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ORIGINAL PAPER

Comparative analysis of multiple disease resistance in ryegrass and cereal crops

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Abstract Ryegrass (*Lolium* spp.) is among the most important forage crops in Europe and Australia and is also a popular turfgrass in North America. Previous genetic analysis based on a three-generation interspecific (*L. perenne* × *L. multiflorum*) ryegrass population identified four quantitative trait loci (QTLs) for resistance to gray leaf spot (*Magneporthe grisea*) and four QTLs for resistance to crown rust (*Puccinia coronata*). The current analysis based on the same mapping population detected seven QTLs for resistance to leaf spot (*Bipolaris sorokiniana*) and one QTL for resistance to stem rust (*Puccinia graminis*) in ryegrass for the first time. Three QTLs for leaf spot resistance on linkage groups (LGs) 2 and 4 were in regions of conserved synteny to the positions of resistance to net blotch (*Drechslera teres*) in barley (*Hordeum vulgare*). One ryegrass genomic region spanning 19 cM on LG 4, which contained three QTLs for resistance to leaf spot, gray leaf spot, and stem rust, had a syntenic relationship with a segment of rice chromosome 3, which contained QTLs for resistance to multiple diseases. However, at the genome-wide comparison based on 72 common RFLP markers between ryegrass and cereals, coincidence of QTLs for disease resistance to similar fungal pathogens was not statistically significant.

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is one of the important forage and turfgrasses in temperate climate zones in the world. In Europe and Australia, along with Italian ryegrass (*L. multiflorum* Lam.), perennial ryegrass is extensively grown for feeding ruminant livestock. In the northern United States perennial ryegrass is one of the most popular turfgrasses due to its fast establishment, fine texture, and dark green color, providing excellent amenities for golf courses and residential areas. Fungal diseases are often one of the most important factors limiting the successful growth of perennial ryegrass, particularly rusts (*Puccinia* spp.) for forage type, and gray leaf spot [*Magnaporthe grisea* (Hebert) Barr] and leaf spot [*Bipolaris sorokiniana* (Sacc.) Shoemaker] for turf type. Therefore, perennial ryegrass cultivars with improved resistance to major fungal diseases would be desirable in many areas.

Rust diseases make significant damage on quality and yield of forage-type perennial ryegrass. The use of host resistance would be a more practical way to manage rust than fungicide applications. Fungicide use decreases profit margins of low cash-input forage production systems and increases the risk of residual effects from fungicide toxins on forage. Therefore, perennial ryegrass cultivars resistant to rust have been intensively sought and molecular markers associated with resistance to crown rust (*Puccinia coronata* Corda) have been identified in ryegrass (Dumsday et al. 2003; Muylle et al. 2005; Sim et al. 2007). However, there are limited reports on the inheritance of resistance to stem rust (*P. graminis* Pers.:Pers. subsp. *graminicola* Z. Urb.) in perennial ryegrass (Rose-Fricker et al. 1986), and no commercial cultivars with high levels of resistance to this pathogen are available.

Foliar, crown and root diseases caused by *Bipolaris*, *Drechslera* and *Exserohilum* species (previously considered to be in the same genus, *Helminthosporium*, due to similar epidemiology and symptoms) are common and widespread on graminaceous plants. Leaf spot fungus (*B. sorokiniana*) mainly affects foliage of perennial ryegrass, and in severe cases damages crowns, resulting in the killing of numerous tillers in a process known as "melting-out". Thinning, withering, and killing of plants greatly reduce the esthetic quality of turf-type perennial ryegrass. Fungicides are often applied to intensively-managed perennial ryegrass on golf courses, nevertheless, the most effective control is to use host resistance. New Kentucky bluegrass (*Poa pratensis* L.) and perennial ryegrass cultivars have been bred to improve resistance to leaf spot and melting-out since the first resistant Kentucky bluegrass cultivar "Merion" was developed in the 1950s (Vargas 1994). However, no resistance genes for leaf spot have been investigated in turfgrasses.

Breeding multiple disease-resistant cultivars is one of the best disease management strategies for perennial ryegrass, although this may take considerable time and a thorough understanding of the genetics of host resistance. Exploitation of genes conferring quantitative resistance as well as qualitative resistance is ideal for managing different races or multiple pathogen species and precluding rapid breakdown of resistance by pathogens. This would be more likely to occur in response to a limited number of combined race-specific resistance genes. Recently genetic linkage and traits maps using molecular markers have been constructed for forage and turfgrasses, providing the basis of marker-assisted selection for breeding resistance to multiple diseases (Hayward et al. 1998; Bert et al. 1999; Forster et al. 2001; Jones et al. 2002; Warnke et al. 2004; Curley et al. 2005), and allowing comparative genome analysis with model cereals (Alm et al. 2003; Sim et al. 2005). Effective utilization of important genetic information available in cereal crops has facilitated a better understanding of the genetic architecture of disease resistance in understudied ryegrass (Sim et al. 2007).

In previous studies, quantitative trait loci (QTLs) for resistance to gray leaf spot (Curley et al. 2005) and crown rust (Sim et al. 2007) have been identified in a three-generation interspecific (*L. perenne* × *L. multiflorum*) ryegrass mapping population (MFA × MFB). The objectives of this study were (1) to identify QTLs for resistance to stem rust and leaf spot based on the same ryegrass population, and (2) to compare locations of QTLs for resistance to these four fungal diseases identified in the ryegrass population with equivalent genomic regions in cereal crops to determine whether loci for disease resistance are coincident in ryegrass and cereal crops.

Materials and methods

Ryegrass mapping population

The three-generation interspecific MFA × MFB ryegrass mapping population used in this study was previously generated by crossing Italian ryegrass cv. Floregon and perennial ryegrass cv. Manhattan (Warnke et al. 2004). The same set of the ryegrass mapping population (originally 169 progenies) has been clonally maintained at two greenhouses located in Madison, WI and Corvallis, OR and was transplanted in the field to enhance viability periodically every 1–2 years. Along with parents (MFA and MFB) and grandparent plants (Manhattan-1, Manhattan-3 and Floregon), individual progeny was selected depending on the availability of plants for different experiments: 152 progenies for leaf spot evaluation in the field, 89 progenies for leaf spot evaluation in the growth chamber, and 156 progenies for stem rust evaluation in the greenhouse. Tillers were split from each original plant and grown in separate plastic containers (10 cm diameter and 10 cm deep) filled with potting mix (Metro-Mix, Sun Gro, Bellevue, WA, USA) in the greenhouse (20 ± 5°C). Plants were watered daily and fertilized monthly with 0.8 g L^{-1} of Peters fertilizer (N:P:K = 20:20:20).

Field evaluation for resistance to leaf spot

The experiment was a randomized complete block design with five replications repeated in 2004 and 2005. Plots were established on the university research field (15 × 24 m) at the O. J. Noer Turfgrass Research and Education Facility, Verona, WI, USA. Plots were re-established in 2005 with new ryegrass plants after the 2004 trial. Each plot was treated with a non-selective herbicide glyphosate (Roundup, Monsanto, St. Louis, MO, USA) to make a weed-free

circular area (30 cm diameter) and 10 cm spacing between circles. Five ramets of each of the152 ryegrass progenies as well as their parents and grandparents were prepared in the greenhouse as described above. Each of the five ramets were randomly assigned to each block and transplanted at the field in April of both years. Plots were treated once with fertilizer (N:P:K = 24:0:14) immediately after transplantation, and mowed once at a height of 5 cm 4 weeks after this event.

Ten young leaves were randomly selected and the percentage of leaf spot area was visually assessed between June and September when natural infection of leaf spot was apparent. The significance in the difference of leaf spot severity among progeny was determined using analysis of variance (ANOVA) of SAS (SAS Institute Inc., Cary, NC, USA).

Growth chamber evaluation for resistance to leaf spot

The same 89 progenies used for constructing the MFA × MFB ryegrass linkage map by Sim et al. (2005) were selected and assayed for susceptibility to leaf spot in a controlled environment chamber. The experiment was conducted twice with a randomized complete block design with four replications. Four ramets of each of the 89 progenies, including their parents and grandparents were grown in containers (5 cm in diameter and 20 cm deep) containing potting mix. Eighty-nine containers of ryegrass clones were randomly arranged in each container rack for each replication and the rack was considered as a block. Plants were cut at a height of 2 cm. When 3-week regrowth promoted three to four new fully expanded leaves from each tiller, the plants were inoculated.

A single spore isolate of *B. sorokiniana* collected from a ryegrass field in Verona, WI, USA, was used for inoculation. Mycelium of this *B. sorokiniana* isolate was grown on V8 medium under a 24 h fluorescent light at 23°C for 3 weeks. Conidia were collected and diluted with 0.02% Tween 20 solution at the concentration of 10^5 spores ml^{-1} and plants were inoculated with *B. sorokiniana* conidia using an atomizer. The inoculated plants were incubated within plastic containers at 23°C and ~100% relative humidity for the first 24 h and then moved to a growth chamber (23°C; 40 ± 10% relative humidity; and 12 h photoperiod). Percent diseased area on 10 leaves randomly selected was visually measured at 7-day post-inoculation. The significance in the difference of leaf spot severity among the progeny was determined using ANOVA of SAS.

Greenhouse evaluation for resistance to stem rust

A total of 156 progenies of MFA × MFB mapping population along with their parents and grandparents were prepared and inoculated with the stem rust pathogen as described previously (Pfender 2001). In brief, plants grown in containers (3.8 cm in diameter and 24 cm deep) were inoculated with a suspension of urediniospores (5×10^6 spores ml^{-1}) collected from a mixture of perennial ryegrass cultivars grown near Corvallis, OR, USA. Plants were incubated in a mist chamber in the dark at 18 ± 5°C for 15 h, and then exposed to light as the leaves gradually dried. Plants were then maintained in a greenhouse with a 14 h photoperiod and temperatures between 13 and 23°C, without allowing the leaves to become wet. Disease assessment was made at 14 days after inoculation. Disease severity was measured with a 0–5 scale corresponding to per-plant pustule counts of 0, 1–2, 3–10, 11–25, 26–100 and >100, respectively. The experiment was conducted twice with a randomized complete block design with four replicates. The significance in the difference of stem rust severity among the progeny was determined using ANOVA of SAS.

QTL analyses

The linkage map of the MFA × MFB mapping population used in this study was modified from the map previously constructed by Sim et al. (2005). An MFA map and an MFB map were constructed independently using the double haploid population type option of JoinMap 3.0 software (Kyazma, Wageningen, Netherlands) because the parents of the ryegrass population were not inbred lines, but generated from interspecific outcrosses with differing segregation ratios of molecular markers.

Markers were grouped into seven linkage groups (LGs) at a logarithm of odds ratio (LOD) value >7.0 and only first or second round maps were selected for a final map construction using the Kosambi mapping function (Kosambi 1944). A total of 152 markers (43 RAPD and 109 RFLP) were mapped on the MFA genetic map and 135 markers (28 RAPD and 107 RFLP) were mapped on the MFB genetic map. The sources of heterologous cDNA probes for RFLP markers were oat (*Avena sativa* L.) (CDO), barley (*Hordeum vulgare* L.) (BCD), rice (*Oryza sativa* L.) (RZ) and creeping bentgrass (*Agrostis stolonifera* L.) (Ast).

Three QTL analyses including Kruskal–Wallis, interval mapping, and multiple QTL mapping (MQM), were performed based on phenotypic data of disease susceptibility to leaf spot and stem rust collected from the field and inoculation experiments using the software program MapQTL5 (Kyazma, Wageningen, Netherlands). Kruskal–Wallis analysis was performed to determine the significant relationship between the marker and phenotype. Interval mapping analysis was conducted to determine putative significant markers associated with the disease resistance phenotype. Lastly, MQM analysis was performed with significant markers as cofactors, which had LOD >2.0 at

the interval mapping and at the same time were significantly associated with the phenotype when tested with the automatic selection option of the MapQTL software. The MQM was repeated by selecting different cofactors until a stable LOD profile was obtained. QTLs for disease resistance were declared when LOD values of the interval mapping or MQM analyses were higher than the genome-wide threshold which was determined with the permutation test of MapQTL (Van Ooijen 1999), and/or significance at the Kruskal–Wallis test ($P \leq 0.05$). The maximum LOD value, location on the genetic map, additive marker allele effect, and the proportion of phenotypic variance attributable to the QTL were determined for each QTL. QTL nomenclature was assigned in the form as q-disease-experiment-LG.

Comparative analysis of multiple disease resistance in ryegrass and cereal plants

The locations of QTLs for resistance to four diseases (leaf spot, stem rust, gray leaf spot and crown rust) identified in the MFA × MFB ryegrass population were compared with those previously identified in cereal crops by means of an integrated ryegrass genetic map and a rice physical map. The integrated ryegrass map was constructed from individual MFA and MFB maps based on common RFLP markers using JoinMap. QTLs for resistance to leaf spot, stem rust, gray leaf spot, and crown rust were located on the integrated genetic map.

The rice physical map was constructed based on sequence information generated by the International Rice Genome Sequencing Project, The Institute of Genomic Research (TIGR; http://www.tigr.org/tdb/e2k1/osa1/index.shtml). Forty-six loci for resistance to net blotch [*Drechslera teres* (Sacc.) Shoemaker related with *B. sorokiniana*], stem rust, rice blast (*M. grisea*) and crown rust previously detected in rice, oat, wheat, rye, ryegrass, or barley (Table 1) were located on the rice physical map, based on sequences of RFLP markers linked to those loci. This rice genome database (TIGR) provides search options by which RFLP marker sequences can be located on rice chromosomes. In addition, locations of rice sequences homologous to 228 resistance gene analogs (RGAs) and 326 transcription factors associated with plant defence were also plotted on the rice physical map. RGAs included nucleotide binding sites-leucine rich repeat (NBS-LRR) and nucleotide binding adaptor shared by APAF-1, qualitative resistance (R) proteins, and CED-4. Defence related transcription factors included WRKY, MYB, and basic leucine zipper (bZIP).

Comparative analysis of genomic regions associated with disease resistance in ryegrass and cereal crops was based on common expressed sequence tags (ESTs)-heterologous RFLP markers present in both the ryegrass genetic map and the rice physical map. To increase the number of common RFLP markers closely linked to QTLs for resistance in cereal crops, appropriate bridge maps were searched at web-based cereal databases: Gramene (http://www.gramene.org) and GrainGenes (http://wheat.pw.usda.gov/GG2/index.sthml).

Statistical analysis

To assess the spatial distribution of QTLs for resistance to leaf spot, stem rust, gray leaf spot and crown rust in ryegrass, the linkage map was binned into three different intervals: 34 intervals of ~15 cM, 101 intervals of ~5 cM, and 163 intervals of ~3 cM. The bin with the peak marker was considered to contain QTL. Data of presence or absence of QTLs in the bins were used to calculate probabilities of coincidence of QTLs assuming independence of the locations of QTLs for disease resistance. Chi-square goodness of fit tests were used to assess whether the coincidence of QTLs was more than would be expected by chance based on the probabilities derived from the model of independence.

In addition, the coincidence of loci for disease resistance in ryegrass and cereals was analyzed at 72 common RFLP markers using Cohen's kappa statistic (Cohen 1960), which tests the ratio of the difference between the probabilities of observed and expected (by chance) agreement in presence or absence of the QTL at the common markers to the probabilities of expected disagreement: Kappa = [-P(Obs) − P(Exp)]/[1 − P(Exp)], where P(Obs) and P(Exp) are the observed and expected probabilities of agreement.

Broad sense heritability (H_b^2) was calculated using the formula $H_b^2 = \sigma_g^2/(\sigma_g^2 + \sigma_e^2/n)$, where σ_g^2 is the genetic variance and σ_e^2 is the error variance divided by the number of clonal replicates (n) of each genotype (Calenge et al. 2004).

Results

Assessment of susceptibilities to leaf spot and stem rust

Significant genotypic effects in the MFA × MFB mapping population were detected in susceptibilities to stem rust in the greenhouse experiment ($P < 0.0001$) and to leaf spot in the growth chamber experiment ($P < 0.0001$) (Table 2). Disease severity between repeated inoculation experiments was significantly different for stem rust ($P < 0.0001$) but not for leaf spot ($P = 0.15$) (Table 2). Interaction effect between the experiment and genotype was borderline significant for stem rust ($P = 0.06$) and was significant for leaf spot ($P = 0.03$) (Table 2). This indicated that

Table 1 Disease resistance loci used for constructing integrated disease resistance maps of ryegrass and rice based on linked RFLP markers

Plant	Disease	Pathogen	Trophic level	No. loci	References
Ryegrass	Leaf spot	*Bipolaris sorokiniana*	Necrotrophic	7	Current study
Ryegrass	Stem rust	*Puccinia graminis*	Biotrophic	1	Current study
Ryegrass	Crown rust	*P. coronata*	Biotrophic	4	Sim et al. (2007)
Ryegrass	Gray leaf spot	*Magnaporthe grisea*	Necrotrophic	4	Curley et al. (2005)
Ryegrass	Crown rust	*P. coronata*	Biotrophic	4	Muylle et al. (2005)
Oat	Crown rust	*P. coronata*	Biotrophic	3	Wight et al. (2004)
Oat	Crown rust	*P. coronata*	Biotrophic	2	Bush and Wise (1996)
Oat	Crown rust	*P. coronata*	Biotrophic	1	Rayapati et al. (1994); Yu et al. (2004)
Oat	Stem rust	*P. graminis*	Biotrophic	2	O'Donoughue et al. (1996)
Rice	Rice blast	*M. grisea*	Necrotrophic	6 of 9	Tabien et al. (2002)
Rice	Rice blast	*M. grisea*	Necrotrophic	9 of 10	Wang et al. (1994)
Rice	Rice blast	*M. grisea*	Necrotrophic	2 of 4	Fukuoka and Okuno (2001)
Rice	Rice blast	*M. grisea*	Necrotrophic	9 of 12	Chen et al. (2003)
Rye	Stem rust	*P. graminis*	Biotrophic	1	Mago et al. (2002)
Wheat	Stem rust	*P. graminis*	Biotrophic	1	Spielmeyer et al. (2003)
Barley	Stem rust	*P. graminis*	Biotrophic	1	Han et al. (1999)
Barley	Net blotch	*Drechslera teres*	Necrotrophic	6 of 12	Richter et al. (1998)

susceptibilities of genotypes to stem rust and leaf spot were not the same at the repeated inoculations.

Significant genotype effect was also found in susceptibilities to leaf spot in the field ($P < 0.0001$) but there was a significant interaction between genotype and year ($P < 0.0001$) (Table 2). Leaf spot pressure at the field plot in 2004 (mean leaf spot severity = 39.0%) was more than two times greater than that in 2005 (mean leaf spot severity = 14.0%). In 2005, the leaf spot severity changed significantly between August and September [P value of scoring (year) <0.0001]. Disease progressed in August (mean leaf spot severity = 14.8%) and decreased by early September (mean leaf spot severity = 11.5%). The susceptibility of each genotype to leaf spot was consistent throughout this time period in 2005 [P value of genotype × scoring (year) = 0.28].

Phenotype distribution indicated that susceptibilities to leaf spot and stem rust were quantitative and transgressive (Fig. 1; Electronic Supplementary Material S1), as is often the case for other disease resistances in ryegrass species (Curley et al. 2005; Sim et al. 2007). The frequency of ryegrass genotypes for leaf spot severity at the growth chamber inoculation and the 2004 field trial where disease pressure was high (mean leaf spot severity >25%) was

Table 2 ANOVA of leaf spot and stem rust severity on the MFA × MFB ryegrass mapping population at the field trial (leaf spot) and inoculation assays in the growth chamber (leaf spot) or greenhouse (stem rust)

Field trial[a]				Inoculation assay[b]						
Source of variation	Leaf spot			Source of variation	Leaf spot			Stem rust		
	df	*F*	*P*		*df*	*F*	*P*	*df*	*F*	*P*
Year	1	5280.54	<0.0001	Exp	1	2.01	0.15	1	228.64	<0.0001
Rep (year)	8	17.82	<0.0001	Rep	3	0.75	0.52	3	12.43	<0.0001
Scoring (year)	3	75.02	<0.0001							
Rep × scoring (year)	12	9.01	<0.0001							
Genotype	151	16.61	<0.0001	Genotype	83	5.07	<0.0001	151	3.72	<0.0001
Genotype × year	143	6.80	<0.0001	Genotype × exp	78	1.36	0.03	141	1.22	0.06
Genotype × scoring (year)	429	1.04	0.28							
Error	2,815			Error	472			794		

[a] Field trial was a randomized complete block design (rep = 5) using 152 progenies (genotype) and conducted in Verona, WI, USA, in 2004 and 2005. Disease severity was measured once and four times (scoring) in 2004 and 2005, respectively

[b] Inoculation experiment was a randomized complete block design (rep = 4) using 89 progenies (genotype) in the growth chamber for leaf spot and 156 progenies (genotype) in the greenhouse for stem rust. The experiment was conducted twice

normally distributed (Fig. 1a, b). The frequency distribution was skewed toward resistance at the 2005 field trial where leaf spot pressure was low (mean leaf spot severity <16%; Fig. 1c, d) but residuals from the ANOVA model (Table 2) were normally distributed. The stem rust severity at the greenhouse inoculation was also normally distributed (Fig. 1e).

When broad sense heritability estimate (H_b^2) of leaf spot resistance was calculated, H_b^2 for the field trials was 0.717 with $\sigma_g^2 = 30.1$ and $\sigma_e^2 = 59.3$, while H_b^2 for the growth chamber experiment was 0.881 with $\sigma_g^2 = 129.2$ and $\sigma_e^2 = 69.6$. Heritability estimate of stem rust resistance evaluated from the greenhouse experiments was 0.833 with $\sigma_g^2 = 1.32$ and $\sigma_e^2 = 1.05$.

Correlation among resistance phenotypes to leaf spot, stem rust, gray leaf spot, and crown rust

The Spearman's rank correlation coefficient (r) was used to measure monotonic association among susceptibilities to leaf spot, stem rust, gray leaf spot, and crown rust as the distribution of the phenotype data would make the application of Pearson's correlation coefficient undesirable or misleading (Sokal and Rohlf 1996). Pairwise comparisons between susceptibilities of ryegrass genotypes to these four different diseases indicated significant negative correlations between leaf spot (inoculation assays and field trials) and crown rust (field trials) (Table 3). However, no significant correlation between leaf spot and either stem rust (inoculation assays) or gray leaf spot (inoculation assays) was found (Table 3).

The susceptibility to leaf spot at the field plots in Verona, WI, USA, in 2004 and 2005 was inversely correlated with the susceptibility to crown rust at the field plots in Verona, WI, USA and Carbondale, IL, USA in 2004 ($r = -0.37$ to -0.17; $P < 0.05$). Some of the pairwise comparisons between susceptibilities to stem rust, gray leaf spot, and crown rust showed moderate correlations at some combinations, but not consistently. For example, the susceptibility to stem rust at the first inoculation test was positively correlated with crown rust at both field locations in Verona and Carbondale ($r = 0.30$ and 0.40, respectively; $P < 0.01$),

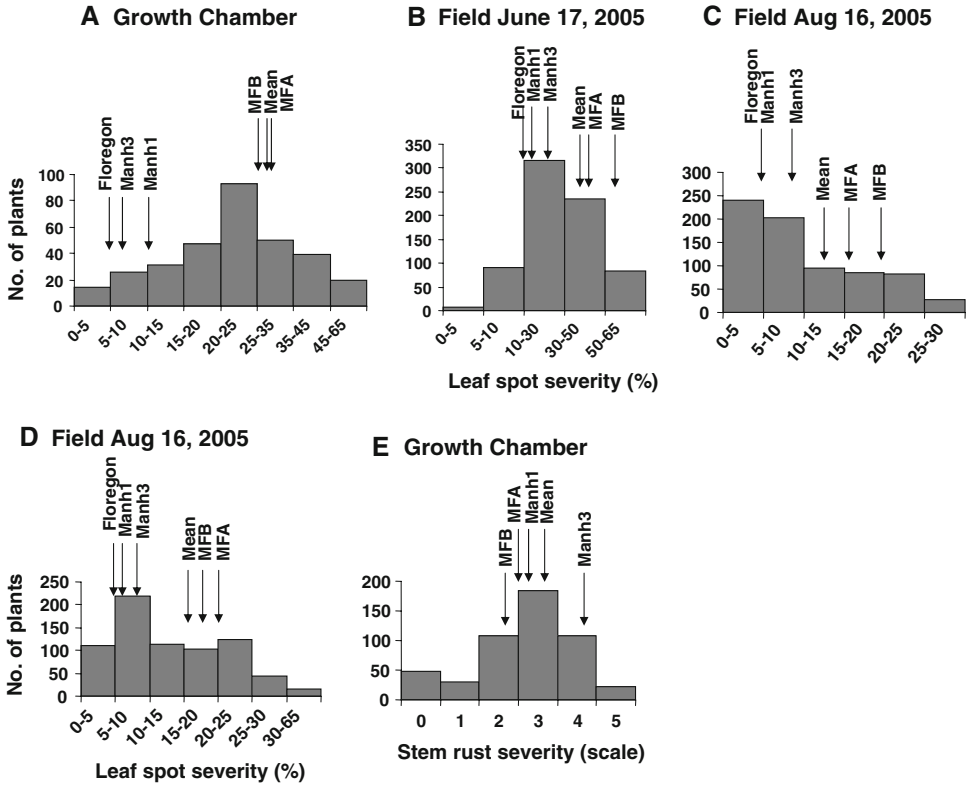

Fig. 1 Frequency distribution of leaf spot (a–d) and stem rust (e) severity in the ryegrass MFA × MFB mapping population. The disease severity is percent diseased area of leaves for leaf spot and a 0–5 disease scale for stem rust corresponding to per-plant pustule counts of 0, 1–2, 3–10, 11–25, 26–100 and >100, respectively. Leaf spot severity was scored at 7 days post-inoculation in the first growth chamber experiment (a LS-GC1), and measured at the field plot in Verona, WI, USA on 17 June 2004 (b LS-WI04), on 16 August 2005 (c LS-WI05b), and on 23 August 2005 (d LS-WI05c). Stem rust severity was scored at 14 days post-inoculation in the second growth chamber experiment (e SR-GC2). Mean disease severities of progeny (*mean*), parents (MFA and MFB), and grandparents (*Manh1* Manhattan1; *Manh3* Manhattan3; and Floregon) are marked on the top of each graph

Table 3 Pairwise correlation of leaf spot (LS), stem rust (SR), gray leaf spot (GLS), and crown rust (CR) severity in the ryegrass mapping population

Experiment[a]	LS-GC1	LS-GC2	LS-WI04	LS-WI05a	LS-WI05b	LS-WI05c	LS-WI05d	SR-GC1	SR-GC2	GLS-GG9	GLS-6082	CR-WI	CR-IL
LS-GC1		<0.0001	0.0002	0.34	0.48	0.67	0.28	0.04	0.71	0.90	0.09	0.88	0.33
LS-GC2	**0.59**		0.04	0.28	0.42	0.23	0.23	0.07	0.72	0.36	0.71	0.73	0.60
LS-WI04	**0.40**	**0.23**		<0.0001	<0.0001	<0.0001	<0.0001	0.74	0.87	0.03	0.18	0.03	0.001
LS-WI05a	0.11	0.12	**0.42**		<0.0001	<0.0001	<0.0001	0.80	0.74	0.50	0.52	0.67	0.01
LS-WI05b	0.08	0.09	**0.48**	**0.68**		<0.0001	<0.0001	0.57	0.86	0.56	0.87	0.05	0.001
LS-WI05c	0.05	0.14	**0.50**	**0.66**	**0.86**		<0.0001	0.74	0.64	0.30	0.39	0.01	0.0001
LS-WI05d	0.12	0.14	**0.40**	**0.45**	**0.73**	**0.75**		0.07	0.31	0.52	0.98	<0.0001	<0.0001
SR-GC1	**0.23**	0.21	−0.03	0.02	−0.05	−0.03	−0.16		<0.0001	0.01	0.10	0.0003	0.01
SR-GC2	0.05	0.04	0.01	−0.03	0.02	−0.04	−0.09	**0.45**		0.11	0.06	0.22	0.88
GLS-GG9[b]	−0.01	−0.10	**−0.18**	0.06	−0.05	−0.09	−0.06	**0.22**	0.15		0.01	0.04	0.35
GLS-6082[b]	−0.19	0.04	−0.11	0.05	0.01	0.07	0.00	0.15	0.17	**0.21**		0.01	0.17
CR-WI[c]	0.02	−0.04	**−0.18**	−0.04	−0.17	**−0.21**	**−0.37**	**0.30**	0.11	**0.17**	**0.21**		<0.0001
CR-IL[c]	0.11	0.06	**−0.27**	**−0.21**	**−0.27**	**−0.32**	**−0.41**	**0.21**	−0.01	0.08	0.12	**0.68**	

Spearman's correlation coefficients (r) are given below the diagonal and P values are given above the diagonal. The significant correlation coefficients ($P < 0.05$) are presented in *bold*

[a] Experiments conducted in this study include field trials for leaf spot in 2004 (LS-Wl04) and 2005 (LS-WI05), inoculation assays for leaf spot in the growth chamber (LS-GC), and inoculation assays for stem rust in the greenhouse (SR-GC). Descriptions of these experiments in Table 4

[b] Gray leaf spot severity was measured at seven days post-inoculation at the growth chamber using a ryegrass isolate (GG9) and a rice isolate (6082) of *Magneporthe grisea* (Curley et al. 2005). The mean disease severity (GLS-GG9) of three separate inoculation experiments using GG9 and the mean disease severity (GLS-6082) from one experiment using 6082 were used for the correlation analysis

[c] Crown rust severity was measured at the field in 2004 (Sim et al. 2007). The mean disease severity (CR-WI) in Verona, WI scored on 28 July and 11 August, and the mean disease severity (CR-IL) in Carbondale, IL, USA, scored on 21 September were used for the correlation analysis

but stem rust at the second inoculation experiment was not significantly correlated with crown rust at all.

The significant phenotypic correlations remained consistent between different datasets of the same disease. Leaf spot susceptibility showed significant positive correlations between two independent growth chamber experiments ($r = 0.59$; $P < 0.0001$) and between the field trials in 2004 and 2005 ($0.40 \leq r \leq 0.50$; $P < 0.0001$). Significant correlation was also noted between stem rust susceptibilities at two different inoculation assays in the greenhouse ($r = 0.45$; $P < 0.0001$); between gray leaf spot susceptibilities in two different inoculation assays at the growth chamber ($r = 0.21$; $P = 0.01$); and between crown rust susceptibilities at the field trials in Verona and Carbondale ($r = 0.68$; $P < 0.0001$). There was a significant correlation of leaf spot susceptibilities between growth chamber experiment and 2004 field trial at $P < 0.05$ but no correlation between growth chamber experiment and 2005 field trial.

Loci for quantitative leaf spot resistance

A total of seven QTLs for leaf spot resistance were detected in the MFA × MFB ryegrass mapping population and were located on LGs 1, 3, 4 and 6 (Table 4). Four marker alleles linked to QTLs contributing to enhanced leaf spot resistance (qLS-GC-6, qLS-WI04-1b, qLS-WI05-4 and qLS-GC1-3) originated from MFB, while three alleles (qLS-GC1-4, qLS-WI04-1a and qLS-GC2WI-4) originated from MFA (Table 4). Particularly, the marker allele for QTL qLS-GC1-4 further originated from the grandparent of the mapping population, Italian ryegrass cultivar "Floregon". The grandparental source of the remaining six QTLs for leaf spot resistance could not be determined in this study.

Four of the seven QTLs (qLS-GC1-4, qLS-GC-6, qLS-WI04-1b and qLS-WI05-4) were significant from at least one dataset by all three analytical methods of Kruskal–Wallis, interval mapping, and MQM. LOD values of these four QTLs were greater than genome-wide thresholds and the P values at the Kruskal–Wallis were <0.001 (Table 4). The remaining three QTLs on LGs 1, 4, and 3 (qLS-WI04-1a, qLS-GC2WI-4 and qLS-GC1-3) did not exceed the genome-wide thresholds but were significant by the Kruskal–Wallis analysis at $P = 0.005$.

Three QTLs on LGs 4 and 6 (qLS-GC2WI-4, qLS-WI05-4 and qLS-GC-6) were detected in more than one dataset and the remaining four QTLs were identified in only one dataset (Table 4). QTL qLS-GC2WI-4 on LG 4 explained 10.6–11.8% of total phenotypic variance and was consistently detected from both the growth chamber

Table 4 Quantitative trait locus (QTL) analysis for leaf spot (LS) and stem rust (SR) disease resistance in the ryegrass MFA × MFB mapping population

QTL	Experiment	Linkage group	Marker interval[a]	Location (cM)	K–W *P* value	MQM			
						LOD	Variance (%)[b]	Additive effect[c]	GW LOD threshold
qLS-WI04-1a	LS-WI04	MFA1	E6.1000-CDO94	0–23.8	0.005	2.20	9.5	−3.87	2.7
qLS-GC1-4	LS-GC1	MFA4	RZ251-CDO541	54.1–65.8	0.001	3.47	18.8	−3.74	2.6
qLS-GC2WI-4	LS-GC2	MFA4	K2.1250-CDO20	0–26.5	0.005	1.89	10.6	2.23	2.7
qLS-GC2WI-4	LS-WI04	MFA4	K2.1250-F14.900	0–14.6	0.005	2.00	11.8	3.98	2.7
qLS-GC2WI-4	LS-WI05b	MFA4	K2.1250-CDO20	0–26.5	0.01	1.94	11.8	2.16	2.6
qLS-GC1-3	LS-GC1	MFB3	BCD1142-BCD927	19.8–30.2	0.005	2.32	10.6	−3.05	2.6
qLS-GC-6	LS-GC1	MFB6	B11.1125-CDO57	33.1–38.4	0.005	2.34	10.7	−3.60	2.6
qLS-GC-6	LS-GC2	MFB6	B11.1125-CDO57	33.1–38.4	0.001	3.26	16.9	−3.45	2.6
qLS-WI04-1b	LS-WI04	MFB1	BCD386-G11.800	25.9–30.2	0.005	2.76	13.4	−4.31	2.7
qLS-WI05-4	LS-WI05a	MFB4	CDO584-CDO241	109.2–117.1	0.0005	2.84	15.7	−1.97	2.5
qLS-WI05-4	LS-WI05b	MFB4	E3.650-CDO504	84.6–103.7	0.01	2.06	10.4	−1.90	2.6
qLS-WI05-4	LS-WI05c	MFB4	CDO504-CDO584	103.7–109.2	0.01	2.00	10.7	−2.09	2.6
qLS-WI05-4	LS-WI05d	MFB4	BCD808-CDO504	98.5–103.7	0.005	2.20	11.7	−1.62	2.5
qSR-GC-4	SR-GC1	MFB4	CDO795-BCD808	83.2–98.5	0.005	1.78	10.6	−0.28	4.1
qSR-GC-4	SR-GC2	MFB4	CDO795-BCD808	83.2–98.5	0.05	2.75	17.4	−0.49	2.3

Results from Kruskal–Wallis (K–W) and multiple QTL model (MQM) analyses were presented. The criteria for inclusion of QTLs were significant (≤0.01) at K–W and/or log-of-odds (LOD) value of the MQM higher than the genome wide (GW) threshold LOD with *P* value = 0.05

[a] Markers bracketing the QTL. Heterologous cDNA probes for RFLP markers originate from oat (CDO), barley (BCD), rice (RZ) and creeping bentgrass (Ast). The remaining markers are RAPD

[b] Proportion of variance explained by the QTL

[c] Additive effect is calculated as half of the mean associated with the "a"-genotype subtracted by half of the mean associated with the "b"-genotype

test and the field trials in 2004 and 2005. QTL qLS-WI05-4 on LG 4 was consistently detected at four continuous ratings from the 2005 field trial. QTL qLS-GC-6 on LG 6 was identified at both growth chamber experiments.

More than one QTL was detected on LGs 1 and 4. Two QTLs (qLS-WI04-1a and qLS-WI04-1b) were closely located <10 cM apart on LG 1 of the integrated parental genetic map (Fig. 2). Three non-coincident QTLs (qLS-GC1-4, qLS-GC2WI-4 and qLS-WI05-4) were located on LG 4 without overlapping (Fig. 2).

Loci for quantitative stem rust resistance

One QTL for stem rust resistance was detected on LG 4 (Table 4). This QTL was significant with the Kruskal–Wallis analysis at both inoculation experiments at $P = 0.005$ and 0.05, respectively (Table 4). The LOD value of this QTL was greater than the genome-wide threshold at one of two inoculation assays. The marker allele of this QTL originated from MFB and perennial ryegrass cultivar "Manhattan", which were parents and grandparents of the mapping population, respectively.

Integration of loci for quantitative disease resistance into the ryegrass genetic map and rice physical map

A total of 16 QTLs for resistance to leaf spot (seven), stem rust (one), gray leaf spot (four), and crown rust (four) were located on the ryegrass genetic map (Fig. 2). A genomic region spanning 19 cM on LG 4 contained QTLs for resistance to three diseases: stem rust (qSR-GC-4), leaf spot (qLS-WI05-4), and gray leaf spot. QTLs for leaf spot resistance (qLS-GC-6) and crown rust resistance were also coincident on LG 6. However, most other QTLs were distributed on six LGs without coincidence. Genome-wide tests of chi-square goodness-of-fit failed to reject the null hypothesis that QTLs are coincident on the ryegrass linkage map by chance. The tests were repeated using three datasets for the presence of QTLs in the bins of 15, 5, or 3 cM interval on the ryegrass linkage map.

A reciprocal view between the ryegrass genetic map and the rice physical map indicated that for most EST–RFLP markers mapped in ryegrass, there is a preponderance of their synteny with orthologous chromosomal segments of rice (Fig. 2). For example, RFLP markers on ryegrass LG 1 are mostly located on rice chromosomes 5

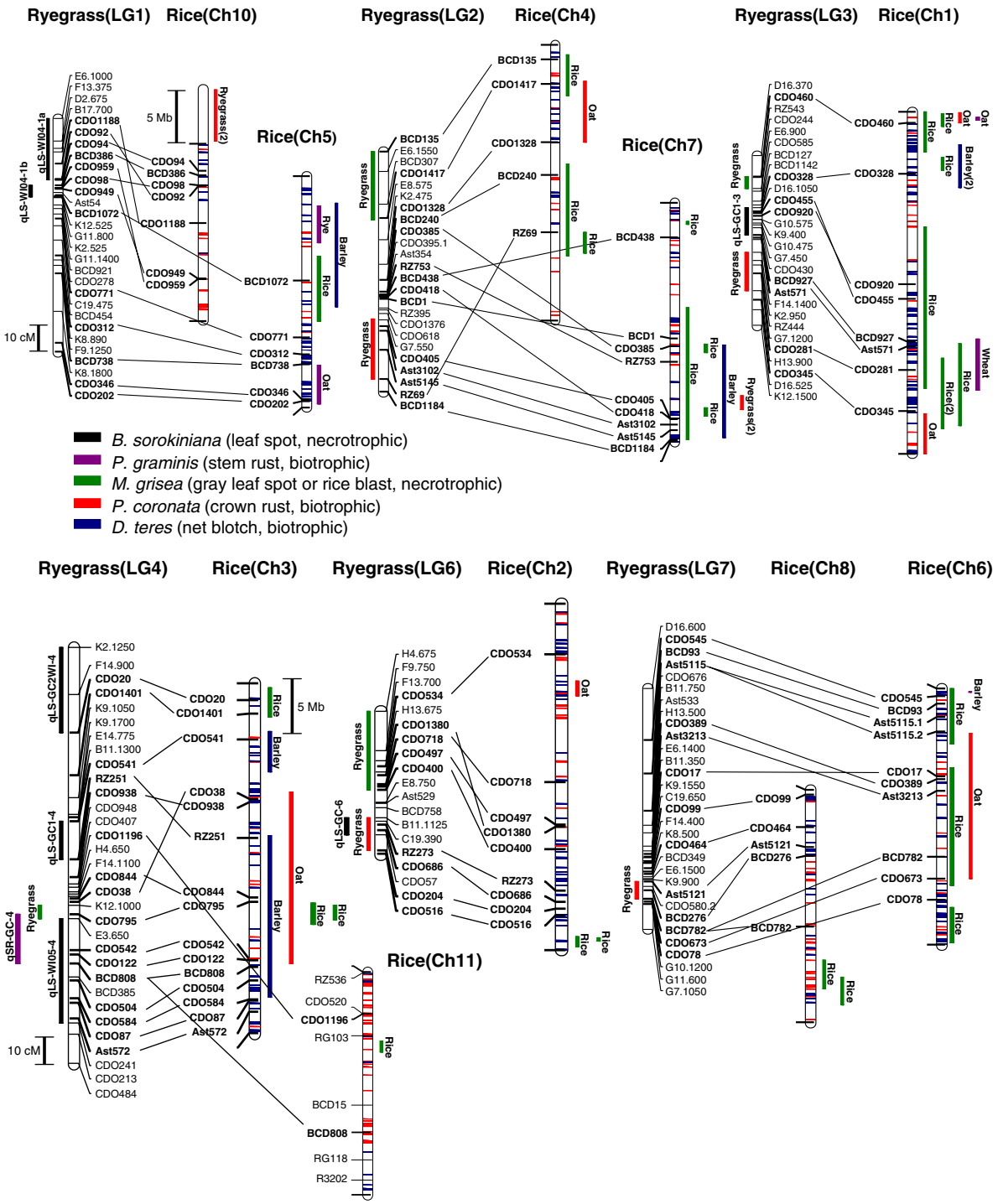

Fig. 2 Integrated disease resistance map of ryegrass and rice using 72 common RFLP markers (*bold*). The sources of cDNA probes for RFLP markers are oat (CDO), barley (BCD), rice (RZ), and creeping bentgrass (Ast), and the remaining markers are RAPD. Disease resistance loci are located on six linkage groups of the ryegrass genetic map and nine corresponding homologous chromosomes of rice. Disease resistance loci are shown as *color-coded bars* based on pathogen species. QTLs for resistance to leaf spot and stem rust determined in this study are located along a side of the ryegrass map, labeled as QTL names (Table 4). Additional QTLs for disease resistance previously identified (Table 1) are located along a side of the ryegrass or rice map, and are labeled with the host plant which is followed by (2) in the case of overlap of two disease resistance loci. In addition, locations of 345 resistance gene analogs (RGAs) (*red color*) and 343 transcription factor genes (*blue color*) associated with plant defence are plotted on the rice chromosomes. RGAs include nucleotide binding sites-leucine rich repeat (NBS-LRR) and nucleotide binding adaptor shared by APAF-1, qualitative resistance (R) proteins, and CED-4. Transcription factor gene families include WRKY, MYB, and basic leucine zipper (bZIP)

Table 5 Number of coincidence of disease resistance loci between ryegrass and cereals based on 72 common RFLP markers

Disease in ryegrass (# of QTLs tested)	Disease in cereals (# of disease resistance loci tested)				
	Rice blast by *M. grisea* (25)	Net blotch by *D. teres* (6)	Crown rust by *P. coronata* (10)	Stem rust by *P. graminis* (5)	No coincidence
Gray leaf spot by *M. grisea* (4)	3	1	3	0	1
Leaf spot by *B. sorokiniana* (7)	3	4*	2	1	2
Crown rust by *P. coronata* (4)	3	1	2	1	1
Stem rust by *P. graminis* (1)	0	1	1	0	0

* Nearly statistically significant difference from random expectation at $P = 0.0544$

and 10 and the markers were aligned in similar order within corresponding orthologous chromosome segments of rice. Therefore, rice physical map could serve as a framework for interspecific comparison of QTLs for disease resistance. A total of 46 loci associated with resistance to rice blast, crown rust, stem rust, and net blotch identified in various Poaceae species (Table 1) were located throughout nine chromosomes of rice because sequences of RFLP markers tightly linked to these loci were available (Fig. 2).

Coincidence of QTLs for disease resistance in ryegrass and cereals (Electronic Supplementary Material S2) was tested for statistical significance using Cohen's kappa (Cohen 1960). QTLs for disease resistance in ryegrass were not coincident with loci for the corresponding disease resistance in cereals at the genome-wide comparison based on 72 common RFLP markers. However, coincidence of QTLs for leaf spot resistance in ryegrass with QTLs for net blotch resistance in barley at the syntenic region was nearly statistically significant ($P = 0.054$; Table 5).

Discussion

The current genetic analysis identified seven QTLs for resistance to leaf spot and one QTL for resistance to stem rust in the same ryegrass mapping population in which QTLs for resistance to gray leaf spot and crown rust were previously reported. Subsequent comparative trait mapping analysis using a ryegrass genetic map and a rice physical map indicated that coincidence of QTLs for disease resistance in ryegrass and cereals was not statistically significant at the genome-wide comparison based on 72 common RFLP markers.

Quantitative trait loci (QTLs) for leaf spot resistance in ryegrass were detected for the first time in this study. Out of them, two QTLs on LGs 4 and 6 were likely to be stable because they were detected in different environmental conditions or experiments. Therefore, they may be utilized for marker assisted breeding for leaf spot resistance in ryegrass. Remaining QTLs for leaf spot resistance seem to depend on evaluation methods (the growth chamber and field assays) and years (2004 and 2005), causing low reproducibility. Detection of QTLs for disease resistance commonly depends on experimental conditions, plant age (Richter et al. 1998; Chang and Hwang 2003), and race composition of the inoculum (Zhu et al. 2003; Portyanko et al. 2005). In growth chamber assays, high moisture and controlled temperature (23°C) after inoculation with a high concentration of a single spore isolate were highly conducive to the development of leaf spot. For the field evaluation, natural pressure of leaf spot varied between 2004 and 2005. Leaf spot pressure in the field was very high in June 2004 due to frequent precipitation events in the early summer but in 2005, the disease was moderate until August because of an unusually dry summer followed by heavy precipitation in the late summer and fall (Electronic Supplementary Material S3). Variation of leaf ages of ryegrass in both field and growth chamber experiments might be minimal since new leaves are continuously generated and disease severity can be scored based on young leaves.

Judicious interpretation of multiple datasets by different analytical approaches is important for QTL mapping (Cogan et al. 2005). Most QTLs for resistance to leaf spot and stem rust were consistent at multiple datasets with various analytical methods despite the relatively low proportion of phenotypic variance explained (10–20%). Indicative QTLs (e.g., qLS-WI04-1a) that were significant at a single dataset with one or two methods, and putative QTLs (e.g., qLS-GC2WI-4) that were only significant with the Kruskal–Wallis analysis were also reported and should be treated with caution. Addition of extra markers to the ryegrass map and an increase in population size will improve the reliability and significance of the QTLs detected.

Many of the alleles for elevated disease resistance detected in the MFA × MFB population seem to originate from the Italian ryegrass cultivar "Floregon", which were grandparents of the mapping population. Marker alleles linked to two QTLs (on LGs 3 and 6) for gray leaf spot resistance (Curley et al. 2005), and to three QTLs (on LGs 2, 3, and 7) for crown rust resistance (Sim et al. 2007) were

previously confirmed to originate from Floregon. One marker allele linked to the QTL (qLS-GC1-4) for leaf spot resistance detected in this study was also inherited from Floregon. Multiple disease resistance in Floregon may be attributable to the breeding history of this cultivar. Selection for tolerance to stresses was performed in Florida and Oregon in which environmental conditions and disease pressure were highly divergent.

A genome-wide comparison of QTLs on the ryegrass map indicated no statistical support (at $P = 0.05$) for colocalization of QTLs for resistance to biotrophic (stem rust and crown rust) and necrotrophic (gray leaf spot and leaf spot) diseases, which have fundamental differences in parasitism. Most QTLs detected in this study are pathogen-specific but two genomic regions on LGs 4 and 6 are associated potentially with multiple disease resistance or clusters of resistance loci for both biotrophs and necrotrophs.

The comparative QTL analysis of resistance to multiple pathogens in ryegrass and cereal crops was highly facilitated by the increased availability of publicly accessible DNA sequence databases of cereal crops. This study used the rice physical map based on sequence information provided by The Institute of Genomic Research (TIGR) rice project website, http://www.tigr.org as a framework to locate and compare candidate resistance genes. Given the high level of conserved syntenic and collinear relationships in Poaceae genomes, molecular markers with known DNA sequences from different grass species can be readily located on the rice physical map as long as their homologous sequences are present in the rice genome.

In addition, complete annotation of pseudomolecules (virtual contigs) on rice chromosomes is of great utility to explore the genetic basis of QTLs for traits of interest. QTLs for disease resistance and both RGAs and defence-related transcription factors appear to be nonrandomly distributed in rice (Fig. 2), as reported previously (Wisser et al. 2005). Some loci for disease resistance occurred in the regions clustered with RGAs and defence-related transcription factors such as the distal region of rice chromosomes 1 and 6 (Fig. 2).

Chromosomal regions of rice can provide initial information about potential orthologous regions associated with QTLs for disease resistance in ryegrass. For example, the ryegrass genome region of LG 4 that contains three QTLs for resistance to leaf spot, gray leaf spot, and stem rust has a syntenic relationship with a segment of rice chromosome 3. This contains QTLs for resistance to multiple pathogens, including rice blast, sheath blight caused by *Rhizoctonia solani* Kühn, and bacteria leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings et al. (Wisser et al. 2005). This rice chromosome segment contains a cluster of biosynthetic pathway-related genes, including those that code for glyceraldehyde-3-phosphate dehydrogenase, ubiquitin, lethal leaf-spot 1, catalase, peroxidase, flavanone 3-hydroxylase, and plant defensin (Wisser et al. 2005). These genes may be logical targets for further dissecting the region of ryegrass LG 4 to see whether the resistance loci in ryegrass correspond to any rice ortholoci.

Two previous QTL mapping studies and the current study based on the same ryegrass mapping population indicate a potential conservation of disease resistance genes in certain syntenic regions between ryegrass and cereals. Curley et al. (2005) reported three QTLs for gray leaf spot resistance on LGs 2, 3, and 4 in ryegrass were located in the syntenic regions of QTLs for rice blast resistance in rice. Sim et al. (2007) also reported that in the syntenic regions where two QTLs on LGs 2 and 7 for crown rust resistance in ryegrass were located, loci for crown rust resistance in a different ryegrass population (Muylle et al. 2005) and oat (Wight et al. 2004) have also been detected. Similarly, three QTLs for leaf spot resistance detected in this study were located in syntenic genomic regions of barley where QTLs for net blotch resistance (Richter et al. 1998) were located.

However, probabilities of finding loci of resistance to similar pathogens between ryegrass and cereals were not statistically significant at the genome-wide comparison. The limitation is that this statistical comparison is based on 72 common heterologous RFLP probes from rice (RZ), oat (CDO), barley (BCD), and creeping bentgrass (Ast). Although there is high conservation of these RFLP markers and their order between ryegrass and cereals, applicability of RFLP markers is limited by the requirement of conserved sequences and the level of genetic polymorphism detected by restriction enzymes. Therefore, not all RFLP probes give similar transferability when applied to different Poaceae species. Oat and barley probes have been known to give higher levels of hybridization with ryegrass than rice probes due to their taxonomic affinity (Sim et al. 2005). Also, macro-colinearity based on RFLP markers does not always predict micro-colinearity which requires extensive sequence information (Sorrells et al. 2003). Paralogs can also lead to selection of locations on the rice physical map other than those defined by ortholoci in this study. However, paralogous sequences of RFLP markers may not affect our statistic analysis for genome-wide comparison of loci for disease resistance since the presence or absence of these resistance loci linked to the RFLP markers is tested without considering the number of paralogs.

Recently, highly transferable PCR-based markers such as EST-derived simple sequence repeats (SSRs) (Saha et al. 2004; Yu et al. 2004) and single nucleotide polymorphism (SNP) (Cogan et al. 2006) have become available and will supplement RFLP-based comparative study in Poaceae. Fine mapping by determination of linkage of candidate genes in conjunction with phenotypic data and DNA

sequences of ryegrass genome regions of interest will provide a better understanding of the molecular architecture of disease resistance. Molecular markers tightly linked with QTLs for disease resistance will facilitate the marker-assisted selection in ryegrass by pyramiding pathogen-specific or multiple disease resistance genes.

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