Theor Appl Genet (2007) 115:9–17
DOI 10.1007/s00122-007-0535-z

ORIGINAL PAPER

Consistent detection of QTLs for crown rust resistance in Italian ryegrass (*Lolium multiflorum* Lam.) across environments and phenotyping methods

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Received: 07 August 2006 / Accepted: 10 March 2007 / Published online: 11 April 2007

Abstract Crown rust, caused by *Puccinia coronata* f. sp. *lolii*, is one of the most important diseases of temperate forage grasses, such as ryegrasses (*Lolium* spp.), affecting yield and nutritional quality. Therefore, resistance to crown rust is a major goal in ryegrass breeding programmes. In a two-way pseudo-testcross population consisting of 306 *Lolium multiflorum* individuals, multisite field evaluations as well as alternative methods based on artificial inoculation with natural inoculate in controlled environments were used to identify QTLs controlling resistance to crown rust. Disease scores obtained from glasshouse and leaf segment test (LST) evaluations were highly correlated with scores from a multisite field assessment ($r = 0.66$ and 0.79, $P < 0.01$, respectively) and thus confirmed suitability of these methods for crown rust investigations. Moreover, QTL mapping based on a linkage map consisting of 368 amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers revealed similar results across different phenotyping methods. Two major QTLs were consistently detected on linkage group (LG) 1 and LG 2, explaining up to 56% of total phenotypic variance (V_p). Nevertheless, differences between position and magnitude of QTLs were observed among individual field locations and suggested the existence of specific local pathogen populations. The present study not only compared QTL results among crown rust evaluation methods and environments, but also identified molecular markers closely linked to previously undescribed QTLs for crown rust resistance in Italian ryegrass with the potential to be applied in marker-assisted forage crop breeding.

Keywords *Lolium multiflorum* Lam. · Disease resistance · *Puccinia coronata* f. sp. *lolii* · Quantitative trait locus (QTL) · QTL × environment interaction

Communicated by T. Lübberstedt.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-007-0535-z) contains supplementary material, which is available to authorized users.

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Introduction

Crown rust, caused by the fungal pathogen *Puccinia coronata* f. sp. *lolii (Pca)*, is one of the most important foliar diseases of forage crops such as ryegrasses (*Lolium* spp.) and fescues (*Festuca* spp.; Yamada et al. 2005). Crown rust epidemics have been reported from temperate regions worldwide (Potter et al. 1990) affecting herbage yield as well as nutritional quality and palatability (Potter 1987). The economic losses associated with crown rust attack have stimulated efforts to reduce disease incidence and severity. The most efficient and environmentally sound method is the development of resistant cultivars. Therefore, resistance to crown rust is a major goal in ryegrass breeding, and targeted phenotypic selection resulted in cultivars with considerable levels of resistance (Suter et al. 2006; Wilkins and Humphreys 2003).

However, in outcrossing species such as perennial and Italian ryegrass exhibiting a gametophytic two-locus system of self-incompatibility (Cornish et al. 1979; Fearon et al. 1983), breeding is complicated by a high degree of heterozygosity resulting in a large genetic variation within as well as among cultivars (Huff 1997; Kölliker et al. 1999). Therefore, further breeding progress is often difficult to obtain. Moreover, disease incidence in field grown plants can be variable due to genotype × environment interactions, which may mask the real genotypic value and hence hinder efficient phenotypic selection. Such interactions have recently been observed in multisite rust evaluations for both Italian and perennial ryegrass cultivars, respectively (Boller et al. 2003). Additionally, evidence for substantial genetic variation within and among crown rust pathogen populations has recently been detected using molecular genetic markers (Dracatos et al. 2006). Therefore, resistance assessment demands accurate phenotypic evaluation protocols to reliably identify the genotypic value of individual plants.

Methods to efficiently select for resistant plants are often based on artificial inoculation in controlled environments. Crown rust evaluations in the glasshouse (Birckensteadt 1990; Clarke et al. 1997; Croft et al. 2000) and using an in situ LST (Lellbach 1994) allow for the rapid and reproducible identification of resistance on entire plants or small leaf segments, respectively. Despite their potential to ensure homogeneous inoculations of pathogen strains obtained from different locations and dates, such methods are only rarely included in targeted resistance breeding because selected pathogen strains used for inoculation may not completely represent natural populations occurring in the field (Aldaoud et al. 2004). Therefore, practical breeding programmes often rely upon multisite field experiments over several years based on natural infection by a wide variety of pathogen races. However, seasonal variation of natural crown rust occurrence may lead to varying selection pressure hampering efficient phenotypic selection.

Molecular genetic markers do not depend on environmental factors and thus provide a powerful tool for continuous and efficient selection. Marker-assisted selection (MAS) enables early selection at the seedling stage resulting in a considerable reduction in the number of plants to be evaluated in the field. Moreover, MAS allows targeted introgression, pyramiding and fixation of resistance genes in cultivars (Armstead et al. 2006; Francia et al. 2005; Rao et al. 2002). Enabling improved simultaneous selection for several traits, MAS has the potential to make valuable contributions to breeding progress (Yamada et al. 2005). Implementation of MAS in forage breeding programmes requires a better understanding of the genetics regarding to number, position and mode of action of QTLs and genes underlying these traits. Phenotypic analysis of crown rust resistance indicated both qualitative (Cruickshank 1957; McVeigh 1975; Schmidt 1980; Wilkins 1975) and quantitative (Hayward 1977; McVeigh 1975; Reheul and Ghesquière 1996) inheritance, which is characteristic for outcrossing species such as ryegrasses. More recent studies identified QTLs of varying magnitude in perennial ryegrass (Dumsday et al. 2003; Muylle et al. 2005a; Muylle et al. 2005b; Roderick et al. 2003; Thorogood et al. 2001), but only a single study to date has assessed crown rust resistance in Italian ryegrass (Fujimori et al. 2004). Furthermore, if investigated in a single environment, QTLs often suffer from limited explanatory power, thus limiting a broad application of the detected resistance in geographically distinct locations. To efficiently identify consistent QTLs, environmental effects have to be minimised. It is therefore necessary to confirm identified QTLs in different environments representing a broad spectrum of pathogen races (Muylle et al. 2005b). Moreover, there is a fundamental lack of knowledge about the congruence of number, position and extension of identified QTLs conferring resistance to crown rust based on different evaluation methods.

Therefore, this study was aimed at (1) comparison of different evaluation methods of crown rust resistance in a *L. multiflorum* mapping population, (2) investigation of the number, contribution and genomic location of QTLs conferring resistance to crown rust and (3) identification of molecular markers linked to QTLs consistently detected in each experiment for implementation of MAS in Italian ryegrass breeding.

Materials and methods

Plant material

A two-way pseudo-testcross population was obtained by a cross between a genotype of the susceptible cultivar Adret and a resistant plant from the ART breeding germplasm (Studer et al. 2006). The 306 F_1 mapping individuals were supplemented with 47 Adret genotypes (PopAdret) and 47 half-sibling individuals derived from open pollination of the resistant parental plant (PopM2289). These reference populations were used to represent the expected range of resistance levels in the parental genotypes of the mapping family that were not available for phenotyping. The resulting 400 plants were grown in 110 mm diameter soil-filled plastic pots in the glasshouse, trimmed to a height of 50 mm every 5–8 weeks and vegetatively propagated into clonal ramets consisting of three to five tillers each.

Phenotypic evaluation of crown rust resistance

Field evaluation was performed at six locations in Switzerland, Germany and the Netherlands, representing different

testing environments. Geographic and climatic characteristics of locations are given in Table 1 (for details see electronic supplementary material). At each location, the experimental design was a 40 × 10 lattice with three clonal replicates of each of the 400 genotypes, which were planted in field nurseries in spring 2004. Crown rust symptoms were scored by local breeders on leaves in autumn 2004, when spread and severity of naturally occurring disease was maximal, using a scale ranging from 1 (no symptoms) to 9 (highly infected).

Glasshouse evaluation was carried out in Hohenheim, Germany, using a lattice design with three replications and artificial inoculation of bulked spore samples collected on various ryegrass genotypes in breeding nurseries of Hohenheim in autumn 2004. Plants were clonally propagated and grown in soil-filled pots for 4 weeks, 48 combined in one tray. Each tray was separately sprayed with 20 ml mineral oil Soltrol 170 (Philips Petroleum, Paris, France) containing 30 mg urediniospores of *Pca*. After 1 h, when the Soltrol 170 was evaporated, plants were kept at 24°C and 90% relative humidity for 24 h in the dark. For the next 12 days, plants were grown using the same temperature and humidity under long day conditions in the glasshouse (photoperiod of 16 h, 350 to 400 $\mu Em^{-2} s^{-1}$), and disease severity was scored 12 days after inoculation according to the scale mentioned above.

LST was performed at ART according to the protocol of Lellbach (1994) with the following modifications. Three replications of leaf segments derived from the youngest mature leaf with a length of 30 to 40 mm were placed on agar (5%) containing 35 ppm benzimidazol (Sigma-Aldrich, St. Louis, MO, USA). Inoculation was performed using urediniospores of a spore collection harvested in autumn 2003 on Italian ryegrass genotypes in Ellighausen, 60 km northeast of ART, which were sprayed on leaf segments with a density of 4 to 6 spores mm^{-2}. After 24 h of incubation in the darkness, leaf segments on media were transferred into continuous light (300 to 400 $\mu Em^{-2} s^{-1}$) and incubated at 20°C for 12 days in the growth chamber. Resistance to crown rust was scored according to the same scale as used in field and glasshouse experiments.

Statistical analysis of phenotypic data

Lattice analysis using the PLABSTAT software, version 2 P (Utz 2000) was performed in order to estimate quantitative genetic parameters summarised in Table 2. Repeatability was calculated by dividing the genotypic variance component σ^2_g with the sum of σ^2_g and the effective mean square of the error. Adjusted means of the 306 mapping individuals were used for location-specific QTL analysis. Multisite ANOVA was based on unadjusted values of the five locations ART, STE, FLE, HOH and ASE using the factors genotype (g), environment (e) and replication (r; nested within environment) and the PLABSTAT model g + e + r:e + ge + ger. The factor environment was considered as random. Heritability was calculated according to the formula $h^2 = \sigma^2_g/(\sigma^2_g + \sigma^2_{ge}/e + \sigma^2_{ger}/er)$ with σ^2_g, σ^2_{ge} and σ^2_{ger} as variance components for the genotype, genotype × environment and genotype × environment × replication interaction. *E* and *r* represent the number of environments and replicates, respectively (Knapp and Bridges 1987).

QTL analysis

For QTL analysis, the linkage map consisting of 368 AFLP and SSR markers (Studer et al. 2006) and the MapQTL software (version 5.0; Van Ooijen 2004) were used. QTL analysis was based on a multiple QTL model (MQM) and on separate parental maps. Automatic cofactor selection (backward elimination, $P > 0.02$) was used for the detection of significantly associated markers as cofactors. LOD significance threshold levels were determined using 5,000 permutations. The 2 LOD interval represents the distance between two map positions given in centiMorgan (cM) obtained at LOD scores 2 U lower than the maximum score (Van Ooijen 2004).

Table 1 Geographical and climatic characteristics of sites used for field evaluation of crown rust resistance

Locations	Altitude (masl)	Geographical location	Long-term average rainfall [mm y^{-1}]	Long-term average temperature [°C]
Reckenholz Zurich, Switzerland (ART)	438	N: 47°25′; E: 08°31′	1,024	8.5
Steinach, Germany (STE)	333	N: 48°57′; E: 12°36′	861	7.2
Flevopolder, the Netherlands (FLE)	0	N: 52°31′; E: 05°29′	849	10.2
Stuttgart Hohenheim, Germany (HOH)	394	N: 48°42′; E: 09°11′	698	8.8
Asendorf, Germany (ASE)	47	N: 52°45′; E: 08°59′	665	9.2
Malchow Poel, Germany (MAP)	7	N: 53°59′; E: 11°24’	802	8.7

More detailed information on climatic conditions of each evaluation site from the date of field planting in spring to resistance scoring in autumn is available as electronic supplementary material

Table 2 Key values of the phenotypic characterisation of resistance to crown rust based on 400 *Lolium multiflorum* genotypes from the F_1 mapping population (306) and two reference populations (2 × 47)

Location/method of evalution	ART	STE	FLE	HOH	ASE	MAP	GHHOH	LST
Genotypic variance[a]	2.16**	2.79**	1.96**	4.59**	0.13**	0.06ns	3.12**	3.71**
Repeatability	0.63	0.82	0.51	0.72	0.48	0.03	0.76	0.70
Mean disease score	2.17	2.19	5.79	1.72	2.15	4.86	2.00	2.60
LSD[b]	1.81	1.25	2.18	1.19	0.60	2.11	1.56	2.20

Resistance was assessed in field experiments at six locations in Switzerland, Germany and the Netherlands (for description of sites see Table 1), in a glasshouse experiment (GHHOH) and using a leaf segment test (LST). Results are based on a lattice design with three replicates per genotype

** $P < 0.01$

ns not significant

[a] Variance components

[b] Least significant difference at $P < 0.05$

Results

Phenotypic characterisation of resistance to crown rust

Crown rust disease scores of the field trials based on all 400 genotypes showed considerable variation within as well as among the six locations (Table 2). Lattice analysis revealed highly significant genotypic variance components for each location with the exception of MAP, which was therefore excluded from further analysis. Repeatability ranged from 0.48 (ASE) to 0.82 (STE). Resistance screening based on artificial inoculation in the glasshouse in Hohenheim (GHHOH) and using the LST at ART resulted in average crown rust disease scores of 2.00 and 2.60, respectively (Table 2). Thus, disease severity of GHHOH and LST was comparable to field locations showing only moderate rust infections. However, significant ($P < 0.01$) genotypic variance components (3.12 and 3.71) as well as repeatabilities of 0.76 and 0.70, respectively, were among the highest values observed in this study.

Average crown rust disease scores were highest for individuals of the reference population derived from the susceptible cultivar Adret (PopAdret; 3.51–4.88), intermediate for the 306 individuals of the mapping population (1.90–2.68) and lowest for the individuals of the reference population derived from the resistant parental plant M2289 (PopM2289; 1.28–2.61; Fig. 1). The susceptible parental population PopAdret as well as the F_1 mapping population showed a broader variation for crown rust resistance than the reference PopM2289. Considering the three subpopulations, glasshouse assessment revealed the lowest mean disease scores, whereas averaged field evaluations revealed the highest values. An exception was PopAdret, which showed highest mean disease scores in the LST. Standard deviations obtained from LST and GHHOH were similar, whereas averaging values over five field locations resulted in a reduction of the standard deviation (Fig. 1).

In general, frequency distribution of crown rust scores deviated in each experiment from a normal distribution and was skewed towards resistance. However, residuals fitted significantly ($P < 0.05$) to a normal distribution (data not shown). Focussing on the F_1 mapping population, no maternal effect, i.e. no significant differences between genotypes grown from seed harvested from the resistant and the susceptible parent, respectively, were identified using field, GHHOH and LST resistance data (data not shown).

Considering only the mapping population consisting of 306 F_1 individuals, quantitative genetic parameters of averaged resistance data observed in the field based on five locations revealed significant ($P < 0.01$) variance components for the genotypes as well as the genotype × environment interaction (Table 3).

Resistance scores assigned to each of the 306 F_1 mapping individuals were positively correlated among the field trials (Table 4). STE, which revealed the highest repeatability, showed moderately correlated resistance scores to the geographically distant location FLE (0.36), higher correlation coefficients to HOH and ASE (0.54 and 0.56, respectively) and with 0.65 the highest correlation to ART in Switzerland. The crown rust scores obtained from the average of field locations were highly correlated to scores obtained from individual sites as well as GHHOH and LST evaluation (0.64 to 0.87).

QTL analysis

Detailed results of QTL analysis based on both parental maps and resistance data derived from individual field assessments, from averaged field experiments as well as GHHOH and LST evaluations are given in Table 5. Two QTLs exceeding the genome wide significance LOD threshold of 2.6 were observed across most experimental sites and screening methods (Fig. 2). One major QTL was consistently detected at the terminal segment of LG 1 explaining up to 56% of total V_p for disease resistance. This

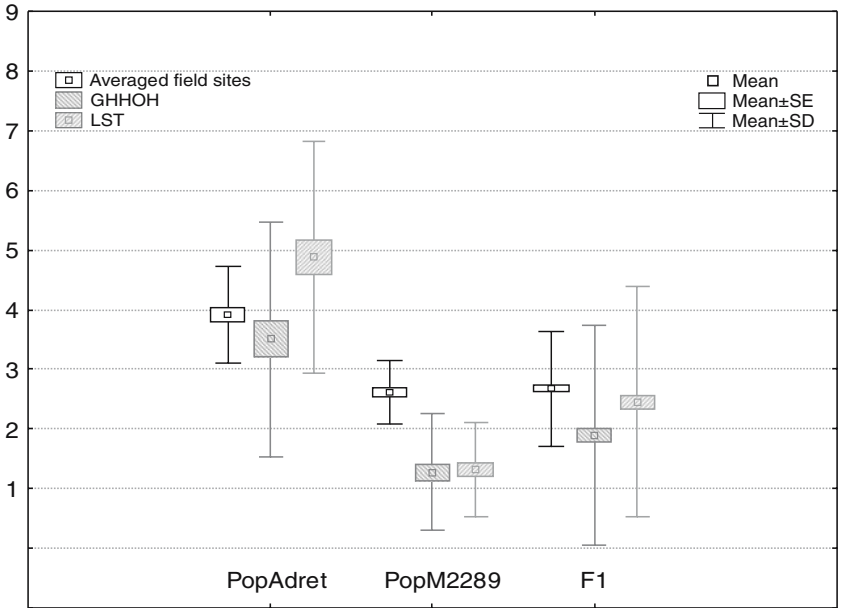

Fig. 1 Mean values, standard errors (SE) and standard deviations (SD) of crown rust disease scores based on averaged results from field experiments at five locations, glasshouse (GHHOH) and leaf segment test (LST) evaluations using a scale ranging from 1 (no symptoms) to 9 (highly infected). Scores of the 306 *F1* mapping individuals were compared to the susceptible (*PopAdret*) and the resistant (*PopM2289*) *Lolium multiflorum* reference population consisting of 47 genotypes each. Results are based on three replicates per genotype

Table 3 Variance components, level of significance and key characteristics of the averaged field resistance data for crown rust based on the locations ART, STE, FLE, HOH and ASE. Results are obtained from 306 F_1 individuals of a *Lolium multiflorum* mapping population

ANOVA	*df*	5 sites[c]
Genotype[a]	305	0.68**
Genotype × environment[a]	1215	0.84**
Heritability		0.75
Mean disease score		2.64
LSD[b]		1.31

** $P < 0.01$

[a] Variance components

[b] Least significant difference at $P < 0.05$

[c] ART, STE, FLE, HOH and ASE, see Table 1

Table 4 Product moment correlation coefficients for pair wise comparisons of crown rust disease scores derived from different field locations and evaluation methods

	ART	STE	FLE	HOH	ASE	GHHOH	LST
STE	0.65**						
FLE	0.39**	0.36**					
HOH	0.31**	0.54**	0.24**				
ASE	0.38**	0.56**	0.28**	0.58**			
GHHOH	0.53**	0.57**	0.29**	0.58**	0.58**		
LST	0.70**	0.79**	0.42**	0.37**	0.44**	0.62**	
Field[a]	0.81**	0.87**	0.66**	0.64**	0.64**	0.66**	0.79**

Calculations are based on 306 F1 genotypes of a *Lolium multiflorum* mapping population using three replicates per genotype

** $P < 0.01$

[a] Crown rust disease scores are based on the average of the field sites ART, STE, FLE, HOH and ASE, see Table 1

QTL was closely linked to the SSR marker NFFA012 (0 to 2 cM) and is therefore of particular interest. A second QTL was identified in all experiments (apart from GHHOH and FLE) at the initial segment of LG 2 (0 to 1 cM from the AFLP marker E35M50_200), which explained up to 35% of V_p (STE). The extensions of the two LOD intervals of the mapped QTL positions depend on LOD values and are given in parenthesis (Table 5). Both major QTLs were inherited from the resistant parent. Their relative importance varied depending on the evaluation environments. The QTL on LG 1 clearly dominated V_p between genotypes observed in the field at ART (56% of V_p) and, to a lesser extent, the artificial inoculation tests GHHOH and LST with 27 and 37% of explained V_p, respectively. On the other hand, the QTL on LG 2 contributed most to V_p at the field sites STE (35%) and HOH (17%). A third QTL was identified on LG 3 (position 3 cM) in the field and glasshouse assessment performed at Hohenheim, Germany (HOH, GHHOH) explaining 11 and 13% of V_p, respectively. All further detected QTLs showed only minimal effects and only marginally exceeded the LOD significance threshold such as the QTLs on LG 4 based on LST (93 cM, 3% explained V_p) and GHHOH (position 74 cM, 5% explained V_p). The GHHOH QTL on LG 4 and two additional minor QTLs on LG 2 and LG 3 observed in FLE were inherited from the susceptible parent.

Discussion

The success of any QTL-based study strongly depends on the magnitude of genetic variation for the target trait observed in the mapping population (Collard et al. 2005).

Table 5 Detailed description of QTLs for crown rust resistance identified in field (for description of sites, see Table 1), glasshouse (GHHOH) and leaf segment test (LST) experiments using a *Lolium multiflorum* mapping population consisting of 306 F_1 individuals (Studer et al. 2006)

	Environment	Linkage group	Position (2 LOD interval) [cM]		Closest marker (distance) [cM]		Maximum LOD score	% variance explained	Source of resistance
Individual field environments	ART	1	99	(96–100)	NFFA012	(1)	54.0	55.5	r
		2	0	(0–9)	E35M50_200	(0)	6.3	4.2	r
	STE	1	100	(96–100)	NFFA012	(0)	28.7	23.8	r
		2	1	(0–4)	E35M50_200	(1)	38.0	34.8	r
	FLE	1	98	(94–100)	NFFA012	(2)	6.0	9.5	r
		2	16	(11–21)	E33M59_124	(2)	3.3	5.1	s
		3	9	(2–18)	P38M50_132	(2)	3.2	4.9	s
	HOH	1	86	(73–94)	P42M61_118	(5)	3.3	3.7	r
		2	1	(0–9)	E35M50_200	(1)	13.9	16.9	r
		3	1	(0–7)	LPSSRK03G05	(1)	9.3	11.0	r
	ASE	1	98	(94–100)	NFFA012	(2)	4.3	6.2	r
		2	0	(0–11)	E35M50_200	(0)	4.8	6.8	r
Averaged field sites[a]		1	100	(97–100)	NFFA012	(0)	33.2	33.1	r
		2	1	(0–6)	E35M50_200	(1)	20.5	18.8	r
Glasshouse (GHHOH)		1	99	(94–100)	NFFA012	(1)	22.1	26.9	r
		3	3	(0–7)	LPSSRK03G05	(3)	10.4	12.5	r
		4	74	(64–87)	E32M48_68	(4)	2.7	4.8	s
Leaf segment test (LST)		1	99	(97–100)	NFFA012	(1)	37.4	36.7	r
		2	0	(0–4)	E35M50_200	(0)	20.0	16.4	r
		4	93	(86–108)	E39M50_144	(7)	3.7	3.1	r

Results are based on multiple QTL model (MQM) mapping using MapQTL and separate parental maps, which were aligned to the ILGI reference map (Jones et al. 2002). The 2 LOD support interval and distance of the closest marker to the respective QTL are indicated in parentheses. Source of resistance indicates the parental map used for QTL analysis, which derived from the resistant (r) and susceptible (s) parental plant, respectively

[a] ART, STE, FLE, HOH and ASE

In the present investigation, an F_1 mapping population (Studer et al. 2006) was assessed for crown rust resistance in laboratory, glasshouse and field experiments and revealed highly significant ($P < 0.01$) genotypic variance components for this trait. In the field, in which natural crown rust infections are generally widespread, five out of six experiments showed a high repeatability. At the only site without significant genotypic variance (MAP), crown rust occurred irregularly in the spaced plant nursery leading to significant ($P < 0.01$) differences of disease incidence among the three replications (data not shown). As a consequence, no significant QTL for crown rust resistance was detected at MAP. Although being evaluated by independent local breeders, disease scores of individual locations were significantly correlated ($P < 0.01$) among each other and with the mean across field trial locations. These findings are in congruence with previous studies, where crown rust disease scores of 18 Italian ryegrass cultivars investigated at 23 experimental sites were strongly correlated (Boller et al. 2003), and a large number of perennial ryegrass genotypes displayed universal reactions to infections of several crown rust isolates (Aldaoud et al. 2004). The high correlations detected may reflect a distinct broad spectrum of crown rust resistance in the targeted germplasm governed mainly by two major QTLs. This supports plant breeder's experiences inadvertently targeting major resistance determinants when performing phenotypic selection based on natural infections.

The detected major QTLs mapped to genomic regions known to control crown rust resistance in *L. perenne*, particularly on LG 1 (Forster et al. 2004; Muylle et al. 2005b) and LG 2 (Dumsday et al. 2003; Muylle et al. 2005b; Roderick et al. 2000; Thorogood et al. 2001). Results of such previous QTL studies allow the comparison of crown rust resistance among mapping populations and plant species by means of comparative mapping. However, such synteny-based approaches require molecular markers mapped in the populations to be compared, which are still limited in number. Therefore, more common markers are needed to confirm syntenies between minor QTLs on LG 4 observed in GHHOH and LST, respectively, and the qualitative resistance genes on LG 4 previously detected by Fujimori et al. (2004). In contrast, the SSR marker LPSSRH03F03 (Jones et al. 2002) closely linked to crown rust resistance in perennial ryegrass (Dumsday et al. 2003) was successfully mapped in the present population at posi-

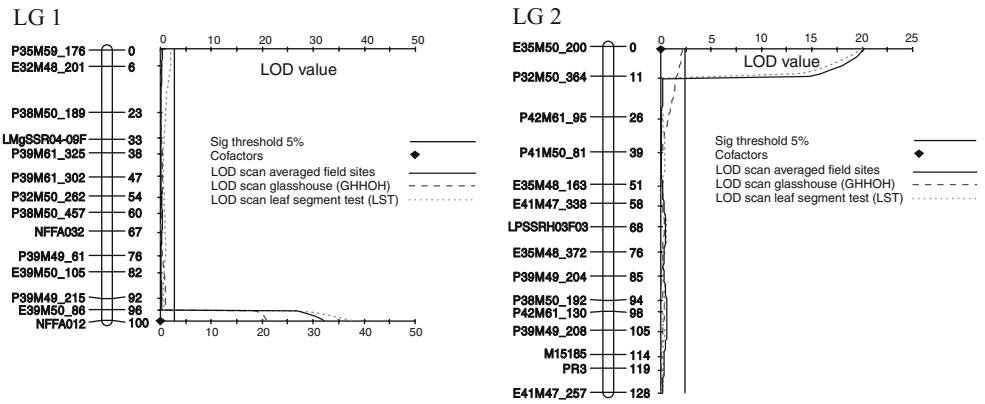

Fig. 2 Linkage map (derived from the resistant parent) used for QTL analysis and the corresponding LOD values of multiple QTL model mapping on *LG 1* and *LG 2*. LOD scans are given for crown rust resistance data derived from averaged results of field experiments at five locations (ART, STE, FLE, HOH and ASE, see Table 1), glasshouse (GHHOH) and the leaf segment test (LST) evaluation. Cofactors designate markers, which absorb the genetic effects of their nearby QTLs and function as a genetic background control

tion 68 cM on LG 2, clearly separated from the major QTL located at one end of the LG. Thus, the detected QTL on LG 2 is likely to describe a distinct source of crown rust resistance when compared to the findings of Dumsday et al. (2003).

In addition to a consistent resistance response under control of the two major QTLs, a few minor QTLs were only partially detected and suggested the existence of location- and method-specific factors influencing crown rust resistance. For the field assessment, partial occurrence of minor QTLs at FLE and HOH as well as modifications of QTL characteristics regarding position and magnitude were reflected in a significant ($P < 0.01$) genotype × environment interaction based on the five field trial locations. Such modifications could be induced by different climatic conditions, such as humidity or temperature, that are reported to cause differences in response of perennial ryegrass cultivars to crown rust infection (Roderick et al. 2000). For example, resistance of the genotypes was most closely correlated ($r = 0.65$) for the two sites revealing the lowest long-term temperature with the highest average of annual rainfall, ART and STE. Similarly, the two sites with the lowest long-term annual rainfall, ASE and HOH, were correlated more closely with each other ($r = 0.58$) than to any other site. In addition, sexual recombination as the primary source of genetic variation has been described for *Pca* on the secondary, aecial host (Simons 1970), enabling the development of race specificity, which was recently reported in perennial ryegrass (Aldaoud et al. 2004). The existence of location-specific pathogen populations varying in virulence was supported by a QTL at the initial segment on LG 3, which was only detected in HOH and GHHOH using spore collections of Hohenheim. This is in congruence with findings in oat (*Avena sativa*), where different races of the crown rust causing pathogen *P. coronata* f. sp. *avenae* were found (Brake et al. 2001).

Multisite field trials based on natural infections were compared to alternative techniques such as GHHOH and LST. Both methods are based on artificial inoculation in controlled environments and thus showed excellent repeatabilities (0.76 and 0.70, respectively) with highly significant genotypic variance components. Compared to the average of field evaluations, LST showed a high correlation of resistance data ($r = 0.79$, $P < 0.01$), which is in congruence with a comparison of the LST within the EUCARPIA multisite field trial, in which Spearman's rank correlation coefficients of 0.79 (perennial ryegrass) to 0.99 (Italian ryegrass) were found (Lellbach 2003). The high correlation among methods and the detection of both major QTLs on LG 1 and LG 2 confirmed LST as a suitable method for resistance evaluation both on phenotypic and on QTL level, whereas only one major QTL on LG 1 was identified in the glasshouse evaluation. This may be a consequence of specific pathogen accessions used for inoculation or of differences in inoculation procedure under described experimental and environmental conditions. Therefore, the development of pathogen isolines will be necessary to unravel effects of specific pathogen compositions. The genetic marker system recently published by Dracatos et al. (2006) may help to characterise genetic diversity of the pathogen and thus offers the possibility of standardising pathogen populations.

Phenotypic evaluation revealed significantly lower mean disease scores of the resistant reference population PopM2289 when compared to the F_1 mapping progeny and the susceptible reference population PopAdret, which indicated successful breeding progress achieved by recurrent selection at ART. In fact, the highest proportions of total V_p explained by the major QTL on LG 1 were detected at ART (field: 56%; LST: 37%), which may be the result of particularly effective selective breeding for major resistance at the location of selection.

Segregation of resistance in the F_1 mapping population as well as the allele composition of the markers most closely linked to the detected major QTLs (SSR marker NFFA012 on LG 1 and AFLP marker E35M50_200 on LG 2, respectively) indicated the resistant mapping parent to be heterozygous for both major QTLs. Hence, homozygosity of the allele associated with crown rust resistance could not be achieved using phenotypic selection. The present study provides molecular markers, which greatly facilitate the fixation of such valuable alleles in outbreeding populations by means of MAS and in addition give access to previously undescribed sources of crown rust resistance in Italian ryegrass. However, successful application of MAS requires QTL validation in different genetic backgrounds and breeding germplasms. The identification of genes underlying QTLs, and consequently the development of functional markers (Andersen and Lübberstedt 2003), would eliminate the risk of loosing the linkage between a marker and a QTL during MAS. This may be achieved by exploiting the high degree of synteny among grasses (reviewed by Devos 2005) and by identification of candidate gene-based markers and resistance gene analogs, which co-locate with the QTLs (Cogan et al. 2006; Ikeda 2005). Subsequent map-based cloning approaches require accurate positioning of QTLs, which depends on the type of population used for mapping, the number of individuals scored and the quality of data (Visscher and Goddard 2004). As small population may lead to underestimation of QTL number and overestimation of explained variance (Schön et al. 2004), a relatively large number of 306 F_1 individuals were assessed in the present study. Although QTLs of small and environmentally sensitive effects generally suffer from large confidence intervals, they may exhibit varying degrees of complex nonadditive behaviour contributing to the genetic basis of crown rust resistance. Often masked by the effects of the two major QTLs and thus not detectable in classical phenotypic selection, molecular markers closely linked to such minor QTLs may represent a valuable tool to effectively complement major resistance detected in the mapping germplasm.

The present study provides not only fundamentals on the relationship of different evaluation methods, but also highlights the significance of multisite field experiments concerning crown rust evaluations for plant breeding in general and QTL studies in particular. Two major QTLs conferring resistance to crown rust were detected on LG 1 and LG 2 and represent attractive candidate gene regions for novel sources of crown rust resistance. The identified molecular markers closely linked to the detected QTLs may substantially contribute to improve disease resistance by means of MAS in one of the most important forage crop species.

Acknowledgments We would like to acknowledge the following breeding institutes and companies for performing field experiments and providing crown rust resistance data: Advanta Flevopolder, the Netherlands; DSV Asendorf, Germany; LSA Stuttgart Hohenheim, Germany; NPZ Hohenlieth, Germany and Saatzucht Steinach, Germany. CRCMPB, Australia and NILGS, Japan kindly granted research licences for the use of SSR markers. We further thank A. Schmidt, P. Streckeisen, Y. Häfele and L. Kren for excellent technical support, Prof. Dr. agr. H.F. Utz and Dr. C.-C. Schön, University of Hohenheim for support regarding field trial design, QTL and statistical analyses. The present study was funded by the Swiss National Science Foundation (grant 3100-065417) and kindly supported by grants from the "Bundesministerium für Verbraucherschutz, Ernährung und Landwirtschaft" (BMVEL) and "Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung e.V." (GFP, grant 01 HS 006).

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