intermediate in rust reaction between the two homozygotes. Rust resistance was associated with inheritance of the male (R2)-specific alleles from the closely linked flanking markers LpRa060 and NFFS232, and from the female (S20) allele of LpSa055. The marker consistently associated with qLpPg3 was LpIC111. Unlike qLpPg1 and qLpPg2, however, resistance was most strongly associated with the heterozygous genotype (C/T) at LpIC111, and alleles from both parents were associated with resistance in linked SSR and RAD markers.

Discussion

Development of RAD tag markers and genotyping-by-sequencing are technologies not previously applied to de novo marker discovery and map construction in a genetically heterogeneous, outcrossing species such as *Lolium*. The utility of RAD markers has been demonstrated for bulk segregant analysis and fine mapping in species where tag sequences can be directly mapped onto reference genomes (Baird et al. 2008; Lewis et al. 2007; Miller et al. 2007; Nipper et al. 2009), and for linkage map construction in a doubled haploid barley population (Chutimanitsakun et al. 2011) where progeny are expected to exhibit homozygosity at all loci. The genetic heterogeneity of *Lolium* presented unique challenges for both marker discovery and genotyping using the RAD system.

The *Lolium* genome yielded approximately 17,000 raw sequence clusters from parental DNA, of which 22% were eliminated due to low (<7 reads) or high (>200 reads) sequence abundance. High abundance sequences are presumed to derive from repetitive DNA. The median sequence depth for the remaining clusters was ~35×, providing sufficient confidence to proceed with marker identification. We tested the effect of grouping putative SNP alleles based on 1 or 2 nt differences between aligned 29-nt cluster sequences and found that the less stringent threshold produced 30% more potential markers while reducing the number of so-called "dominant" markers. "Dominant" RAD tag clusters are those that occur in one parent but are missing in the other, presumably due to sequence variation in *Sbf*I sites. The increase in SNP marker yield at the expense of "dominant" markers, however, indicated that some of the "dominant" RAD tags had cognate alleles among other RAD clusters that were unidentified with a 1-nt threshold because they differed by two or more SNPs. The sequencing depth used for marker discovery in S20 and R2 parents (~1 million sequences per parent) was adequate to discover most RAD tag loci, as the number of non-repetitive clusters obtained (~14,000) was similar to the number of 8-bp *Sbf*I sites expected in diploid *Lolium* based on published information estimating genome size, GC content, and repetitive DNA content (Arumuganathan et al. 1999).

The fraction of RAD tags that is polymorphic in any cross is dependent upon the frequency of nucleotide sequence variability (SNPs and indels) at homologous sites in the DNAs under study. Although knowledge of SNP frequency in *Lolium* is limited, Cogan et al. (2006) reported a rate of one per 54 bp in a sample of 100 genic sequences from two disparate, non-inbred parents (up to four haplotypes), and a study of nucleotide diversity in five *Lolium* genes sequenced from 20 diploid genotypes estimated one SNP per 94 bp between pairs of randomly selected haplotypes (Brazauskas et al. 2010). In the present study, summing the "dominant" clusters (4,763, divided between S20 and R2) and SNP/indel polymorphisms (1733) suggested that approximately one-third of the non-repetitive RAD clusters discovered were polymorphic in one way or another. Considering that each RAD cluster interrogated 31 bp of sequence (*Sbf*I site plus 23 nucleotides of flanking sequence), this result is in rough agreement with published *Lolium* SNP frequency estimates. Although a larger than expected fraction of the RAD tags was classified as "dominant", this may reflect limitations in the criteria used to group clusters, as discussed above.

The ~50 M sequences we obtained from 188 F_1 libraries permitted reliable scoring of only ~25% of the potential RAD tag SNP markers identified in parents. This could be attributed in part to substantial variation in Solexa reads from the progeny libraries. The cause of read number differences is unknown but has been observed in other studies (Baird et al. 2008) and may be due to differences in restriction enzyme digestibility among DNA prepared from different individuals. Greater sequencing depth than used in the present study would allow progeny genotypes to be determined for all polymorphic loci. The full utilization of RAD tag markers and additional benefits of genotyping-by-sequencing will be realized as sequencing costs decline.

Segregation distortion is a feature of most *Lolium* mapping populations (Anhalt et al. 2008; Armstead et al. 2002; Bert et al. 1999; Faville et al. 2004; Gill et al. 2006; Jensen et al. 2005; Jones et al. 2002; Studer et al. 2010; Warnke et al. 2004), and is often attributed to the two-locus (SZ), multiallelic, gametophytic incompatibility system that prevents selfing in *Lolium* (Jones et al. 2002; Anhalt et al. 2008). The S20 × R2 F_1 population exhibited pronounced segregation distortion on LG2, in the vicinity of the Z locus as mapped in the *L. perenne* p150/112 mapping family (Thorogood et al. 2002). Our data suggest that segregating Z alleles contributed by one of the grandparents of the S20 × R2 population were responsible for the distortion we observed, but lack of direct bridging markers shared with the p150/112 population precludes definitive co-localization. We found no distortion on LG1, site of the S locus (Thorogood et al. 2002), but LG3 and LG5 were moderately distorted. Additional loci affecting self-incompatibility and proposed to interact with S and Z alleles have been localized to LG3 and LG5 (Thorogood et al. 2005). Considering that both male and female parents of the S20 × R2 population were drawn from the same 'Kingston' population, it is possible that these parents carried identical alleles at one or more self-incompatibility loci.

We identified three QTL (on LGs 1, 6 and 7) associated with the stem rust reaction of our perennial ryegrass population to a genetically heterogeneous inoculum of *P. graminis* subsp. *graminicola*. The QTL of largest effect was qLpPg1 on LG7, explaining 26–37% of the variance in stem rust reaction in these experiments. Markers with such a strong effect are expected to behave in a nearly Mendelian fashion and to be useful in applied breeding programs (Muylle et al. 2005). We note that plants with resistant alleles at this QTL are not qualitatively resistant to the stem rust inoculum we used, in that very few plants showed a completely resistant response. The quantitative (albeit large) effect we observed could be due to partial resistance against all components of the rust population, or qualitative effects that completely prevent infection by only some pathogen genotypes in the inoculum. Further experiments with single-pustule isolates of this pathogen (Pfender 2009b) are in progress to provide more information. Analysis of resistance in *Lolium* to another rust disease, crown rust (caused by *Puccinia coronata*), also identified a QTL on LG7

(Sim et al. 2007). We do not have in-common markers to precisely compare the location of this LG7 crown rust QTL with our qLpPg1, but both QTL are located in the central portion of the linkage group.

The QTL we identified on LG1, qLpPg2, is less consistent in location than qLpPg1 but its 2-LOD intervals overlap each other among the experiments (Table 4). One allele of the intercross (hk × hk) marker LpIC021, heterozygous in both parents, is associated with resistance. The allele of the testcross (nn × np) marker LpRa060 associated with resistance at this QTL is derived from the male parent, and the resistance-associated allele of testcross (ll × lm) marker Sa055 is derived from the female parent (Table 4). A female testcross (ll × lm) marker, LpSb037, appears to have its distinguishing allele linked in repulsion to the QTL. This contribution of resistance from both parents, and the repulsion linkage of distinguishing alleles, may account for the breadth and inconsistent peak location of qLpPg2. QTL for crown rust have been identified in the telomeric region on LG1 (Muylle et al. 2005; Studer et al. 2007). Our stem rust QTL on LG1, qLpPg2, is located centrally on the linkage group. However, we detected another QTL on LG1 in the female map of Experiment I only (data not shown). The nearest marker is RV0244 (configuration ab × cd), and alleles associated with the resistant phenotype originated in both the male and female parents. This QTL has a significant LOD score of 6.1, is located at 86.5 cM and its 2-LOD interval extends from 64 cM to the telomere. Therefore, it is in the same region of LG1 as the crown rust QTL detected by Muylle et al. (2005) and by Studer et al. (2007).

On LG6 we identified qLpPg3 on male and female maps. The intercross marker LpIC111 is heterozygous in both parents (hk × hk), and the resistant phenotype is associated with the hk genotype in the F_1 population. The nearby testcross markers LpSa059 (female map) and LpRa069 (male map) have distinguishing alleles associated with susceptible and resistant phenotypes, respectively. The female-specific allele of nearby SSR marker NFFA036 (ef × eg configuration) is associated with the resistant phenotype. Resistance at this QTL thus appears to be contributed by sequences originating in both male and female parents. A QTL for crown rust resistance has been identified on LG6 (Sim et al. 2007). Examination of that map and several others that provide indirect bridging to our map suggests that this crown rust QTL may be located about 30 cM from qLpPg3.

In addition to the three QTL presented in Table 4, we detected a local peak (K-W scores of 0.05, non-significant LOD scores of 3.4) near marker LpIC143 in the center region of LG2 on the female map in both experiments. The maps of Armstead et al. (2002) and Gill et al. (2006) provide indirect bridging markers between our map and that of Dumsday et al. (2003), indicating that this peak is in the same region as a crown rust QTL (LpPc1) they identified (Dumsday et al. 2003).

A previous report of QTL analysis for stem rust reaction in *Lolium* (Jo et al. 2008) identified a QTL on LG4, but we did not detect this QTL in the present study. The difference between reports could be due to genetic differences in the inoculum and/or differences in host resistance traits. The phenotyping experiments reported in Jo et al. (2008) were conducted by one of us (Pfender) under similar conditions to those reported in this paper. The inoculum was collected from a mixture of perennial ryegrass cultivars grown in the same geographic region for both investigations, although separated in time by 4 years. There is probably little selection pressure for rapid changes in pathogen population virulence genetic frequencies, because all *Lolium* cultivars in the region are similar in lacking effective stem rust resistance. The mapping population used in the previous study (Jo et al. 2008) was initially developed to investigate a different phenotype (perennial vs. annual habit) and did not have a high level of expression for stem rust resistance. In contrast, the mapping population used in the present study was developed from parents selected for strongly divergent stem rust reaction in the initial testing with a mixed inoculum collected in 2005, and from a cultivar developed in a different geographical location (New Zealand) than the plant population used by Jo et al. (2008).

Genomic organization in cereal grasses of the Triticeae, which are hosts to stem rust fungi closely related to the ryegrass pathogen, shows broad synteny with linkage groups in *Lolium* (Sim et al. 2005). A number of stem rust resistance loci in cereals have been mapped. Examples are: barley loci Rpg1 and Rpg5 mapped to LG 1 and 7, respectively (Brueggeman et al. 2002, 2008); wheat loci Sr31, Sr6 and Sr2 mapped to LGs 1, 2 and 3, respectively (Mago et al. 2005; Tsilo et al. 2009; Spielmeyer et al. 2003); oat loci pg13 and pg9 mapped to LGs 3 and 4, respectively (O'Donoughue et al. 1996). Rust resistance loci appear on all linkage groups in *Lolium*. Stem rust resistance QTL are reported for *Lolium* LGs 1, 6, 7 (this study) and 4 (Jo et al. 2008); crown rust resistance QTL are reported for all *Lolium* LGs (Dumsday et al. 2003; Muylle et al. 2005; Schejbel et al. 2007; Sim et al. 2007; Studer et al. 2007). Despite the broad synteny between linkage groups of *Lolium* and the cereals, one comparative study showed no statistically significant genome-wide correspondence between *Lolium* and cereals in location of genes for resistance to similar pathogens (Jo et al. 2008). The large number and diverse genomic locations of rust resistance genes in *Lolium* and cereals will necessitate analysis of more detailed genomic information than is presently available to determine synteny of these loci.

Co-segregation of linked markers with a trait of interest is a powerful tool for validating QTLs, identified initially in

a segregating population, in additional lines and for marker-assisted selection in breeding schemes (Khedikar et al. 2010; Zhou et al. 2003). Sequence-based markers such as SSRs have been preferred for this function due to their transferability, ease of assay, and co-dominant and highly polymorphic nature. The moderate density maps we developed using a combination of SSR and RAD tag markers enabled localization of a prominent stem rust QTL (qLpPg1) with 2-LOD support intervals only a few cM in length. There are closely associated RAD markers for each of the QTL we identified. Paired-end Solexa sequencing of S20 and R2 parental DNA libraries produced contigs ranging in size from 100 to 400 bp flanking RAD sites (unpublished data). These contigs can be used to develop sequence-based assays (e.g., SNP, indel, CAPs) for the *Lolium* rust-resistance QTL we detected. Although the male parent was more rust resistant than the female parent, both parents contributed alleles associated with resistance (Table 4). Therefore, some of the F_1 individuals had reassortment combinations with more resistance-associated alleles, and other individuals had fewer resistance-associated alleles, than either parent. For example, each parent had genotype hk for the marker LpIC021 (Table 4), and the average rust phenotypes of F_1 genotypes hh and kk were more and less resistant, respectively, than the average phenotype of the hk genotype. Genetic markers for alleles such as these could be useful in selection of transgressive segregants with improved resistance.

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References

Anhalt UCM, Heslop-Harrison P, Byrne S, Guillard A, Barth S (2008) Segregation distortion in *Lolium*: evidence for genetic effects. Theor Appl Genet 117:297–306

Armstead IP, Turner LB, King IP, Cairns AJ, Humphreys MO (2002) Comparison and integration of genetic maps generated from F_2 and BC_1-type mapping populations in perennial ryegrass. Plant Breed 121:501–507

Arumuganathan K, Tallury SP, Fraser ML, Bruneau AH, Qu R (1999) Nuclear DNA content of thirteen turfgrass species by flow cytometry. Crop Sci 39:1518–1521

Baird NA, Etter PD, Atwood TS, Currey MC, Shiver AL, Lewis ZA, Selker EU, Cresko WA, Johnson EA (2008) Rapid SNP discovery and genetic mapping using sequenced RAD markers. PLoS One 3:e3376

Bert PF, Charmet G, Sourdille P, Hayward MD, Balfourier F (1999) A high-density molecular map for ryegrass (*Lolium perenne*) using AFLP markers. Theor Appl Genet 99:445–452

Brazauskas G, Pasakinskiene I, Asp T, Lubberstedt T (2010) Nucleotide diversity and linkage disequilibrium in five *Lolium perenne* genes with putative role in shoot morphology. Plant Sci 179:194–201

Brueggeman R, Rostoks N, Kudrna D, Kilian A, Han F, Chen J, Druka A, Steffenson B, Kleinhofs A (2002) The barley stem rust-resistance gene Rpg1 is a novel disease-resistance gene with homology to receptor kinases. PNAS 99:9328–9333

Brueggeman R, Druka A, Nirmala J, Cavileer T, Drader T, Rostoks N, Mirlohi A, Bennypaul H, Gill U, Kudrna D, Whitelaw C, Kilian A, Han F, Sun Y, Gill K, Steffenson B, Kleinhofs A (2008) The stem rust resistance gene Rpg5 encodes a protein with nucleotide-binding-site, leucine-rich, and protein kinase domains. PNAS 105:14970–14975

Calenge F, Faure A, Goerre M, Gebhardt C, Van de Weg WE, Parisi L, Durel CE (2004) Quantitative trait loci (QTL) analysis reveals both broad-spectrum and isolate-specific QTL for scab resistance in apple progeny challenged with eight isolates of *Venturia inaequalis*. Phytopathology 94:370–379

Chutimanitsakun Y, Nipper RW, Cuesta-Marcos A, Cistue L, Corey A, Filichkina T, Johnson EA, Hayes PM (2011) Construction and application for QTL analysis of a restriction site associated DNA (RAD) linkage map in barley. BMC Genomics 12:4

Cogan NOI, Ponting RC, Vecchies AC, Drayton MC, George J, Dracatos PM, Dobrowolski MP, Sawbridge TI, Smith KF, Spangenberg GC, Forster JW (2006) Gene-associated single nucleotide polymorphism discovery in perennial ryegrass (*Lolium perenne* L.). Mol Gen Genomics 276:101–112

Dumsday JL, Smith KF, Forster JW, Jones ES (2003) SSR-based genetic linkage analysis of resistance to crown rust (*Puccinia coronata* f. sp *lolii*) in perennial ryegrass (*Lolium perenne*). Plant Pathol 52:628–637

Faville MJ, Vecchies AC, Schreiber M, Drayton MC, Hughes LJ, Jones ES, Guthridge KM, Smith KF, Sawbridge T, Spangenberg GC, Bryan GT, Forster JW (2004) Functionally associated molecular genetic marker map construction in perennial ryegrass (*Lolium perenne* L.). Theor Appl Genet 110:12–32

Fischer BM, Salakhutdinov I, Akkurt M, Eibach R, Edwards KJ, Töpfer R, Zyprian EM (2004) Quantitative trait locus analysis of fungal disease resistance factors on a molecular map of grapevine. Theor Appl Genet 108:501–515

Gill GP, Wilcox PL, Whittaker DJ, Winz RA, Bickerstaff P, Echt CE, Kent J, Humphreys MO, Elborough KM, Gardner RC (2006) A framework linkage map of perennial ryegrass based on SSR markers. Genome 49:354–364

Grattapaglia D, Sederoff R (1994) Genetic linkage maps of *Eucalyptus grandis* and *Eucalyptus urophylla* using a pseudo-testcross mapping strategy and RAPD markers. Genetics 137:1121–1137

Hampton JG (1986) Fungicidal effects on stem rust, green leaf area and seed yield in 'Grasslands Nui' perennial ryegrass. NZ J Exp Agric 14:7–12

Jensen LB, Muylle H, Arens P, Andersen CH, Holm PB, Ghesquiere M, Julier B, Lubberstedt T, Nielsen KK, De Riek J, Roldan-Ruiz I, Roulund N, Taylor C, Vosman B, Barre P (2005) Development and mapping of a public reference set of SSR markers in *Lolium perenne* L. Mol Ecol Notes 5:951–957

Jo YK, Barker R, Pfender W, Warnke S, Sim SC, Jung G (2008) Comparative analysis of multiple disease resistance in ryegrass and cereal crops. Theor Appl Genet 117:531–5434

Jones ES, Mahoney NL, Hayward MD, Armstead IP, Jones JG, Humphreys MO, King IP, Kishida T, Yamada T, Balfourier F, Charmet G, Forster JW (2002) An enhanced molecular marker based genetic map of perennial ryegrass (*Lolium perenne*) reveals comparative relationships with other Poaceae genomes. Genome 45:282–295

Kantety RV, Rota ML, Matthews DE, Sorrells ME (2002) Data mining for simple sequence repeats in expressed sequence tags from barley, maize, rice, sorghum and wheat. Plant Mol Biol 48:501–510

Khedikar YP, Gowda MVC, Sarvamangala C, Patgar KV, Upadhyaya HD, Varshney RK (2010) A QTL study on late leaf spot and rust

revealed one major QTL for molecular breeding for rust resistance in groundnut (*Arachis hypogaea* L.). Theor Appl Genet 121:971–984

Lauvergeat V, Barre P, Bonnet M, Ghesquiere M (2005) Sixty simple sequence repeat markers for use in the *Festuca–Lolium* complex of grasses. Mol Ecol 5:401–405

Lewis ZA, Shiver AL, Stiffler N, Miller MR, Johnson EA, Selker EU (2007) High-density detection of restriction-site-associated DNA markers for rapid mapping of mutated loci in Neurospora. Genetics 177:1163–1171

Mago R, Miah H, Lawrence GJ, Wellings CR, Spielmeyer W, Bariana HS, McIntosh RA, Pryor AJ, Ellis JG (2005) High-resolution mapping and mutation analysis separate the rust resistance genes Sr31, Lr26 and Yr9 on the short arm of rye chromosome 1. Theor Appl Genet 112:41–50

Mehlenbacher SA, Brown RN, Nouhra ER, Gokirmak T, Bassil NV, Kubisiak TL (2006) A genetic linkage map for hazelnut (*Corylus avellana* L.) based on RAPD and SSR markers. Genome 49:122–133

Miller MR, Dunham JP, Amores A, Cresko WA, Johnson EA (2007) Rapid and cost-effective polymorphism identification and genotyping using restriction site associated DNA (RAD) markers. Genome Res 17:240–248

Muylle H, Baert J, Van Bockstaele E, Petris J, Roldan-Ruiz I (2005) Four QTLs determine crown rust (*Puccinia coronata* f. sp. *lolii*) resistance in a perennial ryegrass (*Lolium perenne*) population. Heredity 95:348–357

Nipper RW, Atwood TS, Boone JQ, Gribbin JM, Johnson EA (2009) SNP discovery in *Zea mays* using sequenced restriction-site associated DNA markers. Acta Hortic 859:129–133

O'Donoughue LS, Chong J, Wright CP, Fedak G, Molnar SJ (1996) Localization of stem rust resistance genes and associated molecular markers in cultivated oat. Phytopathology 86:719–727

Pfender WF (2001) A temperature-based model for latent period duration in stem rust of perennial ryegrass and tall fescue. Phytopathology 91:111–116

Pfender WF (2003) Prediction of stem rust infection favorability, by means of degree-hour wetness duration, for perennial ryegrass seed crops. Phytopathology 93:467–477

Pfender WF (2009a) A damage function for stem rust of perennial ryegrass seed crops. Phytopathology 99:498–505

Pfender WF (2009b) Demonstration of pathotype specificity in stem rust of perennial ryegrass. Phytopathology 99:1185–1189

Rose-Fricker CA, Meyer WA, Kronstad WE (1986) Inheritance of resistance to stem rust (*Puccinia graminis* subsp. *graminicola*) in six perennial ryegrass (*Lolium perenne*) crosses. Plant Dis 70:678–681

Saha MC, Mian R, Eujayl I, Zwonitzer JC, Wang L, May GD (2004) Tall fescue EST-SSR markers with transferability across several grass species. Theor Appl Genet 109:783–791

Saha MC, Mian R, Zwonitzer JC, Chekhovskiy K, Hopkins AS (2005) An SSR- and AFLP-based genetic linkage map of tall fescue (*Festuca arundinacea* Schreb.). Theor Appl Genet 110:323–336

Schejbel B, Jensen LB, Xing Y, Lubberstedt T (2007) QTL analysis of crown rust resistance in perennial ryegrass under conditions of natural and artificial infection. Plant Breed 126:347–352

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotechnol 18:233–234

Sim S, Chang T, Curley J, Warnke SE, Barker RE, Jung G (2005) Chromosomal rearrangements differentiating the ryegrass genome from the Triticeae, oat, and rice genomes using common heterologous RFLP probes. Theor Appl Genet 110:1011–1019

Sim S, Diesburg K, Casler M, Jung G (2007) Mapping and comparative analysis of QTL for crown rust resistance in an Italian × perennial ryegrass population. Phytopathology 97:767–776

Sim S, Yu J, Jo Y, Sorrells ME, Jung G (2009) Transferability of cereal EST-SSR markers to ryegrass. Genome 52:431–437

Spielmeyer W, Sharp PJ, Lagudah ES (2003) Identification and validation of markers linked to broad-spectrum stem rust resistance gene Sr2 in wheat (*Triticum aesetivum* L.). Crop Sci 43:333–336

Steiner JJ, Poklemba CJ, Fjellstrom RG, Elliot LF (1995) A rapid one-tube genomic DNA extraction process for PCR and RAPD analyses. Nucleic Acids Res 23:2569–2570

Studer B, Boller B, Bauer E, Posselt UK, Widmer F, Kolliker R (2007) Consistent detection of QTL's for crown rust resistance in Italian ryegrass (*Lolium multiflorum* Lam.) across environments and phenotyping methods. Theor Appl Genet 115:9–17

Studer B, Kolliker R, Muylle H, Asp T, Frei U, Roldan-Ruiz I, Barre P, Tomaszewski C, Meally H, Barth S, Skot L, Armstead IP, Dolstra O, Lubberstedt T (2010) EST-derived SSR markers used as anchor loci for the construction of a consensus linkage map in ryegrass (*Lolium* spp.). BMC Plant Biol 10:177

Thorogood D, Kaiser WJ, Jones JG, Armstead I (2002) Self-incompatibility in ryegrass 12. Genotyping and mapping the *S* and *Z* loci of *Lolium perenne* L. Heredity 88:385–390

Thorogood D, Armstead IP, Turner LB, Humphreys MO, Hayward MD (2005) Identification and mode of action of self-compatibility loci in *Lolium perenne* L. Heredity 94:356–363

Tsilo TJ, Chao S, Jin Y, Anderson JA (2009) Identification and validation of SSR markers linked to the stem rust resistance gene Sr6 on the short arm of chromosome 2D in wheat. Theor Appl Genet 118:515–524

Van Ooijen JW (2004) MapQTL5, software for the mapping of quantitative trait loci in experimental populations. Kyazma B.V., Wageningen, Netherlands

Van Ooijen JW (2006) JoinMap 4, Software for the calculation of genetic linkage maps in experimental populations. Kyazma B.V., Wageningen, Netherlands

Van Tassell CP, Smith TPL, Matukumalli LK, Taylor JF, Schnabel RD, Lawley CT, Haudenschild CD, Moore SS, Warren WC, Sonstegard TS (2008) SNP discovery and allele frequency estimation by deep sequencing of reduced representation libraries. Nat Methods 5:247–252

Warnke SE, Barker RE, Jung G, Sim SC, Rouf Mian MA, Saha MC, Brilman LA, Dupal MP, Forster JW (2004) Genetic linkage mapping of an annual × perennial ryegrass population. Theor Appl Genet 109:294–304

Wilcox PL, Amerson HV, Kuhlman EG, Liu BH, O'Malley DM, Sederoff RR (1996) Detection of a major gene for resistance to fusiform rust disease in loblolly pine by genomic mapping. Proc Natl Acad Sci USA 93:3859–3864

Zhou WC, Kolb FL, Bai GH, Domier LL, Boze LK, Smith NJ (2003) Validation of a major QTL for scab resistance with SSR markers and use of marker-assisted selection in wheat. Plant Breed 122:40–46