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ORIGINAL PAPER

Genetic mapping reveals a single major QTL for bacterial wilt resistance in Italian ryegrass (*Lolium multiflorum* Lam.)

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Abstract Bacterial wilt caused by *Xanthomonas translucens* pv. *graminis* (*Xtg*) is a major disease of economically important forage crops such as ryegrasses and fescues. Targeted breeding based on seedling inoculation has resulted in cultivars with considerable levels of resistance. However, the mechanisms of inheritance of resistance are poorly understood and further breeding progress is difficult to obtain. This study aimed to assess the relevance of the seedling screening in the glasshouse for adult plant resistance in the field and to investigate genetic control of resistance to bacterial wilt in Italian ryegrass (*Lolium multiflorum* Lam.). A mapping population consisting of 306 F_1 individuals was established and resistance to bacterial wilt was assessed in glasshouse and field experiments. Highly correlated data ($r = 0.67$–0.77, $P < 0.01$) between trial locations demonstrated the suitability of glasshouse screens for phenotypic selection. Analysis of quantitative trait loci (QTL) based on a high density genetic linkage map consisting of 368 amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) markers revealed a single major QTL on linkage group (LG) 4 explaining 67% of the total phenotypic variance (Vp). In addition, a minor QTL was observed on LG 5. Field experiments confirmed the major QTL on LG 4 to explain 43% (in 2004) to 84% (in 2005) of Vp and also revealed additional minor QTLs on LG 1, LG 4 and LG 6. The identified QTLs and the closely linked markers represent important targets for marker-assisted selection of Italian ryegrass.

Keywords Disease resistance · Linkage mapping · *Lolium multiflorum* · Quantitative trait loci (QTL) · *Xanthomonas translucens* pv. *graminis*

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Introduction

Italian ryegrass (*Lolium multiflorum* Lam.), a forage crop of key importance in temperate regions worldwide, is negatively affected by the bacterial wilt-inducing pathogen *Xanthomonas translucens* pv. *graminis* (*Xtg*). First observed in the early 1970s (Egli et al. 1975), bacterial wilt was recognised as a major disease of many forage grass species (Paul and Smith 1989) and is prevalent in most contemporary pastures and meadows of Europe, the USA and Australasia (Leyns 1993). Substantial yield losses and reduced persistency in *Lolium* and *Festuca* species have been reported in temperate grassland (Channon and Hissett 1984), with Italian ryegrass considered to be the most susceptible species (Schmidt and Nüesch 1980). As host plant infection occurs through stomata and epidermal wounds, mowing facilitates infection and spread of bacterial wilt by contaminated equipment. Several measures of disease control have been considered, among them disinfection of contaminated mowing equipment and inoculation with epiphytic

bacteria to induce disease resistance. However, these methods showed only limited success in the field (Schmidt 1988a, b). As chemical pest and disease control is highly undesirable in grassland management, breeding for resistant cultivars remains the only practicable means of disease control.

Targeted resistance breeding based on artificial inoculation of seedlings in the glasshouse and subsequent phenotypic selection was initiated shortly after discovery of the causal agent of bacterial wilt and resulted in Italian ryegrass cultivars with considerable levels of disease resistance (Lehmann et al. 2000). However, despite rapid initial progress in breeding for resistance, further improvement appeared to be difficult in advanced generations and highly susceptible plants could still be observed after many cycles of recurrent selection (Michel 2001). In a recurrent selection programme with meadow fescue (*Festuca pratensis* Huds.), improvement of resistance to bacterial wilt stagnated after four to six cycles of selection without achievement of complete resistance (Boller et al. 2001).

Resistance to *Xanthomonas* spp. has been extensively studied in major crop species, in which race specificity based on gene for gene interactions have been frequently observed (reviewed by Baker et al. 1997). In rice, for which cultivar development is based on repetitive self-fertilisation resulting in nearly homozygous lines corresponding to a single genotype, 24 race specific major genes for resistance to bacterial blight have been recognised (Gnanamanickam et al. 1999; Rao et al. 2002). In addition, both quantitative (Lopez et al. 2005; Mutlu et al. 2005; Verdier et al. 2004) and qualitative (Delannoy et al. 2005; Essenberg et al. 2002; Yang and Francis 2005) resistance mechanisms have been observed in various dicotyledonous plant species such as cassava (*Manihot esculenta* Crantz), cotton (*Gossypium* spp.), pinto bean (*Phaseolus vulgaris* L.), tomato (*Lycopersicum esculentum* Mill.) and passion fruit (*Passiflora edulis* f. *flavicarpa*). In contrast to inbreeding species, cultivars of outbreeding forage grass species such as Italian ryegrass are generally based on multiple crosses among several selected parents and subsequent random mating, resulting in highly heterogeneous populations consisting of many individual genotypes (Brummer 1999). Therefore, disease resistance in ryegrass is generally based on interactions of a large number of diverse host genotypes with pathogen populations of mostly unknown genetic composition, rendering difficult prediction of the genetic principles of resistance. For example, several QTLs of varying magnitude (Dumsday et al. 2003; Muylle et al. 2005a, b; Thorogood et al. 2001) as well as single genes conferring qualitative resistance (Fujimori et al. 2004) have been identified for crown rust caused by *Puccinia coronata* f. sp. *lolii* and there is growing evidence for the existence of specific pathotypes (Aldaoud et al. 2004).

Although continuous variation for bacterial wilt symptoms in breeding populations indicates quantitative inheritance, little is known about the genetic control of resistance to bacterial wilt in *L. multiflorum*. Moreover, there is a lack of knowledge of the relationship between seedling resistance observed in the glasshouse and adult plant resistance in the field. Therefore, a detailed investigation of the genetic control of bacterial wilt in *L. multiflorum* and its phenotypic evaluation in glasshouse and field experiments is needed for improvement of targeted breeding efforts for this important trait.

Genetic linkage map construction and QTL analysis are valuable tools for genetic dissection of complex traits (Yamada et al. 2005) and may permit identification of genetic markers closely linked to genes and QTLs controlling important traits. The exploitation of such markers by means of marker-assisted selection (MAS) can complement traditional breeding tools and may help to implement more efficient breeding strategies in outcrossing species (Newbury 2003). Therefore the aims of our study were (1) to assess the relationship of the phenotypic characterisation of resistance to bacterial wilt of seedlings in the glasshouse and adult plants in the field, (2) to construct a high density genetic linkage map, (3) to detect the number of QTLs or genes conferring resistance to bacterial wilt and (4) to evaluate their contribution to resistance and genomic location for the development of genetic markers closely linked to resistance for MAS implementation in breeding programmes.

Materials and methods

Plant material

A two-way pseudo-testcross population (Grattapaglia and Sederoff 1994) derived from a reciprocal cross between two highly heterozygous *L. multiflorum* genotypes with contrasting levels of resistance to bacterial wilt was established. The resistant genotype was selected from advanced breeding germplasm (Agroscope Reckenholz-Tänikon ART, Zurich, Switzerland) while the susceptible genotype was selected from the cultivar Adret (Verneuil Recherche, Verneuil-Etang, France). An equal number of 200 seeds were harvested from both parental genotypes, germinated on wet filter

paper (Schleicher and Schuell, Dassel, Germany) for 3 days, transferred into soil filled pots and grown in the glasshouse for DNA extraction and clonal propagation. After excluding plants resulting from self-fertilisation, which were identified using a fully informative simple sequence repeat (SSR) marker, the mapping population consisted of 306 F_1 individuals, 153 derived from each parent.

Phenotypic characterisation of bacterial wilt resistance

Assessment of resistance to bacterial wilt in the glasshouse was performed in four completely randomised replications at Agroscope Reckenholz-Tänikon ART. Six-week-old seedlings were clonally propagated by separating single tillers, which were transferred into soil filled pots. After 4 weeks, four plants per genotype were inoculated using the leaf clipping technique of Kauffman et al. (1973), whereas the remaining uninfected clones were maintained for field evaluation. Pathogen inoculum for infection of the plants was produced on yeast dextrose calcium agar (YDCA) as described by Rechsteiner et al. (2006) on petri dishes for 3 days at 28°C. Bacteria of the isolate *Xtg* 29 (Kölliker et al. 2006) were washed from the agar and diluted with 0.8% saline solution to a final concentration of 10^9 colony forming units (CFU)/ml. Plants were cut to 4 cm stubble height with scissors dipped into the bacterial suspension and allowed to regrow under 32°C/24°C (day/night temperatures), long day conditions (16 h light, [> 100 $\mu Em^{-2}s^{-1}$]) and an average of 90% relative humidity. Disease severity was scored 9, 14 and 28 days after inoculation according to a symptom severity scale ranging from 1 (no symptoms) to 9 (plant died). Disease scores obtained 28 days after infection showed the highest variance among the F_1 genotypes and were used for further analysis. Assessment of resistance to bacterial wilt in the glasshouse was repeated using the same bacterial isolate and a subset of 60 genotypes in order to confirm its reliability.

Field resistance to bacterial wilt was assessed at Agroscope Reckenholz-Tänikon ART (441 m above sea level) with total rainfall of 131 mm and an average temperature of 23°C (day) and 14°C (night) during 33 days of field evaluation. In spring 2004, three uninfected replicates per genotype of the clonally propagated mapping population were planted into a field nursery using an α-design and a 0.5×0.3 m plant-to-plant spacing. Infection was performed in July 2004 (9 weeks after planting) using 0.3 l/m^2 of pathogen inoculum (10^9 CFU/ml), which was continuously sprayed onto the blade of a front mower using a backpack sprayer to ensure homogeneous dispersal on cut plant surfaces. Disease progress was monitored in the field 18, 24, 28 and 33 days after infection using the same scale as used in the glasshouse. Although surviving plants largely recovered from infection, they again showed disease symptoms in the following season in July 2005 and disease scoring was repeated without prior inoculation.

Statistical analyses of phenotypic data

Analysis of variance and estimation of variance components of glasshouse data were performed using the general linear model (GLM) of the STATISTICA software (version 6.1, StatSoft, Tulsa, OK, USA) considering the factor genotype as random. Least square means (LSM) were used for QTL analysis. For field data, lattice analysis was calculated using the PLABSTAT software, version 2 P (Utz 2000). The adjusted entry means obtained were subsequently used for QTL analysis. Repeatability was calculated according to the formula $r = \sigma_g^2/\left(\sigma_g^2 + \sigma_e^2\right)$, where σ_g^2 represents the variance component of the genotype and σ_e^2 the effective error mean square from single ANOVA (Falconer and Mackay 1996).

Marker analyses and genetic linkage map

Fresh leaf tissue of the parental plants and 306 F_1 individuals was used for DNA extraction using the DNeasy 96 plant kit (Qiagen, Hilden, Germany). In total, 233 SSR primer pairs were screened for polymorphisms within a subset of the mapping population consisting of the resistant and the susceptible parental plant as well as 14 F_1 genotypes using primer specific PCR protocols and 3% Metaphore™ gel electrophoresis. References to primers are given in Table 1. SSR primers reproducibly detecting polymorphisms within the subset were applied to the whole mapping population. PCR reactions were conducted in a total volume of 10 µl containing 20 ng of genomic DNA, 1× PCR buffer (Invitrogen, Carlsbad, CA, USA), 0.2 mM of each dNTP (Invitrogen), 1.5 mM $MgCl_2$, 0.25 U of Taq DNA polymerase (Invitrogen) and 200 nM of the respective forward (Cy5® labelled) and reverse primer. Amplification conditions consisted of an initial denaturation step of 4 min at 94°C followed by 35 cycles of 30 s denaturation at 94°C, 30 s annealing according to the primer specific annealing temperature and 30 s extension at 72°C. After a final extension of 7 min at 72°C, size of amplification products was determined by gel electrophoresis on a 6% polyacrylamide gel using an ALF Express DNA-Sequencer

Table 1 Names, numbers and references of SSR loci used for the final map of the *L. multiflorum* segregating F_1 population

Marker denomination	Number of markers mapped	References
LPSSRHXX242 to LPSSRK15H05	9	CRCMPB, Australia[a]
LMgSSR02-01B to LMgSSR04-09F	4	NILGS, Japan[b]
NFFA012 to NFFA109	6	Saha et al. (2004)
M15185, PR3, PR25, PRG, PR10	5	Kubik et al. (2001)
B1-C9, B3-B8, B1-A8, B3-B6	4	Lauvergeat et al. (2005)

[a]Primer sequences kindly provided under research licence by the Cooperative Research Centre for Molecular Plant Breeding (CRCMPB), Glen Osmoud, Australia

[b]Primer sequences kindly provided under research licence by the National Institute of Livestock and Grassland Science (NILGS), Nishinasuno, Tochigi, Japan

(Amersham Pharmacia Biotech, Freiburg, Germany). Marker segregation among the 306 F_1 individuals was scored manually.

AFLP analysis was performed according to the protocol of Vos et al. (1995) with the modifications reported by Kölliker et al. (2003) using eight *Pst*I/*Mse*I (P32M49, P32M50, P35M59, P38M50, P39M49, P39M61, P41M50 and P42M61) and nine *Eco*RI/*Mse*I (E32M48, E35M48, E35M50, E38M59, E39M48, E39M50, E41M47, E41M59 and E42M59) primer combinations with three selective nucleotides on each primer. AFLP fragments were analysed on an ABI Prism 3100 genetic analyser using POP4 polymer and 36 cm capillaries (Applied Biosystems, Foster City, CA, USA). Amplification patterns were scored for presence and absence of fragments using the Genotyper 3.6 software and polymorphic loci were denominated using standard AFLP nomenclature (Keygene, Wageningen, the Netherlands).

SSR and AFLP data were converted into JoinMap® (version 3.0; Van Ooijen and Voorrips 2001) compatible genotype codes using the cross pollination (CP) segregation type indicating a population resulting from a cross between two heterozygous diploid parents.

The genetic linkage map based on 306 F_1 individuals was constructed using JoinMap®. Marker segregation ratio was tested using the χ^2 test implemented in JoinMap® and markers deviating from the expected segregation ratio at the level of $\alpha = 0.001$ were considered as highly distorted and excluded from map calculation. Separate parental maps were calculated and combined to a biparental consensus map using markers segregating in both parents, which served as allelic bridges. For linkage group determination, a LOD threshold of 4.0 or lower was used. Map calculations were performed using a LOD larger than 1.0, a ripple value of 1.0 and a jump threshold of 5.0. Genetic distances were estimated using the Kosambi mapping function (Kosambi 1944).

QTL analysis

QTL analysis of both glasshouse and field resistance data was performed using MapQTL (version 5.0; Van Ooijen 2004). This program allows for the detection of QTLs in cross pollinating species such as Italian ryegrass (CP population type) using a multiple QTL model (MQM). QTL detection was optimised for each experiment as follows: molecular markers significantly associated with resistance response were first detected using the separated parental maps with the F_1 genotypes coded as a double haploid (DH) population. The preselected markers identified using PlabQTL (Utz and Melchinger 1996) and biparental SSRs were primarily used to construct a map with markers evenly distributed over the genome and with an average locus distance of 5–10 cM. After a first interval mapping analysis, marker density in putative QTL regions was increased. Final QTL analysis was performed using backward cofactor selection and MQM mapping with optimised maps. LOD significance threshold levels, which were specific for each experiment, were determined using 5,000 permutations.

Results

Phenotypic characterisation

Average bacterial wilt disease scores in the glasshouse increased from 3.90 (9 days) to 6.63 (28 days). At the 28 day time point, least square means (LSM) of the genotypes varied from 3.25 to 9.00 with a mean of 6.63 and a standard deviation of 1.56 (24% of the mean, Table 2). The experiment showed a repeatability of

Table 2 Key values of the phenotypic characterisation of resistance to bacterial wilt of a *L. multiflorum* mapping population consisting of 306 F_1 individuals

Least square means (LSM)	Glasshouse	Field 2004	Field 2005
Mean	6.63	2.76	3.08
Minimum	3.25	1.00	0.95
Maximum	9.00	6.17	8.50
Standard deviation	1.56	1.04	2.21
Repeatability	0.84	0.64	0.80

Resistance was assessed in glasshouse and field experiments and results are based on least square means of three (field) and four (glasshouse) replicates per genotype

0.84 and resistance data were highly correlated with data obtained from a repeated glasshouse assessment of a subset of 60 F_1 genotypes using the same bacterial isolate ($r = 0.78$–0.82, $P < 0.01$). The frequency distribution of genotype LSM was bimodal and skewed towards the resistant genotypes (skewness: –0.01, kurtosis: –1.19), but residuals fitted to a normal distribution (data not shown). ANOVA revealed a highly significant difference among F_1 genotypes, but no significant effect of maternal plants on the disease score was observed (Table 3). The ANOVA explained 88% of the observed variance.

In the field trial in July 2004, average disease scores for bacterial wilt developed from 1.80 (18 days) to 2.46 (24 days) and reached a maximum value of 2.74 33 days after inoculation, when genotypic variability for resistance was highest. Scores obtained 33 days after inoculation showed a repeatability of 0.64 and LSM ranged from 1.00 to 6.17 with a mean of 2.76 and a standard deviation of 1.04 (38% of the mean). The frequency distribution of LSM was skewed towards resistance (skewness: 0.63, kurtosis: –0.02), but residuals were normally distributed. One year after inoculation, i.e. in July 2005, all F_1 genotypes were regrown in the field and showed again extensive variation for bacterial wilt resistance without prior pathogen inoculation. LSM of the disease scores varied from 0.95 to 8.50, with a mean of 3.08 and a standard deviation of 2.21 (72% of the mean). ANOVA of field data collected in 2004 and 2005 explained 67 and 87% of total variance, respectively, and revealed highly significant differences among F_1 genotypes (Table 3). No evidence for the influence of maternal factors on field resistance was observed in the different data sets. Product moment correlation among the three data sets was highest for field data from 2004 and field data from 2005 ($r = 0.78$, $P < 0.01$) and reached r values of 0.67 ($P < 0.01$) and 0.77 ($P < 0.01$) for glasshouse and field data of 2004 and 2005, respectively.

Genetic linkage mapping

Of the 233 SSR primer pairs screened, 51 (22%) amplified products which were clearly polymorphic within the subset of the mapping population. The low proportion of polymorphisms detected may be explained by the limited resolution of agarose gel electrophoresis used for the initial screening. Among the 51 polymorphic SSR markers, 20 were biparental (both parents were heterozygous for that locus) while the remaining 31 primer pairs produced monoparental markers with only one parent heterozygous for that locus (Table 4). The latter type of SSR marker was scored as a dominant feature. The 17 AFLP primer combinations yielded 12–30 markers each with a mean of 22 and a total of 367 (Table 4). No difference in the number of markers per primer combination or in the distribution of markers within the linkage groups (LG) was observed between *Pst*I/*Mse*I and *Eco*RI/*Mse*I primer combinations. Ten AFLP and five SSR markers, i.e. 3.6% of all marker, deviated significantly ($P < 0.001$) from expected Mendelian segregation ratios. These markers showed no clustering and were randomly distributed across the genome (data not shown). After exclusion of these highly distorted markers as well as individuals and markers, which showed more than 15% missing values, 368 loci and 297 individuals were finally used to construct a genetic map with a total map length of 804 cM and an average distance between loci of 1.9 cM (Fig. 1). The resulting

Table 3 *F*-values, levels of significance and proportion of phenotypic variance explained (R^2) by ANOVA for the phenotypic evaluation of a *L. multiflorum* mapping population consisting of 306 F_1 genotypes

	Glasshouse		Field 2004		Field 2005	
	df	*F*	*df*	*F*	*df*	*F*
Maternal plant[a]	1	1.3 (NS)	1	3.3 (NS)	1	2.2 (NS)
Genotype (maternal plant)[b]	304	22.0***	304	6.3***	304	12.7***
Replication	3	51.1***	2	22.4***	2	0.6 (NS)
Error	915		610		610	
R^2		0.88		0.67		0.87

Resistance to bacterial wilt was assessed in glasshouse and field experiments with three (field) and four (glasshouse) replicates per genotype

NS not significant

***$P \leq 0.001$

[a]Progenies were obtained from a reciprocal cross; one half of the genotypes were harvested from the resistant and the other half from the susceptible parental plant, respectively

[b]Nested analysis; genotype within motherplant

Table 4 Number of SSR and AFLP loci used for the construction of a genetic linkage map in *L. multiflorum*

	Bi-parental loci[a]			Mono-parental loci[b]		Total
	abxcd[c]	efxeg[c]	hkxhk[c]	lmxll[c]	nnxnp[c]	
SSR	9	11 (2)	9 (2)	14 (1)	8	51 (5)
AFLP *Eco*RI/*Mse*I	–	–	12	76 (2)	100 (3)	188 (5)
AFLP *Pst*I/*Mse*I	–	–	14	75 (3)	90 (2)	179 (5)
Total	9	11 (2)	35 (2)	165 (6)	198 (5)	418 (15)

The number of distorted markers is indicated in parentheses

[a]Loci heterozygous in both parents

[b]Loci heterozygous in one parent

[c]Segregation types according to JoinMap (Van Ooijen and Voorrips 2001)

seven LGs contained 38–68 markers (mean of 53) with at least two biparental SSR markers present on each LG. Length of LGs ranged from 87 to 154 cM, with an average of 115 cM.

QTL analysis

QTL analysis based on glasshouse data revealed two QTLs exceeding the genome-wide significance threshold (Table 5). On LG 4, a major QTL was detected at position 52 cM explaining 67% of total phenotypic variance (Vp) for disease resistance. This QTL is closely linked to the marker LPSSRK05A11 at a distance of less than 1 cM. A second QTL was identified on LG 5 at position 32 cM, explaining 7.4% of total Vp. Field data of 2004 revealed four significant QTLs. A major QTL on LG 4 was found at position 56 cM explaining 43% of Vp. The AFLP marker P38M50_252 mapped at position 56 cM and was consequently most closely linked to this major QTL. Additionally, three QTLs on LG 1, LG 4 and LG 6 were detected explaining 3–11% of total observed variance for resistance. Using field data from 2005, only one major QTL on LG 4 was identified 1.3 cM away from AFLP marker P38M50_252 at position 55 cM, explaining 84% of Vp. The major QTL on LG 4 was consistently observed in all experiments at a map position between 52 and 56 cM, explaining 43–84% of resistance variance while minor QTLs on LG 1, LG 4, LG 5 and LG 6 were not consistently found in glasshouse and field experiments (Table 5).

Discussion

Reliable phenotypic analysis is crucial not only for QTL studies, in which the association of molecular markers with the target trait is analysed, but also for plant breeding in general, for which the method used for selection must accurately reflect the expected phenotype of plants under cultivation. The artificial pathogen inoculation in the glasshouse used in this study showed an excellent repeatability of 0.84 and was therefore also employed in a field experiment. In the field, average disease scores for bacterial wilt (2.76 in 2004 and 3.08 in 2005, respectively) were clearly lower when compared to the glasshouse experiment (6.63). This may be primarily explicable due to the controlled conditions in the glasshouse, which make it possible to optimise temperature and relative humidity for growth and dispersal of bacterial wilt (Imaizumi et al. 1999). Moreover, plants inoculated in the field showed more vigorous constitution due to optimal field growth and mechanical depth of cut was lower in the field when compared to manual cutting in the glasshouse. The assumption that adult plants generally show enhanced levels of resistance to bacterial wilt was rejected based on the repetition of the glasshouse screening using a subset of 60 adult genotypes, which resulted in similar genotypic disease scores (data not shown). Despite lower mean disease scores, repeatability values obtained in the field were high (0.64 in 2004 and 0.80 in 2005) and the scores of both years were highly correlated ($r = 0.78$, $P < 0.01$). In addition, field data showed high and significant correlation to data obtained in the glasshouse ($r = 0.77$ and 0.67, $P < 0.01$). Thus, resistance screening in the glasshouse permitted prediction of response in the field and may hence provide a suitable method for improvement of bacterial wilt resistance in forage crop breeding programmes.

The congruence of glasshouse and field data was highlighted by the extent and location of highly coincident QTLs on linkage group (LG) 4. This QTL location appeared to be stable across each experiment and was closely linked to the nearest genetic marker with distances less than 1.3 cM. Although the linked AFLP markers require conversion into sequence specific PCR-based markers and confirmation in independent populations, they provide a promising tool for

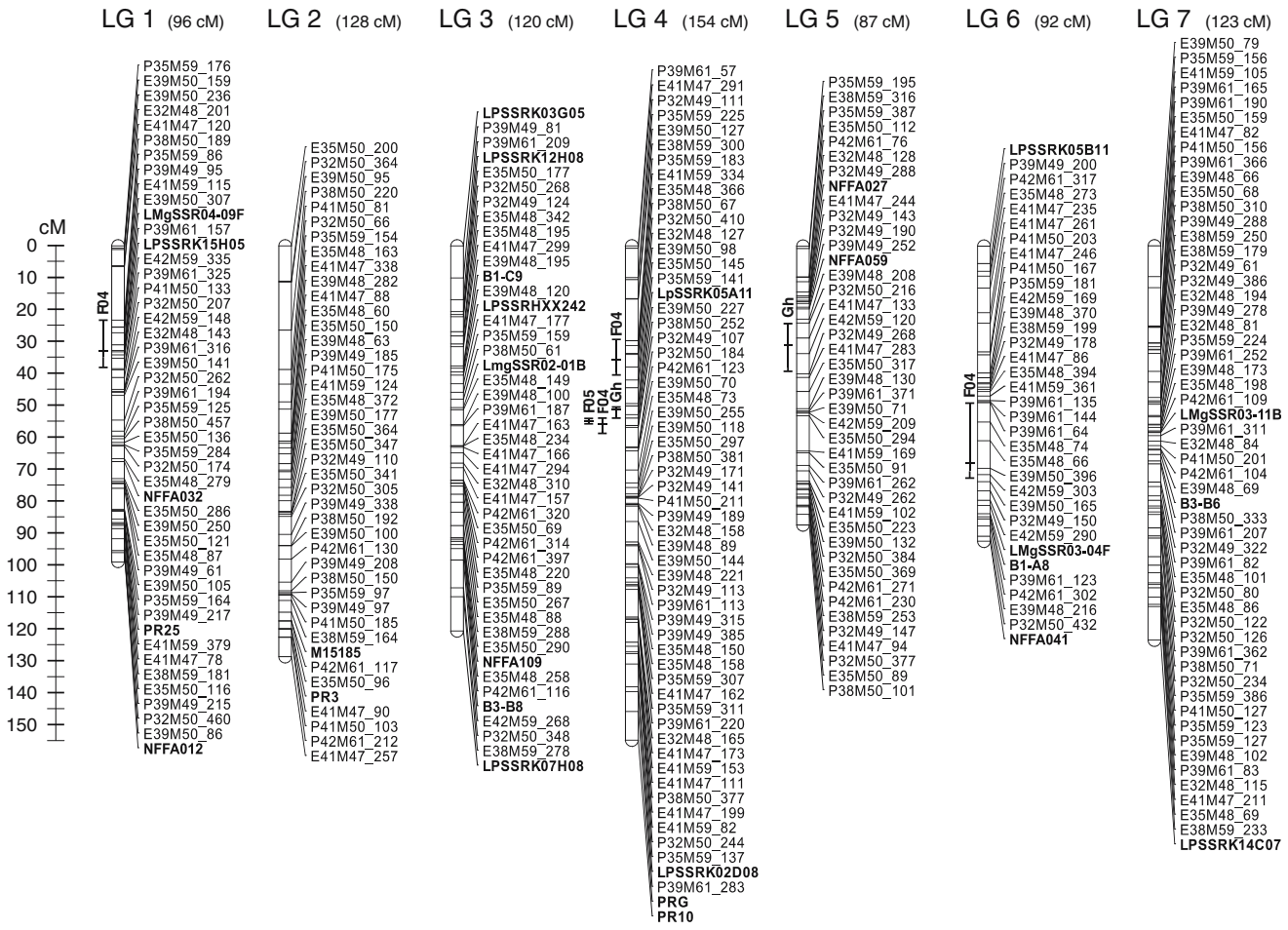

Fig. 1 Genetic linkage map of a *L. multiflorum* population based on 297 F_1 genotypes and 368 markers. The seven linkage groups have been aligned with the ILGI reference map (Jones et al. 2002a). Locus names of SSR markers (*bold*) consist of the original denomination (Table 1). For AFLP markers, the standard primer combination code (Keygene, Wageningen, the Netherlands) was used followed by the allele size in relative migration units. Scale units are given in centiMorgan (*cM*). Positions of QTLs for bacterial wilt resistance were calculated using MQM mapping based on least square means of resistance data from glasshouse (*Gh*) and field experiments (F04, F05). The maximum LOD score position of each QTL is indicated with a *horizontal line*, *bars* represent the interval between two positions obtained at LOD scores two units lower than the maximal score. For better readability, only one of multiple markers mapping to the same position is shown

Table 5 Position and description of QTLs for bacterial wilt resistance identified in glasshouse and field experiments using MQM mapping and a *L. multiflorum* mapping population consisting of 306 F_1 genotypes

Environment	Linkage group	Position (95% CI) [cM]	Closest marker (distance) [cM]	Maximum LOD score	Percentage of variance explained	Source of resistance
Glasshouse	4	52 (51–54)	LpSSRK05A11 (0.6)	70.6	66.8	Resistant parent
	5	32 (24–39)	E41M47_133 (3.0)	11.1	7.4	Resistant parent
Field 2004	1	27 (22–38)	LMgSSR04-06F (5.6)	3.9	2.9	Resistant parent
	4	36 (29–41)	P32M50_410 (2.3)	5.5	10.8	Resistant parent
	4	56 (54–58)	P38M50_252 (0.3)	38.3	43.3	Resistant parent
	6	68 (49–73)	E39M50_196 (1.5)	7.1	7.3	Resistant parent
Field 2005	4	55 (54–56)	P38M50_252 (1.3)	65.9	83.8	Resistant parent

Confidence intervals (CI) and distances of the closest marker to the respective QTLs are indicated in parentheses

the further improvement of bacterial wilt resistance in *L. multiflorum* by means of marker assisted selection (MAS).

In addition to the major QTL on LG 4, which was consistently observed in each experiment, several minor QTLs with small effects on phenotypes were detected in the glasshouse and in the field experiment in 2004. Such minor QTLs suffer from limited precision regarding positioning (Schön et al. 2004), resulting in substantial confidence intervals (Table 5). However, they may exhibit varying degrees of complex epistatic behaviour contributing to the genetic basis of the trait under analysis (Erickson 2005) and thus complement the major resistance observed in this study. The effect of an existing major QTL often masks minor QTLs and the latter are therefore not detectable in classical phenotypic selection. Thus, selection based on markers closely linked to minor QTLs provides an effective means to introgress such resistance traits into breeding germplasm.

In addition to reliable phenotypic characterisation, precision and accuracy of QTL analysis based on segregating populations largely depend on map quality and population size. Although the AFLP and genomic SSR markers used in this study are not functional markers and thus may not be directly associated with the trait of interest (Faville et al. 2004), they yielded a high density linkage map with an average distance between marker loci of 1.9 cM (Fig. 1). As functionally associated candidate gene-based markers are only available for a limited number of species and traits, such a linkage map still provides a valuable tool for the molecular characterisation of agronomic traits. Preselection of AFLP primer combinations and the use of both *Pst*I and *Eco*RI as rare cutting restriction enzymes resulted in a high level of polymorphism with markers evenly distributed over the genome. In contrast to other studies (Bert et al. 1999; Castiglioni et al. 1999), no pronounced clustering of *Eco*RI/*Mse*I based AFLP markers in centromeric regions was observed. The total map length of 804 cM was comparable to the length of maps developed from *L. perenne* (692–930 cM; Bert et al. 1999; Hayward et al. 1998; Jones et al. 2002a, b) but significantly shorter then the *L. multiflorum* map of Inoue et al. (2004), which had a total map length of 1,244.4 cM. This discrepancy may be due to the substantially larger number of AFLP markers incorporated causing map extension (Harushima et al. 1998; Stam 1993) and the use of the Haldane instead of the Kosambi map function. Segregation distortion may negatively influence the stability of genetic maps as well as the accuracy of QTL analyses. Only 15 DNA markers (3.6%) deviated significantly from Mendelian segregation ($P = 0.001$). These markers were randomly distributed across linkage groups not indicating any relationship between observed segregation distortion and the self-incompatibility loci. The parents may consequently be considered to be completely heterogeneous at the S and Z self-incompatibility loci (Thorogood et al. 2002, 2005). Distorted alleles originated from both pollen donor and recipient, eliminating unequal selection pressures on pollen or egg cells during fertilisation as a potential source of segregation distortion. Therefore, the observation of segregation distortion of five SSR and ten AFLP markers is likely to be due to inaccurate assignment of segregation patterns of SSR and size homoplasy of AFLP markers (O'Hanlon and Peakall 2000; Vekemans et al. 2002).

Small populations may lead to underestimation of QTL number, overestimation of explained variance (Schön et al. 2004) and limited precision (Visscher and Goddard 2004). The relatively large number of analysed individuals (306) and the detection of a small number of QTLs of generally large magnitude support the reliability of the results obtained, since traits with a high number of QTLs with small effects are particularly prone to overestimation (Schön et al. 2004). Resistance to crown rust in *L. multiflorum* may be partially influenced by the presence of maternal effects, due to variation in mitochondrial and choroplast DNA (Adams et al. 2000). Such factors could severely confound the interpretation of QTL analysis. However, for bacterial wilt resistance, no maternal effects were detected, indicating that nuclear genetic variation is responsible for variability of this trait.

Generally, a gradual variation of disease response indicates a quantitative inheritance of the resistance. Although gradual variation was also observed in the current study, the frequency distributions of the resistance scores observed in the different experiments rather suggested the existence of one or few genes with major effects. The presence of a single major QTL on LG 4 from analysis of both glasshouse and field experiments strongly supports this assumption. This is in accordance with the findings in other species, for which resistance to *Xanthomonas* spp. was predominantly associated with a few major genes or QTLs (Brinkerhoff 1970; Mutlu et al. 2005; Verdier et al. 2004; Yang and Francis 2005). Although in the present study, resistance to bacterial wilt in *L. multiflorum* appeared to be mainly influenced by one major QTL, complete resistance was rare and generally a broad variation for resistance was observed. Therefore, general defence mechanisms, which limit bacterial penetration and colonisation of vascular tissue through

mechanisms like lignification, callose deposition, suberisation and accumulation of phenolic compounds (Kpémoua et al. 1996) seem to be responsible for the partial resistance observed. This observation is in accordance with a survey of 30 *Xtg* isolates, for which limited virulence variation and no indication for the existence of race specific resistance interactions were observed (Kölliker et al. 2006). However, in rice, also a member of the Poaceae, 24 race specific resistance genes to *Xanthomonas* spp. have been identified (Gu et al. 2005; Rao et al. 2002). Among them, the broad spectrum resistance gene *Xa21* is of particular interest, as it is located on LG 11, which is considered syntenic to *L. multiflorum* LG 4 (Devos 2005), where the major QTL for *Xtg* resistance was identified in the present study. Sequence availability of several *Xanthomonas* spp. resistance genes from other species, resistance gene analogs identified in Italian ryegrass (Ikeda 2005) and the use of conserved sequences characteristic for many resistance genes (Meyers et al. 1999) offer further opportunities for the molecular characterisation of bacterial wilt resistance using a candidate gene-based approach (Andersen and Lübberstedt 2003).

To the best of our knowledge, this is the first study on molecular dissection of bacterial wilt resistance in forage grasses. Screening for resistance to bacterial wilt using artificial inoculation in the glasshouse was shown to be a reliable tool to efficiently select for improved resistance under field conditions. The major QTL on LG 4 consistently detected in the glasshouse as well as in the field indicated that the resistance was controlled by one major locus influenced by several minor QTLs. The identified markers closely linked to the detected QTLs represent a valuable basis for MAS to improve disease resistance in *L. multiflorum*.

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