Theor Appl Genet (2008) 117:203–219
DOI 10.1007/s00122-008-0766-7

ORIGINAL ARTICLE

Discovery and genetic mapping of single nucleotide polymorphisms in candidate genes for pathogen defence response in perennial ryegrass (*Lolium perenne* L.)

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Received: 12 November 2007 / Accepted: 3 April 2008 / Published online: 1 May 2008

Abstract Susceptibility to foliar pathogens commonly causes significant reductions in productivity of the important temperate forage perennial ryegrass. Breeding for durable disease resistance involves not only the deployment of major genes but also the additive effects of minor genes. An approach based on in vitro single nucleotide polymorphism (SNP) discovery in candidate defence response (DR) genes has been used to develop potential diagnostic genetic markers. SNPs were predicted, validated and mapped for representatives of the pathogenesis-related (PR) protein-encoding and reactive oxygen species (ROS)-generating gene classes. The $F_1(NA_6 \times AU_6)$ two-way pseudo-test cross population was used for SNP genetic mapping and detection of quantitative trait loci (QTLs) in response to a crown rust field infection. Novel resistance QTLs were coincident with mapped DR gene SNPs. QTLs on LG3 and LG7 also coincided with both herbage quality QTLs and candidate genes for lignin biosynthesis. Multiple DR gene SNP loci additionally co-located with QTLs for grey leaf spot, bacterial wilt and crown rust resistance from other published studies. Further functional validation of DR gene SNP loci using methods such as fine-mapping and association genetics will improve the efficiency of parental selection based on superior allele content.

Communicated by T. Lübberstedt.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-008-0766-7) contains supplementary material, which is available to authorized users.

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Introduction

Genetic resistance to foliar pathogens is an important determinant of successful meat, milk and wool production based on fodder crops (Vanderplank 1984). Perennial ryegrass (*Lolium perenne* L.) is the most widely cultivated pasture species in temperate grazing zones of the world, due to high digestibility and nutritive content as well as superior re-growth capacity following intensive defoliation by herbivores (Fulkerson et al. 1994; Kimbeng 1999; Potter 1987; Tas et al. 2004). The majority of current perennial ryegrass varieties are cultivated as monocultures, leading to potential increased disease pressure on susceptible host cultivars. Specific pathogens of forage varieties include crown rust (*Puccinia coronata* Corda *f.sp. lolii* Brown), stem rust (*Puccinia graminis* Persoon f.sp. *lolii* Guyot et Massenot) and bacterial wilt (*Xanthomonas campestris* pv. *graminis*). All these diseases cause significant reductions in both green and dry matter yield by

acting as a sink for water soluble carbohydrate in the host-leaf blade (Price 1987). Grey-leaf spot (*Magnaporthe grisea* [T.T.Hebert] Yaegashi et Udagawa) causes significant damage to turf varieties used for both sporting and amenity applications.

Molecular breeding for genetic control of resistance is the most economically and environmentally efficient solution for disease management, reducing requirements for fungicide use. Historically, development of cultivars with major gene-based resistance has been identified as the most important method for disease control (Vanderplank 1984). However, prior experience in the breeding of cereals for rust disease resistance suggests that race-specific resistance is not always durable, due to the rapid evolution of pathogen populations (Anikster and Wahl 1979; Burdon 1987; Kimbeng 1999; Potter et al. 1990; McDonald and Linde 2002). Current breeding strategies for both cereals and forage grasses have involved the use of minor genes in addition to major genes to provide a more robust form of genetic resistance (Kimbeng 1999).

Major gene resistance mechanisms have frequently been attributed to genes encoding nucleotide binding site-leucine rich repeat (NBS-LRR) proteins which mediate recognition of pathogens by the host plant (Collins et al. 2001; Ellis et al. 2000; Morel and Dangl 1997). In contrast, functions encoded by DR genes are expressed following recognition and signal transduction and typically contribute to quantitative variation of resistance (Ryals et al. 1996). DR proteins are thought to be essential for host survival when challenged by virulent pathogen strains in the absence and presence of gene-for-gene-based resistance (Faris et al. 1999). Candidate DR genes have been identified from a broad range of monocotyledonous (Hejgaard et al. 1992; Koiwa et al. 1997; Molina et al. 1999) and dicotyledonous (De Bolle et al. 1993; Peumans and Van Damme 1995) plant species. Gene classes such as thionins (Bloch et al. 1998; Chandrashekar and Satyanarayana 2006), defensins (Hernández et al. 2005), WRKY transcription factors (Christian et al. 2007; Eulgem et al. 1999), peroxidases,and catalases (Smith et al. 1989) and oxalate oxidases (Torres et al. 2002, 2005) contribute to disease resistance in closely related cereal species susceptible to many of the same pathogen genera that infect perennial ryegrass. Specific co-locations between DR gene loci and resistance QTLs have been reported (Giese et al. 1993; Nodari et al. 1993; Leonards-Schippers et al. 1994; Ferreira et al. 1995; Faris et al. 1999; Geffroy et al. 2000). Variation in the amino acid sequence of DR genes such as those encoding oxalate oxidase, peroxidase, catalase and chitinase has been associated with increased latency period of leaf rust infection and disease resistance QTLs in wheat (Anguelova-Merhar et al. 2001; Faris et al. 1999; Messmer et al. 2000) and other cereals.

Progress in the understanding of disease resistance in perennial ryegrass has been limited compared to that of major cereal species due to the obligate outbreeding nature of the reproductive system and consequent complexity of genetic analysis. The major focus to date has been on crown rust resistance, for which major and minor QTLs have been detected in different mapping populations on all LGs (Dumsday et al. 2003; Muylle et al. 2005; Studer et al. 2007; Thorogood et al. 2001, Sim et al. 2007; Schejbel et al. 2007). In addition, QTLs for grey leaf spot and bacterial wilt resistance have been detected on LGs 3, 4, 6 of perennial ryegrass and Italian ryegrass (*Lolium multiflorum* Lam.) populations (Curley et al. 2005; Studer et al. 2006). QTLs for disease resistance are commonly detected within similar chromosomal regions, suggesting that certain genomic locations may confer multiple pathogen resistance. The small number of common markers between various genetic maps of ryegrass species, in combination with a relatively small number of published trait dissection studies, has until now limited the validity of this inference. Nonetheless, the perennial ryegrass p150/112 reference genetic map (Jones et al. 2002a, b) provides the basis for alignment of trait specific genetic maps and assessment of co-location between disease trait QTLs and candidate genes for disease resistance.

Perennial ryegrass genetic maps based on functionally associated polymorphisms were previously constructed using restriction fragment length polymorphisms (RFLP) (Faville et al. 2004) and SNPs (Cogan et al. 2006). SNP discovery has been based on an in vitro approach applicable to any gene or gene class, involving template gene identification, amplicon generation from the parents of a two-way pseudo-testcross mapping population, sequence analysis, SNP identification and validation in the progeny of the mapping family (Cogan et al. 2006; Ponting et al. 2007). Gene-associated SNPs are suitable as diagnostic markers for superior allele content in forage species, subject to functional validation through linkage-based QTL mapping and association genetics studies (Cogan et al. 2006; Dobrowoski and Forster 2006). DR gene-associated SNP loci may hence be evaluated as contributors to phenotypic variation for durable disease resistance against a broad range of pathogens.

The objectives of this study were to develop molecular markers for a diverse collection of candidate genes involved in durable quantitative resistance and to determine functional significance based on co-location with disease resistance QTLs. SNP markers for a number of representative DR gene classes have been assigned to genetic maps and locations have been compared to novel

QTLs for crown rust field infection, as well as published QTLs for resistance to multiple pathogens.

Materials and methods

Plant materials

Perennial ryegrass genomic DNA was extracted from parents and progeny of the $F_1(NA_6 \times AU_6)$ (Faville et al. 2004) and p150/112 (Jones et al. 2002b) mapping families using the CTAB method (Fulton et al. 1995). A genetic mapping panel was constructed using both parental genotypes and 141 F_1 $(NA_6 \times AU_6)$ progeny and the heterozygous parent and 23 F_1 progeny from p150/112, selected for maximised recombination.

Candidate gene identification

Information for a proprietary perennial ryegrass EST collection (c. 44,700 sequences, corresponding to c. 15,000 unigenes: Sawbridge et al. 2003) was incorporated into the customised Bioinformatics and Advanced Scientific Computing (BASC) database system (Love et al. 2005; Erwin et al. 2007). Sequence annotation was performed against template genes from related Poaceae species in GenBank, including components of the rice genome. Candidate DR sequences were selected on the basis of lowest wuBLASTX similarity levels (Altschul et al. 1997), using a threshold value of $E < 10^{-10}$, and gene ontology analysis using Pfam (Bateman et al. 2002). Supporting functional data was obtained through use of candidate DR ESTs as query sequences in BLASTN to identify putative orthologous wheat ESTs within the wEST SQL database (http://wheat.pw.usda.gov/wEST/blast), a proportion of which were mapped to deletion bins (http://wheat.pw.usda.gov/cgi-bin/westsql/map_locus.cgi: Qi et al. 2003).

DNA sequence information for candidate DR genes used in this study is freely available on request from the corresponding author.

In vitro discovery and validation of gene-associated SNPs

Locus amplification primers (LAPs) were designed using DR gene EST templates, PCR was performed, genomic amplicons were cloned and sequenced and DNA sequences were aligned essentially as described by Cogan et al. (2006). Predicted SNPs were initially validated as showing Mendelian segregation using 10 $F_1(NA_6 \times AU_6)$ individuals and then genotyped across the full mapping panel of 141 $F_1(NA_6 \times AU_6)$ progeny through single nucleotide primer extension (SNuPe) (Cogan et al. 2006).

Genetic map construction

Integration of SNP loci into the existing $F_1(NA_6 \times AU_6)$ parental genetic maps was performed as described by Faville et al. (2004) and Cogan et al. (2006).

Phenotypic analysis of crown rust resistance

A spaced plant field trial consisting of $F_1(NA_6 \times AU_6)$ progeny (156 individuals) was established at DPI-Hamilton in October 2005, to evaluate the effects of a natural crown rust infection. The field experiment was planted in a resolvable incomplete block row × column design, with check plots of the NA_6 parent running diagonally across the design to account for spatial variance. Each progeny genotype position within the trial consisted of up to 4 perennial ryegrass plants within the same unit (depending on persistence) and all units were replicated six times. Each individual was scored for both leaf and plant crown rust resistance for all six replicates. Leaf infection scoring was based on a 0–5 (non-linear) visual scale representing percentage spatial coverage from resistant (0% rust infection) to susceptible (70%) phenotypes of the most severely infected leaf, whilst plant infection score was based on the severity of infection throughout the plant canopy for each genotype and was assessed using the same scoring scale.

QTL analysis

Following genetic map construction using MAPMAKER 3.0, a sub-set of marker loci was selected to provide even coverage of the genome with marker intervals of approximately 5 cM, and consensus map distances were subsequently used. Single marker regression (SMR) was initially used to identify significant variation associated with selected genetic markers. Simple interval mapping (SIM: Lander and Botstein 1989) and composite interval mapping (CIM) methods were used to identify and confirm the presence of QTLs. All analyses were performed using the QTL Cartographer 2.5. software (Basten et al. 1994). The maximum log-of-odds (LOD) score of association between the genotype and trait data was calculated for SIM and CIM, and QTL location predictions were accepted for SIM for values greater than a threshold value of 2.5. Permutation analysis (1,000 iterations) was used to establish an experiment-wise significance value at the 0.05 confidence level defined as a minimum LOD threshold for each trait in CIM (Churchill and Doerge 1994; Doerge and Churchill 1996). For each form of interval analysis, the maximum LOD value, additive marker allele effects, proportion of phenotypic variance attributable to the QTL and the extent of drops by 1 and 2 LOD units from the maximum value on the genetic map were tabulated.

Statistical analysis of the crown rust QTL phenotypic data

Plant and leaf infection scores were obtained for each of the six experimental replicates and statistically analysed for mean score and standard deviation per F_1 genotype, and normal distribution and broad sense heritability of each trait through use of REML in Genstat V8. Experimental datapoints were spatially adjusted to account for the check plots within the field trial.

Comparative genetic mapping

Crown rust (Sim et al. 2007) and grey leaf spot (Curley et al. 2005) resistance QTLs were previously assigned to the parental maps of the MFA × MFB pseudo-F_2 (Ψ-F_2) interspecific (perennial ryegrass × Italian ryegrass [*L. multiflorum* Lam.]) genetic mapping population, which contain heterologous RFLP markers in common with the p150/112 reference map (Jones et al. 2002a). QTLs for bacterial wilt resistance (Studer et al. 2006) were assigned to the biparental consensus genetic map of an Italian ryegrass two-way pseudo-test cross population based on parental genotypes from the advanced breeding germplasm (ABG) pool of Agroscope FAL Reckenholz, Zürich, Switzerland, and from cultivar Adret, respectively. The F_1(ABG × Adret) map contains genomic DNA-derived SSR (LPSSR) markers (Jones et al. 2001) in common with the p150/112 reference map. Comparison of marker locus order between the p150/112 and $F_1(NA_6 \times AU_6)$ genetic maps was also performed through the presence of common LPSSR loci (Jones et al. 2002b). The location of the *Lp*Pc1 crown rust resistance locus identified from analysis of the perennial ryegrass $F_1(Vedette_6 \times Victorian_9)$ two-way pseudo-test cross population (Dumsday et al. 2003) was directly extrapolated onto the p150/112 genetic map through common LPSSR loci.

Coincidence between disease resistance QTLs and herbage quality QTLs was also based on alignment with the p150/112 genetic map, following trait-dissection within this population as described by Cogan et al. (2005).

Results

DR candidate gene identification

A total of 13 candidate EST sequences were selected (Table 1), including representatives from the PR protein-encoding (*Lp*PERa, *Lp*THc, *Lp*THb, *Lp*CHIjb, *Lp*GLUCk, *Lp*TLa, *Lp*DEFa, *Lp*ERa and *Lp*WRKY14) and cellular ROS-generating (*Lp*NOX, *Lp*GLR, *Lp*CAT and *Lp*OXO) gene classes. For example, *Lp*GLR, *Lp*CAT, *Lp*NOX and *Lp*OXO gene selection was based on sequence annotation informed by physiological knowledge of resistance mechanisms during early stages of infection of Poaceae species by both biotrophic and necrotrophic fungal pathogens. Perennial ryegrass ESTs putatively orthologous to functionally defined wheat genes were identified by comparative sequence analysis. The *Lp*CAT, *Lp*OXO and *Lp*PERa genes showed highest similarity to mapped wheat ESTs located within the same deletion bins as gene sequences previously shown to co-locate with disease resistance loci or QTLs (ESM 1) (Huang et al. 2003; Kuraparthy et al. 2007; Ling et al. 2003). Interspecific sequence diversity in coding sequences was low, with an average of 89% sequence identity between putative CAT, PER and OXO orthologues (ESM 2). Comparative sequence analysis also revealed conservation of intron and exon number and boundary sequences for all intron-containing DR genes (data not shown).

Perennial ryegrass DR gene SNP discovery

DR genes were subjected to in vitro SNP discovery through comparison of genomic amplicons from the $F_1(NA_6 \times AU_6)$ mapping population parents, and subsequent validation using selected F_1 progeny (Table 1). Genomic DNA of 10,637 bp cumulative length was resequenced from the template genes, 7 of which contained between 1 and 5 introns. A total of 219 SNPs were detected, at an overall frequency of 1 per 49 bp. Average SNP frequency within introns was 1 per 38 bp, and the equivalent value for exons was 1 per 57 bp (Table 1). Eight (62%) DR genes (*Lp*GLUCk, *Lp*DEFa, *Lp*ERa, *Lp*OXO, *Lp*CAT, *Lp*CHIjb, *Lp*WRKY14 and *Lp*TLa) contained fewer than 10 SNPs in total, while 2 (17%) contained 50 or more SNPs (Table 1). Contigs obtained from two distinct genes of the same functional class (*Lp*THb and *Lp*THc) differed considerably in both structure and polymorphism content (data not shown).

A sub-set of 43 predicted SNPs were used to design SNuPe assays to interrogate nucleotide variants from each of the 13 variable genes, of which 28 were identified within the NA_6 parental genotype, 12 within the AU_6 parental genotype and 3 were insertion–deletion (indel) polymorphisms identified within the NA_6 parent. A total of 27 (63%) of the SNP loci were validated as segregating in a subset of 10 F_1 progeny, with representation from each gene. Details of SNP validation, including SNuPe assay primer information for SNPs progressed to genetic map assignment, are shown in Table 2.

Genetic mapping of DR gene SNPs

Fifteen validated SNP loci from 12 DR genes were integrated into the $F_1(NA_6 \times AU_6)$ framework parental

Table 1 Summary information for selected candidate putative defense response genes targeted for SNP discovery

Gene name	Functional classification based on sequence annotation (wuBLASTX)	Source species for annotation	E value	Intron number	SNP discovery			Genetic mapping	
					Predicted SNP number	SNP incidence (per bp)	Number of mapped SNPs (F_1[NA_6 x AU_6])	Mapped SNP locus	Map position (LG/cM position)
*Lp*PERa	Peroxidase class III	*T. aestivum*	3.5×10^{-112}	0	21	61	1	xlppera-1041ag	LG2 (16.3)
*Lp*THb	Putative defensin	*H. vulgare*	2×10^{-24}	0	50	12	1	xlpthb-317ag	LG1 (110.8)
*Lp*THc	Gamma-thionin	*H. vulgare*	2×10^{-26}	1	18	20.3	1	xlpthc-148at	LG7 (25.6)
*Lp*GLUCk	1,4 β-glucanase	*N. lolii*	5×10^{-90}	1	6	124	1	xlpgluck-394ct	LG5 (95.2)
*Lp*DEFa	Putative defensin	*T. aestivum*	2×10^{-26}	1	9	77	1	xlpdefa-233ct	LG6 (116.5)
*Lp*ERa	Putative elicitor response	*O. sativa*	8.4×10^{-44}	3	9	190	1	xlpera-376ct	LG2 (139)
*Lp*OXO	Oxalate oxidase	*L. perenne*	2×10^{-68}	0	2	292	1	xlpoxo-123ag	LG4 (90.2)
								xlpoxo-171ag	LG4 (90.2)
*Lp*GLR	Cytosolic glutathione reductase	*T. aestivum*	5.8×10^{-58}	5	32	26	1	xlpglr-1435ct	LG6 (171.5)
*Lp*CAT	Catalase	*T. aestivum*	6.4×10^{-100}	0	6	49	1	xlpcat-561cg	LG4 (182.1)
								xlpcat-353gt	LG6 (86.5)
*Lp*CHIjb	Chitinase	*S. bicolor*	6.3×10^{-14}	0	3	52	1	xlpchijb-240cg	LG5 (80.1)
*Lp*TLa	Thaumatin-like protein	*H. vulgare*	3×10^{-59}	0	3	102	0	–	
*Lp*NOX	NADPH oxidase	*O. sativa*	5×10^{-68}	4	55	226	1	xlpnox-735ag	LG1 (145.7)
								xlpnox-1131at	LG1 (145.7)
*Lp*WRKY14	WRKY DNA binding transcription factor 14	*O. sativa*	1×10^{-26}	0	5	63	1	xlpwrky-166ag	LG3 (89.3)

Bioinformatic annotations including source species, *E* value and functional classification represent the latest wuBLASTX matches to GenBank accessions. Sequence and mapping information is shown for each gene, documenting intron number, SNP number and incidence, mapped SNP locus nomenclature (after Cogan et al. 2006) and genetic map position

Table 2 Summary information for DR gene SNP loci showing segregation in the $F_1(NA_6 \times AU_6)$ genetic mapping population

Gene name	SNuPe primer designation	Forward LAP sequence (5′–3′)	Reverse LAP sequence (5′–3′)	SNuPe interrogation primer sequence (5′–3′)	Validation in subset of 10 ($F_1[NA_6 \times AU_6]$) F_1 s	Expected SNP variant	Base scored	Validated in full ($F_1[NA_6 \times AU_6]$) sibship
*Lp*PERa	xlppera-1041ag	CGGCGACACCAACATCAACG	GGCCTTATTCATTCACGTGA	TGTTGAAGAGCACCTGGTCGGA	NA_6	A/G	G	Yes
	xlppera-1105cg	CGGCGACACCAACATCAACG	GGCCTTATTCATTCACGTGA	GAACTTCGCGTCCAGCG	NA_6	C/G	C	Yes
	xlppera-978cg	CGGCGACACCAACATCAACG	GGCCTTATTCATTCACGTGA	TTGTCGAAGGCGTTGGCGGT	NA_6	C/G	G	No
*Lp*THb	xlpthb-317ag	GCAGAGCCACAACTTCAAG	CCGAGAAACACACAACTACG	GCTAGCCCATCAGCAGTTC	NA_6	A/G	G	Yes
	xlpthb-27ct	AGCAAGCGAAAGCAGTGAG	AACGGGAGCAGAAGCGGAG	CAAGCGAAAGCAGTGAGCT	Both	C/T	Both	Yes
	xlpthb-145ct	AGCAAGCGAAAGCAGTGAG	AACGGGAGCAGAAGCGGAG	TCGYTGGTTCTTGGTGCTC	AU_6	C/T	T	No
	xlpthb-309ct	GCAGAGCCACAACTTCAAG	CCGAGAAACACACAACTACG	GCTTCCARCGCAAGTGCTT	Both	C/T	Both	No
*Lp*THc	xlpthc-148at	GGAGTTCAAGCCCAAGGCAA	ACTAGGGTACATAGAGGTGAG	ATCGAGAGAGTAGCACCATCCCA	AU_6	A/T	A	Yes
	xlpthc-284at	CGTGCGTCTCGGACAAGATCT	ACTAGGGTACATAGAGGTGAG	TGCCAGAAGAAGTGCTAGACACA	AU_6	A/T	A	Yes
*Lp*GLUCk	xlpgluck-394ct	GGCGTTAATTTCGGCGGCTG	TGCGAATCGTGTTCAGGCCA	CATCGGGTACTGGTCCTA	AU_6	C/T	T	Yes
*Lp*DEFa	xlpdefa-233ct	GCCAGTAGTAGCAGCAAGCAG	CCGGCTAGCAGACCCTCTTG	ACCATCAGTTCATCACCCATTTACAG	NA_6	TT/CC (Indel)	TT	Yes
*Lp*ERa	xlpera-376ct	TCACACCGGGAGGACCAAGA	GAACTTGAGACCAATTCTGAT	GGGAGAATTGCGGTGCTGTTTATGT	NA_6	C/T	C	Yes
	xlpera-300cg	TCACACCGGGAGGACCAAGA	GAACTTGAGACCAATTCTGAT	CGAATTCTGCTATGATCTCTCT	NA_6	C/G	C	No
	xlpera-407	TCACACCGGGAGGACCAAGA	GAACTTGAGACCAATTCTGAT	AACGATTTGTCAGCGAGACTCC	AU_6	G/T/A	GT	No
	xlpera-1242	AACCTTTGTGTTCACGACCT	GAACTTGAGACCAATTCTGAT	GCTCACTTCCTTTGCCTGCACTCACCAAA	AU_6	G/C	C	Yes
*Lp*OXO	xlpoxo-123ag	ACTTGTGCGTGTCCATGAA	ACAATAAAAGTACAAGCCTCG	TGCCGATGATTCCGACGAG	Both	A/G	Both	Yes
	xlpoxo-171cg	ACTTGTGCGTGTCCATGAA	ACAATAAAAGTACAAGCCTCG	TCTGGGAACAAGCTATACTC	Both	C/G	Both	Yes
*Lp*GLR	xlpglr-1435ct	ACCTTACACCTGTTGCTCT	GCTGTTCTTCATTGGATTGA	ATTGCACTATCATTCCCCT	NA_6	C/T	T	Yes
	xlpglr-132gt	GATGAGATGAGAACAATTGTC	AGAGCAACAGGTGTAAGGT	GGTCAAAACAAACTGCAGGC	AU_6	G/T	G	Yes
*Lp*CAT	xlpcat-561cg	GACCCACGACATCTCCCACCTGA	CCGGGTGGTGCGAGAAGAAG	CGGGTGGTGCGAGAAGAAGTC	NA_6	C/G	G	Yes
*Lp*CAT	xlpcat-353gt	GACCCACGACATCTCCCACCTGA	CCGGGTGGTGCGAGAAGAAG	CCGTCATCGTCCGCTTCT	AU_6	G/T	G	Yes
*Lp*CHIjb	xlpchijb-240cg	CTGCAGCCAGTTCGGCTTCTG	TGGATTTGGACTTGATTGCGTTGAAG	CCACACCTCCGCTACCA	NA_6	C/G	C	Yes
*Lp*NOX	xlpnox-735ag	TGACTGGACACGAGAGCT	CAATATGCTGATGAAAGGAG	GCAGACGAGACAACCAAAAAA	NA_6	A/G	A	Yes
	xlpnox-1057gta	CTCCTTTCATCAGCATATTG	CTAGGTCAGCAATCTCAT	TTAAAGCCCTCGATTGCA	NA_6	G/T/A	GT	No
	xlpnox-1131at	CTCCTTTCATCAGCATATTG	CTAGGTCAGCAATCTCAT	GTTCTTATTGTAGTTTGGGG	NA_6	A/T	T	Yes
*Lp*WRKY14	xlpwrky-166ag	CGACGATATGCTGATGTACG	CGAGCAGATAAACTCATGGGG	CACTGCCATGACATGAGCAT	AU_6	A/G	A	Yes
*Lp*TLa	xlpxrky-132ct	CGACGATATGCTGATGTACG	CGAGCAGATAAACTCATGGGG	GACATGTGGGACATGACATG	AU_6	C/T	T	Yes
	xlptla-471ct	ATCGGCATGGGCTTCTCTTG	GATTGTCCCACTCATGGTTGTA	GAGGCTGCCCAGACAGGG	NA_6	C/T	T	Yes

Note: All LAP pairs and SNuPe interrogation primers were designed to operate at common annealing temperatures of 55 and 50°C, respectively

genetic maps (Table 2) and 5 (33%) of these SNPs were also polymorphic in the p150/112 population (data not shown). Only single representatives of co-locating SNP loci are represented on the maps. The NA_6 parental genetic map contains 9 loci on 5 linkage groups (LGs), while the AU_6 parental genetic map contains 5 loci on 5 LGs. Two SNP loci each from the *Lp*NOX and *Lp*OXO genes were fully coincident on the same LG (Table 1). The *Lp*OXO GA SNP at nucleotide coordinate 123 is heterozygous in both parents and provides a bridging locus between the two maps on LG4 (Fig. 1, Table 1). Two distinct *Lp*CAT SNPs are located on different LGs in each map and presumably correspond to allelic variants of differing paralogous sequences. The DR gene loci failed to cluster, with no obvious correlation between functional class and chromosomal location. SNPs from 4 genes (*Lp*ERa, *Lp*PERa, *Lp*DEFa and *Lp*GLUCk) co-located with a corresponding RFLP marker derived from the same EST sequence, but the *Lp*THc SNP locus on LG7 was distantly located from the cognate RFLP locus. Individual SNPs from the *Lp*NOX,

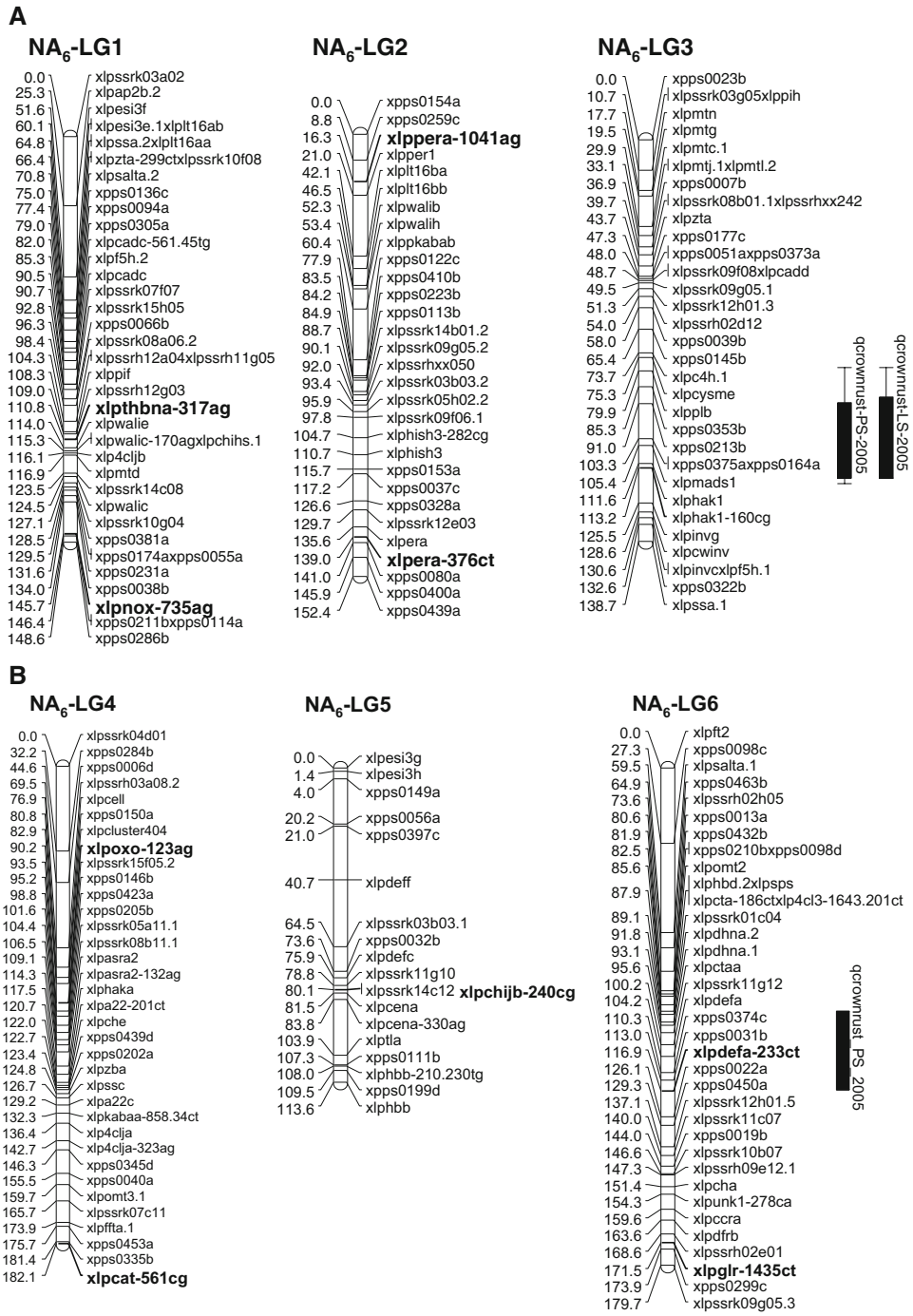
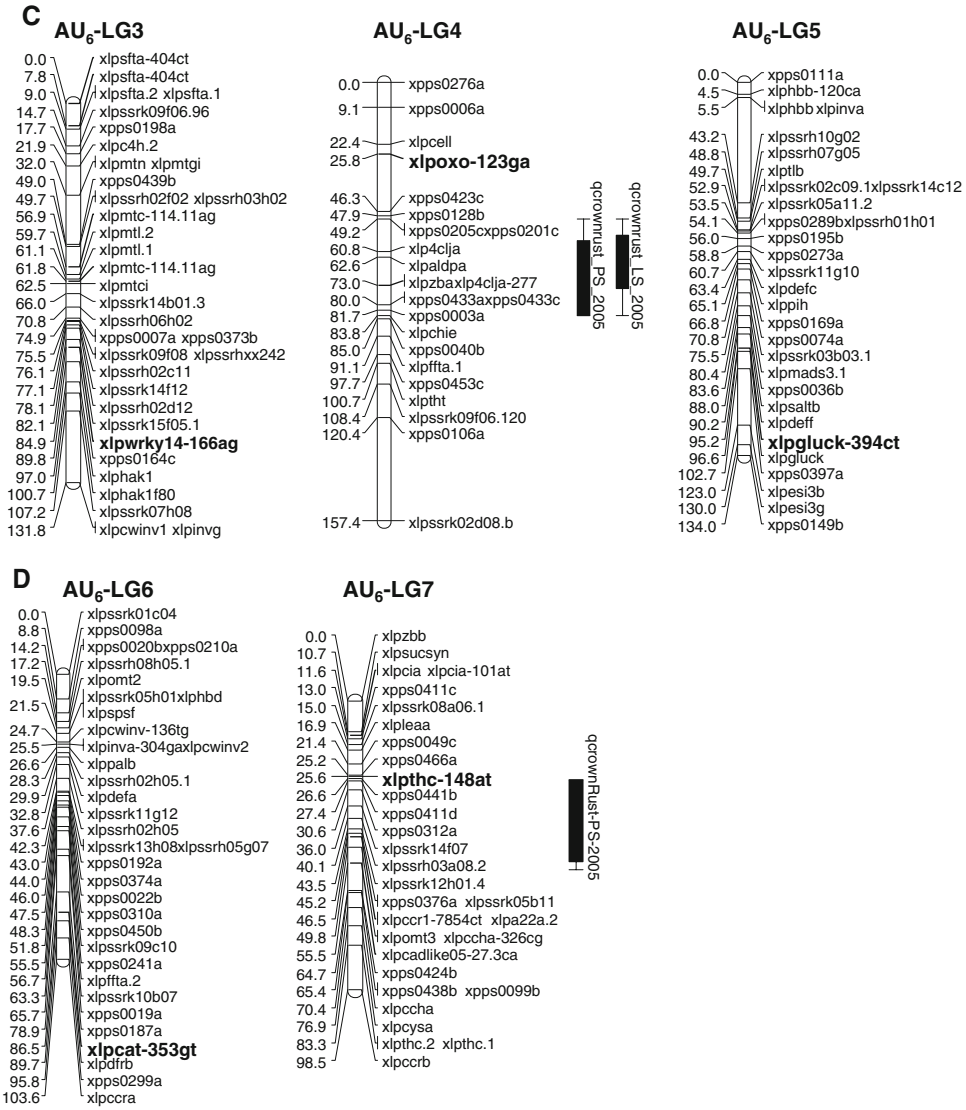


Fig. 1 Location of mapped DR gene SNP loci (*bold*) and QTLs for crown rust resistance measured as both leaf and plant infection score on the NA_6 (**a**, **b**) and AU_6 (**c**, **d**) parental maps of the $F_1(NA_6xAU_6)$ mapping population. The nomenclature for DNA-derived and cDNA-derived simple sequence repeat (*SSR*) markers, single nucleotide polymorphic (*SNPs*) markers and restriction fragment length polymorphisms (*RFLP*) is as described in Faville et al. (2004) and Cogan et al. (2006). QTL nomenclature has been adapted from Cogan et al. (2005) in the form of qtrait-season-year, e.g. q_crownrust-LS-2005 (LS and PS are defined as leaf and plant infection score traits, respectively). All QTLs depicted were detected using CIM. Bars and lines represent 1 and 2 drops from the maximum LOD value

Fig. 1 continued



*Lp*THc, *Lp*CAT, *Lp*GLR and *Lp*GLUCk genes were assigned to the p150/112 genetic map at locations corresponding to the equivalent loci on the $F_1(NA_6 \times AU_6)$ genetic maps, except for the *Lp*THc SNP which mapped closer to the centromeric region of LG7 (data not shown).

QTL analysis of crown rust resistance in the $F_1(NA_6 \times AU_6)$ mapping population

The phenotypic data for both traits (leaf and plant infection score) were analysed to determine population mean, standard deviation, frequency distribution and broad sense heritabilities. Each of the measured traits was normally distributed and both leaf score and plant score were skewed toward the resistant range based on mean phenotype (coefficients of skewness $= -0.05$ and -0.0137, respectively). Significant variation ($P < 0.001$) was detected between all genotypes of the $F_1(NA_6 \times AU_6)$ mapping population and broad sense heritability was very similar for both traits (Table 3, Fig. 2). A total of 2 and 4 QTLs were detected for leaf and plant infection score, respectively, both exceeding the genome-wide LOD threshold score of 2.5 using CIM following 1,000 permutations. In each instance, the leaf score QTLs coincided with plant score QTLs (Table 4, Fig. 1).

SMR analysis was performed between phenotypic scores for both traits and marker genotypic data, and in all instances significantly associated ($P < 0.001$) marker loci were exclusively located in chromosomal regions containing QTLs. A total of three QTLs detected as significant (LOD score > 2.5) using SIM were not significant using CIM. These include two leaf score QTLs on NA_6 LG6 and one on AU_6 LG7, and are of potential interest in future candidate gene marker co-location studies (Table 4).

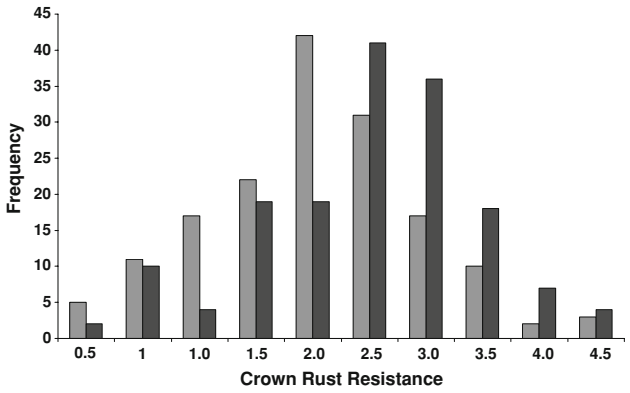


Fig. 2 Composite frequency histograms for trait distribution data: **a** plant score (*light grey*) **b** leaf score (*dark grey*) for the 2005 Hamilton spaced field trial

Association of crown rust resistance QTLs with candidate DR genes

QTLs which mapped near the bottom of NA_6 LG3 were significantly ($P < 0.001$) associated by SMR with the EST-SSR locus xpps0375a (located at 103.3 cM) (Fig. 1a). Alignment of the parental maps indicates that the xlpwrky14-166ag SNP locus on AU_6 LG3 is located 5 cM distal to the EST-SSR locus xpps0164 (Fig. 4a), which co-locates with xpps0375a on the NA_6 parental map. The QTL near to the centromeric region of NA_6 LG6 was significantly associated ($P < 0.01$) with the SNP locus xlpdefa-233ct. In addition, the QTL detected in the centromeric region of AU_6 LG7 was significantly associated ($P < 0.01$) with the SNP locus xlpthc-148at, although the marker is located approximately 1 LOD unit drop from the maximum score location.

Association of DR gene SNP loci with multiple pathogen resistance QTLs using comparative mapping

Comparative mapping analysis between disease resistance QTLs from published studies and the DR gene SNP loci identified in this study was performed using bridging markers between each genetic map. A total of 5 SNP loci corresponding to *Lp*ERa, *Lp*PERa, *Lp*DEFa, *Lp*CAT and *Lp*OXO co-located with QTLs for crown rust, grey leaf spot and bacterial wilt resistance. The SNP loci xlpera-376ct and xlppera-1041ag were assigned to the top and bottom of LG2 on the NA_6 parental map, respectively (Fig. 1a). The elicitor response gene locus was found be located within the same chromosomal region as a QTL for grey leaf spot resistance based on common bridging markers with the p150/112 map and the Ψ-F_2(MFA × MFB) parental genetic maps (Fig. 3a). The peroxidase gene locus is located in the upper region of LG2, in which crown rust QTLs are situated based on alignment with Ψ-F_2(MFA × MFB) maps through the xcdo405 RFLP locus, but lack of further bridging markers prevents more detailed analysis (Fig. 3a). The *LpPc*1 major crown rust resistance QTL is also located in this genomic region. Assignment of *Lp*CAT and *Lp*DEFa SNPs to the p150/112 map and comparison with the Ψ-F_2(MFA × MFB) maps revealed that these SNPs were located within the same chromosomal region as a grey leaf spot resistance QTL and a crown rust resistance QTL, respectively (Fig. 3b). A SNP within the oxalate oxidase gene is located in close proximity to several QTLs for bacterial wilt resistance in the central region of LG4 on the F_1(ABG × Adret) map, based on the bridging genomic DNA-derived SSR locus xlpssrk05a11.1 (Fig. 3c).

Coincidence of crown rust resistance and herbage quality QTLs

QTLs for crown rust resistance detected in this study co-located with QTLs for herbage quality within the p150/112 population, as well as molecular genetic marker loci derived from candidate genes involved in lignin biosynthesis. The LG3 NA_6 QTL for crown rust resistance was located in the same chromosomal region as QTLs for in vivo dry matter digestibility, neutral detergent fibre, estimated metabolisable energy and crude protein based on bridging markers between the NA_6, AU_6 and p150/112 genetic maps (Fig. 4a). Similar results were observed for the AU_6 LG7 resistance QTL which co-located with multiple QTLs for similar herbage quality traits (Fig. 4b). The AU_6 LG7 QTL also coincides with a cluster of SNP and RFLP markers derived from candidate genes involved in lignin biosynthesis (xlpccr1-7854ct, xlpomt3 and xlpcad-like05-27.3ca). SMR analysis identified a significant association ($P < 0.001$) between markers xlpssrk14f07, xpps0376a and xlpcch-326cg and the AU_6 LG7 QTL. Alignment with the p150/112 map reveals further evidence for loci encoding the lignin biosynthesis genes cinnamoyl alcohol dehydrogenase (*Lp*CAD2), caffeic acid *o*-methyl-transferase (*Lp*OMT1) and cinnamoyl CoA reductase (*Lp*CCR1) within this region, based on proximity to the xlpssrk1407 locus (Fig. 4b).

Discussion

Candidate DR gene SNP discovery and genetic mapping

DR gene loci provide plausible candidates for quantitative resistance mechanisms due to cumulative and non-specific modes of action (Faris et al. 1999; Kruger et al. 2003;

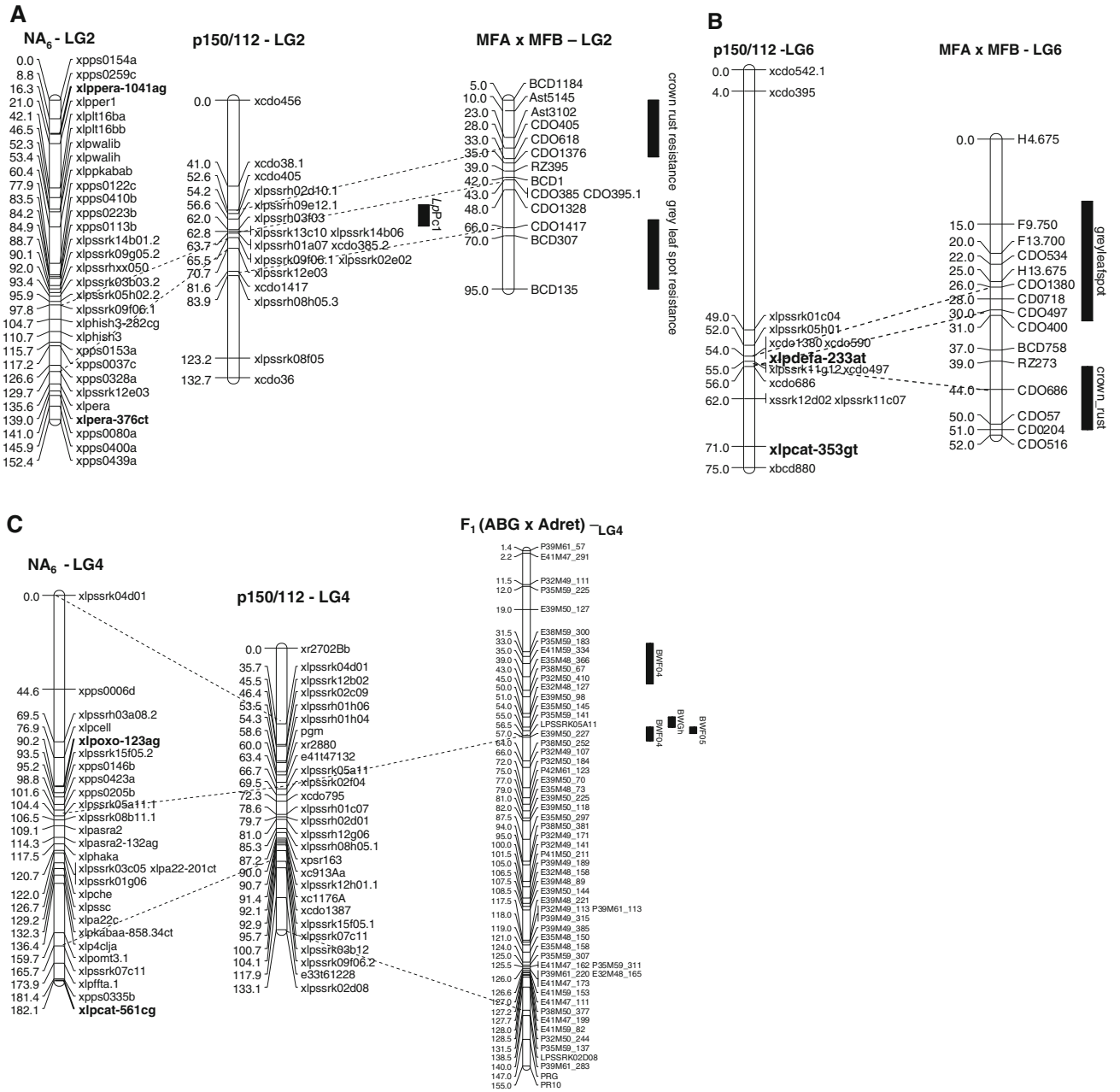


Fig. 3 Comparative mapping analysis between DR gene SNP loci and QTLs for disease resistance from other published studies. Marker nomenclature for the NA_6, AU_6 and p150/112 is as described in Faville et al. (2004) and Cogan et al. (2006). The location of the *LpPc*1 crown rust resistance locus is as described by Dumsday et al. (2003). Genetic marker and QTL nomenclature for the MFA × MFB Ψ-F_2 population maps and the F_1(ABG × Adret) map are as described by Sim et al. (2007) and Studer et al. (2006), respectively. BW denotes QTLs for bacterial wilt resistance. AFLP markers were excluded from the F_1(ABG × Adret) map for the purpose of comparative analysis

Molina et al. 1999). Bioinformatic discovery of candidate perennial ryegrass DR gene ESTs has provided an abundant source of SNP markers for use in associating allelic variation with disease resistance QTLs. Multiple SNPs were obtained for all representative gene classes, confirming the suitability of this marker class for genetic analysis of outbreeding species. SNP incidence for the DR genes in this study was very similar to that observed (1 per 54 bp) in a larger sample of candidate genes involved in abiotic stress tolerance, herbage quality and other traits (Cogan et al. 2006). Although there was marked variation in SNP frequencies between different DR gene classes, further analysis across diverse germplasm samples is required to determine whether some DR genes show

Table 3 Summary of statistical parameters for field measurement of crown rust resistance (assessed as plant and leaf infection score)

	Phenotypic trait	
	Plant score	Leaf score
Statistical parameter		
Mean	2.398	2.799
Minimum value	0	0
Maximum value	5	5
Standard deviation	1.293	1.309
Error		
Within plot	0.051	0.076
Between plot	1.025	1.072
Probability–genotype	<0.001***	<0.001***
Genotypic variance	0.605	0.581
Broad sense heritability (H)	0.361	0.351

P values were deemed significant at the 5% confidence interval ($P < 0.05$*, $P < 0.01$**, $P < 0.001$***)

anomalously high levels of conservation. Variability in SNP polymorphism between DR genes may also be attributable to heterogeneity in the nucleotide diversity of both coding and non-coding DNA (Andersen and Lubberstedt 2003; Cho et al. 2000; Gupta and Rustgi 2004; Zhu et al. 2003). SNP polymorphism was significantly higher within introns: for instance, only 20% of nucleotide variation in the *Lp*NOX and *Lp*GLR genes was located in exonic regions. Higher exonic SNP frequencies observed for the *Lp*THb, *Lp*THc and *Lp*ERa genes were largely attributable to complex haplotypic structures arising from putative paralogous sequence amplification.

The level of attrition from SNP discovery to genetic map assignment in this study was less than previously reported (Cogan et al. 2006), probably due to low levels of haplotypic complexity and effective gene-specific SNuPe primer design. Multiple SNPs for both the *Lp*NOX and *Lp*OXO genes were assigned to identical map locations, confirming origin from the genetic locus. A total of 5 eligible SNP loci co-located within a 5–10 cM interval with previously mapped RFLP markers developed from the same EST clone. Variation between the precise chromosomal locations of the two marker types is probably due to the lower fidelity of data scoring inherent to RFLPs as compared to SNPs, leading to missing datapoints within a relatively small population size. However, it is also possible that some non-coincident RFLP and SNP loci may arise from paralogous sequences adjacent on the same linkage group. As an example, the xlpthc-148at SNP locus is located c. 66 cM from the xlpthc RFLP locus, probably due to independent mapping of paralogous members of the thionin multigene family. Previous isolation and characterisation of cDNAs corresponding to leaf-specific thionins

Table 4 Summary of QTL analysis for crown rust resistance traits (leaf and plant infection score) in the $F_1(NA_6 \times AU_6)$ genetic mapping population

Phenotypic trait	QTL Identifier	LG	SMR $P < 0.01$ (cM)	Parental genetic map	Most closely linked marker	SIM				CIM			
						Maximum LOD score	Position (cM)	a[a]	R[b]	Maximum LOD score	Position (cM)	a[a]	R[b]
Leaf score	qcrown rust_LS_2005	3	101.8–132	NA_6	xlphak1-180cg	2.89	106.9	−0.570	0.093	2.65	106.9	−0.503	0.072
	qcrown rust_LS_2005	6	59.5–100.2	NA_6	xlpssrh02h05	2.76	73.6	−0.530	0.079	2.12	73.6	−0.423	0.047
	qcrown rust_LS_2005	4	59.7–82.4	AU_6	xlpffta.1	2.92	68.31	−0.561	0.098	2.64	66.3	−0.486	0.066
	qcrown rust_LS_2005	7	10.8–15.2	AU_6	xlpssrh03a08.2	2.80	4.4	−0.530	0.079	2.00	4.4	−0.427	0.048
Plant score	qcrown rust_PS_2005	3	101.8–132	NA_6	xpps0375a, xlphak1-180ag	3.23	102.9	−0.570	0.094	3.07	102.9	−0.508	0.075
	qcrown rust_PS_2005	6	59.5–116.4	NA_6	xlpffta.1	2.82	95.8	−0.530	0.081	2.45	93.8	−0.456	0.059
	qcrown rust_PS_2005	4	0–37, 59.7–84.4	AU_6	xlpssrh03a08.2	3.27	66.3	−0.569	0.095	2.88	68.3	−0.501	0.070
	qcrown rust_PS_2005	7	10.8–15.2	AU_6	xlpssrk07b07	2.76	42.5	−0.525	0.080	0.43	40.5	0.433	0.064
	qcrown rust_PS_2005	7	10.6–59.8	AU_6	xpps0376	2.84	42.5	−0.524	0.081	2.59	40.5	−0.472	0.064

[a] Additive effect of substituting alternative alleles at marker locus

[b] Proportion of variance explained by QTL

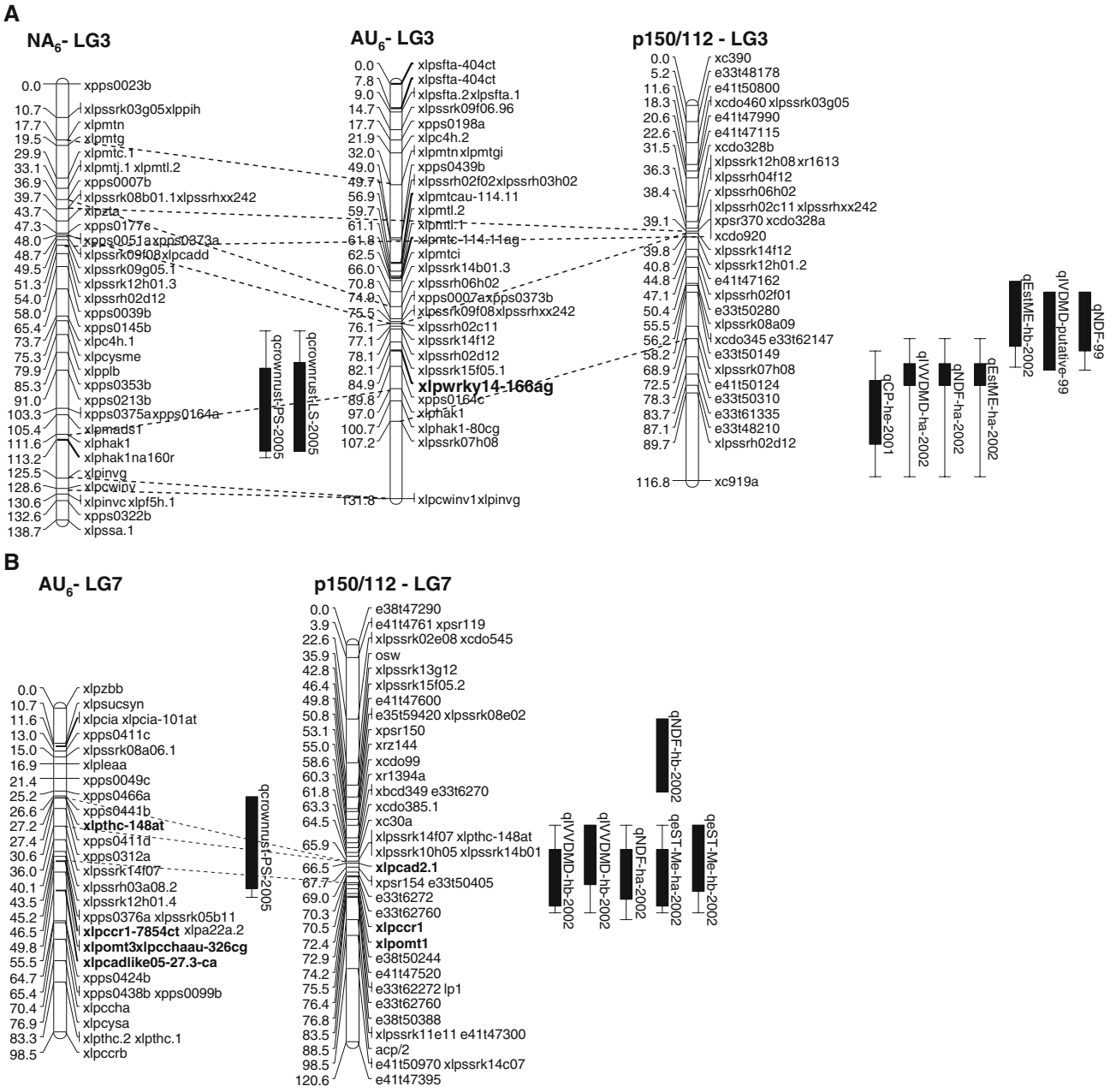


Fig. 4 Coincidence of crown rust resistance QTLs with herbage quality trait QTLs digestibility as described by Cogan et al. (2005). Comparative mapping analysis between the p150/112 reference genetic map and LG7 of the $F_1(NA_6 \times AU_6)$ AU_6 parental map (**a**) and LG3 of the $F_1(NA_6 \times AU_6)$ NA_6 parental map (**b**) was based on common RFLP markers and genomic-derived SSR markers (Jones et al. 2002b)

in barley suggests that the presence of multiple genomic copies (Bohlmann and Apel 1987). Gene paralogy was also observed during map assignment of catalase gene-derived SNPs. Assays were designed for polymorphisms at different base coordinates in each parental genotype from the same template contig, generating distinct chromosomal positions on the parental maps of the $F_1(NA_6 \times AU_6)$ population. Genetic mapping of wheat DR genes has frequently identified multiple copies of specific DR gene classes (catalases, oxalate oxidases) mapping to distinct chromosomal positions (Faris et al. 1999; Li et al. 1999), suggesting that two different isoforms of the catalase gene have been mapped within this study.

Trait dissection for crown rust resistance

Three previously unidentified chromosomal regions containing crown rust resistance QTLs have been identified on

LGs 3, 4 and 6. Six of the 9 detected QTLs were identified using all three analytical methods (CIM, SIM and SMR). Trait dissection studies of crown rust resistance within ryegrass populations have to date identified genetic control by both major and minor QTLs on all 7 LGs using both field infection and artificial inoculation trials (Dumsday et al. 2003; Muylle et al. 2005; Studer et al. 2007; Thorogood et al. 2001; Sim et al. 2007; Schejbel et al. 2007). In several studies (Dumsday et al. 2003; Muylle et al. 2005), QTLs of large magnitude were identified, accompanied by bimodal trait frequency distributions. Such QTLs may correspond to major gene effects, due to the action of receptor-like resistance gene classes such as the NBS–LRR gene family. Only QTLs of low magnitude were observed in this study, possibly due to a lack of race-specific resistance genes matching avirulence loci within Hamilton pathogen isolates. Certain chromosomal regions (upper LG2, lower–central LG1 and central LG7) have been detected under different environmental conditions through analysis of more than one mapping population (Muylle et al. 2005; Sim et al. 2007; Schejbel et al. 2007), suggesting that not only major but also minor gene effects may be responsible for low magnitude (<20% V_p) QTLs. However, the low number of common markers between genetic maps renders such comparisons difficult.

Correlation of genomic regions controlling quantitative disease resistance and DR gene SNP loci

SNP loci corresponding to multiple DR genes were located within the same chromosomal regions as previously published QTLs for resistance to multiple pathogens (crown rust, grey leaf spot and bacterial wilt) (Curley et al. 2005; Studer et al. 2006; Sim et al. 2007; Schejbel et al. 2007). Additionally, mapped SNP loci derived from representatives of the WRKY and thionin gene families were identified in similar genomic locations as newly described crown rust resistance QTLs. Previous studies located a total of 68% of all WRKY cDNA sequences within the disease resistance QTL-containing component of the rice genome, suggesting that such genes are important for the regulation of Poaceae defence reponses (Wisser et al. 2005). Crown rust resistance QTLs have been mapped to the pericentromeric region of LG7 in the current study, and also through analysis of the Ψ-F_2(MFA × MFB) population (Sim et al. 2007). The SNP locus xlpthc-148at, which is adjacent to this QTL, may be correlated with this source of resistance due to the proposed gene function of thionins in related plants (Chandrashekar and Satyanarayana 2006), The *Lp*DEFa SNP locus coincided with an F_1(NA_6 × AU_6) crown rust resistance QTL, as well as the grey leaf spot resistance QTL described previously, although caution must be exercised in extrapolation of location through the p150/112 reference map, due the use of bin mapping-based maximally recombinant individuals.

Peroxidase expression has been positively correlated with increased resistance to wheat stem rust (*P. graminis* f.sp.*tritici*) (Seevers et al. 1971). The peroxidase (class III) enzymatic activity has been shown to be important in the responses to rice blast pathogen (Sasaki et al. 2004), powdery mildew in barley (Kruger et al. 2003) and leaf rust in wheat (Faris et al. 1999). The *Lp*PERa gene locus is more likely to coincide with the crown rust resistance QTL on the top of the Ψ-F_2(MFA × MFB) LG2 genetic map than the *LpPc*1 crown rust resistance locus, based on the position of a limited number of bridging RFLP and SSR loci (Sim et al. 2007). A crown rust QTL designated *LpPc*3 was also identified in the upper region of LG2 in the map derived from the F_1(SB2 × TC1) population (Muylle et al. 2005), but detailed comparison is once again complicated by limited numbers of common markers. In wheat, a peroxidase (*Ta*PER) gene RFLP marker was found to co-locate with a QTL for leaf rust resistance accounting for 33% of phenotypic variance (Faris et al. 1999). Based on current knowledge of Poaceae comparative relationships (Jones et al. 2002a) *Ta*PER is located in a region of conserved synteny with the mapped *Lp*PERa SNP locus on the NA_6 parental genetic map. Furthermore, bioinformatic and sequence analysis has shown that perennial ryegrass and wheat peroxidase genes are highly similar at the nucleotide level and hence putatively orthologous.

Comparative analysis also indicates that the *Lp*CAT SNP locus maps to a region in perennial ryegrass which is in conserved synteny to the *Lr3* locus on 6BL which confers resistance to wheat leaf rust (*P. triticana* f.sp. *tritici*) (Sacco et al. 1996). DR gene mapping in hexaploid wheat additionally detected coincidence of a *Ta*OXO RFLP locus with a QTL for wheat leaf rust on chromosome 4A accounting for 10% of phenotypic variance (Faris et al. 1999), which is also in a region of conserved synteny with the *Lp*OXO SNP locus on LG4. Translational genomics hence provides further support for functionality of perennial ryegrass DR genes.

Trait association between herbage digestibility and crown rust resistance

Coincident QTLs were detected for both herbage quality and crown rust resistance on LG3 and LG7, suggesting that genes involved in cell wall development such as lignin biosynthesis, may be associated with disease resistance mechanisms. Although herbage quality QTLs coincided with a cluster of lignin biosynthesis genes on LG7 (Cogan et al. 2005) no candidate genes for lignification have so far been mapped to LG3. Comparative genomic analysis between wheat and perennial ryegrass identified putative

orthologues of *Lp*CCR1, *Lp*OMT1 and *Lp*CAD2 which physically map to the long arms of all wheat homeologous chromosomes 3 (Cogan et al. 2005). Previous studies of conserved synteny within the Poaceae (Jones et al. 2002a; Sim et al. 2005; Devos 2005; Gale and Devos 1998) suggest that similar genes are likely to be located in this genomic region of perennial ryegrass. Trait dissection analysis of the MFA × MFB Ψ-F_2 population identified a crown rust resistance QTL in the centre of LG7 in a similar position as the QTL detected in this study, suggesting that this genomic region may be important for resistance in multiple germplasm sources (Sim et al. 2007).

Candidate genes involved in lignin biosynthesis have also been tentatively associated with host defence mechanisms to invasion by biotrophic pathogens (Kawasaki et al. 2006). In other forage species such as smooth bromegrass, crown rust resistance was positively correlated with high lignin content (Delgado et al. 2002) and in closely related cereal species such as wheat, the inhibition of enzymes involved in lignin production increased the susceptibility to stem rust (*P. graminis* f.sp. *tritici*) (Moerschbacher et al. 1990). Structural studies of genes involved in increased cell wall lignification in response to pathogen invasion have obtained contrasting results for perennial ryegrass. The sequences of *Lp*CAD1 and *Lp*CAD2 show close phylogenetic affinity to *A. thaliana* and rice CAD genes involved in plant defence responses (Lynch et al. 2002). However, no increase in the level of expression of *Lp*OMT1 and *Lp*OMT2 genes were detected in response to wounding and crown rust infection (Heath et al. 1998). Allelic variation, leading to changes in amino acid primary sequence, and hence protein function, may instead contribute to observed phenotypic variation of disease susceptibility. A role for perennial ryegrass lignin biosynthesis genes as candidates for quantitative disease resistance has yet to be determined, but the current results suggest that further investigation may be worthwhile. This could involve crown rust resistance evaluation of different transgenic lines with down-regulation of lignin biosynthesis genes such as *Lp*CAD2, *Lp*CCR1 and *Lp*OMT1.

Potential for genic SNP-based marker assisted selection

The data obtained in this study shows that QTL analysis may be performed for disease resistance traits in perennial ryegrass and that molecular genetic marker loci linked to the relevant genomic regions may be defined. The ability to infer direct coincidence between DR gene SNP loci and mapped disease resistance QTLs is limited by two factors: low numbers of common markers across genetic maps, and the known extent of QTL-containing regions for lower heritability traits when measured in small mapping populations. However, this study has demonstrated that extrapolation through the reference genetic map reveals substantial co-location of DR gene loci in genomic regions shown consistently to contain resistance QTLs for multiple pathogens. The SNP locus assays described in this study will highly assist future mapping studies to refine and extend these relationships. Definitive data on the functional significance of selected DR genes would require the use of large-scale fine-mapping populations, which are still in development for perennial ryegrass (Shinozuka et al. 2008), and association mapping approaches (Mackay 2001). The extent of linkage disequilibrium (LD) in out-crossing forage species is not large, typically spanning the length of an average plant gene (Ponting et al. 2007; Xing et al. 2007). Validation of diagnostic DR gene markers will hence depend on the use of highly characterised association mapping panels (Ponting et al. 2007) to directly correlate SNP haplotype with disease resistant host phenotypes (Dobrowoski and Forster 2006). Given success, such markers will be valuable for direct selection of superior allele content (Sorrells and Wilson 1997) in commercial perennial ryegrass breeding programs.

Acknowledgments This work was supported by funding from the Victorian Department of Primary Industries, Dairy Australia Ltd., the Geoffrey Gardiner Dairy Foundation, Meat and Livestock Australia Ltd. and the Molecular Plant Breeding Cooperative Research Centre (MPB CRC). The authors thank Dr. Charles Brummer (University of Georgia, USA) and Prof. Michael Hayward (Rhydgoch Genetics, Aberystwyth, UK) for careful critical reading of the manuscript and Dr Guoyou Ye for assistance with statistical and QTL analyses.

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