Theor Appl Genet (2007) 114:1427–1435
DOI 10.1007/s00122-007-0528-y

ORIGINAL PAPER

Identification of QTL for resistance and susceptibility to *Stagonospora meliloti* in autotetraploid lucerne

J. M. Musial · J. M. Mackie · D. J. Armour · H. T. T. Phan · S. E. Ellwood · K. S. Aitken · J. A. G. Irwin

Received: 1 December 2006 / Accepted: 16 February 2007 / Published online: 14 March 2007

Abstract In eastern Australia and California, USA, one of the major lethal fungal diseases of lucerne (*Medicago sativa*) is Stagonospora root and crown rot, caused by *Stagonospora meliloti*. Quantitative trait loci (QTL) involved in resistance and susceptibility to *S. meliloti* were identified in an autotetraploid lucerne backcross population of 145 individuals. Using regression analysis and interval mapping, we detected one region each on linkage groups 2, 6 and 7 that were consistently associated with disease reaction to *S. meliloti* in two separate experiments. The largest QTL on linkage group 7, which is associated with resistance to *S. meliloti*, contributed up to 17% of the phenotypic variation. The QTL located on linkage group 2, which is potentially a resistance allele in repulsion to the markers for susceptibility to *S. meliloti*, contributed up to 8% of the phenotypic variation. The QTL located on linkage group 6, which is associated with susceptibility to *S. meliloti*, contributed up to 16% of the phenotypic variation. A further two unlinked markers contributed 5 and 8% of the phenotypic variation, and were detected in only one experiment. A total of 517 simple sequence repeat (SSR) markers from *Medicago truncatula* were screened on the parents of the mapping population. Only 27 (6%) SSR markers were polymorphic and could be incorporated into the autotetraploid map of *M. sativa*. This allowed alignment of our *M. sativa* linkage map with published *M. truncatula* maps. The markers linked to the QTL we have reported will be useful for marker assisted selection for partial resistance to *S. meliloti* in lucerne.

Keywords Alfalfa · Molecular markers · QTL mapping

Communicated by J. E. Bradshaw.

J. M. Musial (✉) · J. M. Mackie · D. J. Armour · J. A. G. Irwin
School of Integrative Biology,
The University of Queensland,
Brisbane, QLD 4072, Australia
e-mail: j.musial@uq.edu.au

H. T. T. Phan · S. E. Ellwood
Australian Centre for Necrotrophic Fungal Pathogens,
State Agricultural Biotechnology Centre,
Health Sciences, Murdoch University,
Perth, WA 6150, Australia

K. S. Aitken
CSIRO Plant Industry,
Queensland Bioscience Precinct,
St Lucia, QLD 4067, Australia

Introduction

Lucerne is an important forage legume both in Australia, where up to 3.5 Mha are grown (Pearson et al. 1997), and worldwide where areas were estimated at 32 Mha (Michaud et al. 1988). Fungal root and crown rots are major biotic constraints on lucerne productivity and persistence (Leath 1989; Irwin 1989). In eastern Australia and California, USA, one of the major fungal diseases of lucerne is Stagonospora root and crown rot, caused by *S. meliloti*. Erwin et al. (1987) reported root and crown rot caused by *S. meliloti* as one of the most important components contributing to stand decline in California. In Australia, Irwin et al. (2004) showed *S. meliloti* to be widespread in southern New South Wales, where the largest lucerne areas in Australia prevail. In addition to a root and crown rot, characterised by red blotching of the woody tissue, *S. meliloti* also causes characteristic buff coloured leaf spots and stem lesions (Jones and Weimer 1938).

Working at the cultivar level (Erwin et al. 1987), and at the level of the individual plant (Irwin et al. 2004), a direct correlation has been established between root and foliar reaction to *S. meliloti*. It is advantageous to assess disease reaction to *S. meliloti* using foliar response as results can be obtained within 10–14 days versus 2–3 months for root reactions (Irwin et al. 2004). Inheritance of foliar resistance to *S. meliloti* was studied in an S_1, F_1 and a backcross population ((D × W116) × D), where the susceptible parent (D) was used as the recurrent parent (Irwin et al. 2004). The inheritance of resistance to *S. meliloti* appeared to be conditioned by a single dominant gene, based on segregations observed in S_1 and F_1 populations, but not in the backcross population where an excess of susceptible individuals was obtained.

The more complex nature of polyploid inheritance patterns had meant that mapping studies are less advanced (Mather 1936; Fisher 1947) in comparison to diploid studies. Despite having 45 documented diseases (Stuteville and Erwin 1990) and over 100 insect pests (Manglitz and Ratcliffe 1988), apart from resistance to downy mildew (Obert et al. 2000), *Phytophthora medicaginis* (Musial et al. 2005) and *Colletotrichum trifolii* (Irwin et al. 2006), there are no reports of molecular markers linked to disease and pest resistance in either diploid or autotetraploid lucerne.

Molecular markers provide a powerful means of determining chromosomal locations of genes that control both simply inherited and complex traits, and in marker assisted selection for disease resistance in breeding programs (Tanksley et al. 1989). The use of molecular markers in autotetraploid lucerne ($2n = 4x = 32$), of importance to Australian agriculture, has considerable potential for lucerne (Irwin et al. 2001), in particular for the identification of clones with multiple resistances to pests and diseases. The development of single- (Wu et al. 1992) and double-dose (Hackett et al. 1998) molecular markers has led to the development of genetic maps in many polyploid species, including lucerne. Eight genetic maps have been constructed for either diploid *M. sativa* (Brummer et al. 1993; Kiss et al. 1993; Echt et al. 1994; Tavoletti et al. 1996; Kalo et al. 2000) or autotetraploid *M. sativa* (Brouwer and Osborn 1999; Julier et al. 2003; Musial et al. 2005).

Thoquet et al. (2002) and Choi et al. (2004a) have generated genetic maps for *Medicago truncatula*. *M. truncatula* is useful as a model legume as it is diploid, outbreeding, and has a rapid reproductive cycle and small genome. Choi et al. (2004a) produced a comparative map between *M. truncatula* and diploid *M. sativa*. Evidence suggests there is a high degree of nucleotide sequence conservation and order between these two species, which leads to the potential for inference of genome information between these and other related legume species.

This paper reports research to identify and map QTL conditioning resistance and susceptibility to *S. meliloti* in an autotetraploid lucerne clone (W126) in which resistance to *C. trifolii* races 1, 2 and 4 has also been mapped (Mackie et al. 2007). Using SSR markers previously mapped in *M. truncatula* (Mun et al. 2006, Gutierrez et al. 2005 and http://www.medicago.org), we have aligned the QTL with their syntenic loci in *M. truncatula*.

Materials and methods

Plant materials

The same backcross (BC) population (but different clonal propagules) used to identify QTL for response to *C. trifolii* races 1, 2 and 4 (Mackie et al. 2007) was also used to identify QTL for response to *S. meliloti*. The lucerne clones used as parents, and their reactions to *S. meliloti*, are shown in Table 1. A single F_1 plant (WA647) from the cross W126 (resistant) × D (susceptible) was identified as resistant to all races of *C. trifolii*, and to foliar inoculation with *S. meliloti*. This plant was backcrossed to the susceptible recurrent parent (D) and a BC population of 145 individuals was generated. The F_1 individual (WA647) used to generate the BC population, and each BC individual, was confirmed as resulting from a cross by studying the parents and their DNA banding patterns using randomly amplified polymorphic DNA (RAPDs). The parents, WA647 and each BC individual were clonally propagated, as necessary, from stem cuttings.

Phenotypic characterisation of the BC population for foliar reaction to *S. meliloti*

Inoculum was prepared by growing cultures (*S. meliloti* isolate BRIP 28281) on V8 agar. The inoculation procedures employed were as described in Irwin et al. (2004). The 14-day-old regrowth on each of the BC individuals,

Table 1 Phenotypic characterisation of parental clones W126 and D, and an F_1 plant (WA647) from the cross W126 × D following foliar inoculation with *Stagonospora meliloti*

Clone	Disease reaction	Disease rating[a]
W126	Watersoaked spots on stems, no macroscopic leaf reaction	1
D	Death of tillers due to extensive coalescing stem and leaf lesions	5
WA647	Narrow necrotic flecks on leaves, petioles and stems with no fruiting bodies	2

[a] Rating system of Irwin et al. (2004), where 1 = resistant, 5 = highly susceptible

WA647, W126 and D were atomised to run-off with 2×10^6 spores ml^{-1}. Plants were incubated under natural light in a moist chamber at 22–26°C for 72 h, and grown for a further 16 days on a glasshouse bench at the same temperature range, then assessed for disease reaction following the disease rating described in Irwin et al. (2004) (Test Sm1). All plants from the above test were cut back, then 14-day-old regrowth was again inoculated; plants were treated as described above. Plants were assessed 19 days after inoculation, as previously described (Test Sm2). The final pooled disease rating (Test Smp) assigned to each individual was the highest rating recorded in the two tests.

DNA extraction

Leaf material (regrowth less than 4 weeks old) for DNA isolation and subsequent marker analysis was harvested from W126, D, WA647, and 145 BC individuals grown in a glasshouse. The methods as described in Musial et al. (2005) were applied.

AFLP analysis

Amplified Fragment Length Polymorphism (AFLP) analysis was performed according to Vos et al. (1995) and Musial et al. (2005). In total 28 primer combinations were used to genotype the mapping population, chosen from a pre-screening of 189 primer combinations on W126, D and WA647. The AFLP marker name refers to the primers used: the first two letters refer to the selective nucleotides of the *Eco*RI primer, the remaining letters refer to the selective nucleotides of the *Mse*I primer and the number refers to the band scored. AFLP marker names preceded with a '2' denote duplex markers (segregating 5:1).

SSR analysis

Each of 517 *M. truncatula* SSR primer pairs (Table 2) was screened for ability to amplify clear and polymorphic amplicon(s) in parental and F_1 DNAs. These primers were designed from all eight *M. truncatula* linkage groups using sequences from Mun et al. (2006), Gutierrez et al. (2005) and http://www.medicago.org. PCRs were carried out as described in Nelson et al. (2006). Two different methods were used to genotype the BC population depending on types of polymorphism identified. SSR primers that generated amplicons with distinct length (>10 bp) polymorphisms were separated directly on 3.5% agarose gels and visualised under UV-light after staining with ethidium bromide. Small differences in amplicon size (<10 bp) were resolved on an ABI3730 capillary sequencer using fluorescently labelled primers and the GeneScan™ -500 LIZ® size standard (Applied Biosystems USA). Genotyping was performed using the AB GeneMapper program (Applied Biosystems USA).

DNA marker analysis

DNA markers, which were present as a single band in W126 and WA647, but absent from D, were identified and genotyped in our BC population of 145 individuals. Markers were tested to determine their segregation ratios and those that segregated either 1:1 (simplex markers) or 5:1 (duplex markers) were selected to generate the linkage map. This procedure allowed the development of a coupling and repulsion phase linkage map of markers located on the 16 chromosomes of the F_1, which derived from W126, as per Brouwer and Osborn (1999).

Segregation analysis, map construction and QTL analysis

For each segregating marker, a chi-square (X^2) analysis ($P < 0.05$) was performed to test for deviation from the expected segregation ratio (1:1 for simplex markers or 5:1 for duplex markers). Markers segregating at a presence to absence ratio of less than 2.24:1 were considered to be SDRFs (Brouwer and Osborn 1999). This ratio gives equal chi-square values for both 1:1 and 5:1 hypotheses (Mather

Table 2 Simple sequence repeat (SSR) marker identification for comparative mapping in an autotetraploid lucerne backcross (BC) population ((W126 × D) = WA647) × D, where WA647 is a single resistant F_1 plant from the cross W126 × D

Source	Screened	Amplified	Polymorphic[a]	Mapped[b]
M. truncatula SSR (Mun et al. 2006)	98	44	24	7
http://www.medicago.org	200	152	94	17
M. truncatula SSR (Guiterrez et al. 2005)	138	68	36	3
Lupin EST-SSR (Nelson et al. 2006)	81	18	2	0
Total	517	282	156	27

[a] SSR markers were designated polymorphic if they produced amplicon(s) of different lengths between parental DNA (W126 and D) and WA647

[b] Informative markers (absent from D, present in W126 and WA647 and segregating in the BC population) were used to genotype the BC population; simplex and duplex markers were used to generate the genetic linkage map

1951). Selection of only these markers potentially excludes any null alleles from analysis. Linkage analysis on the AFLP and SSR markers (simplex and duplex) was performed using the computer package TetraploidMap (Hackett et al. 2006). TetraploidMap arranges the markers into the expected number of linkage groups by calculating the X^2 value of independence between markers. The expected number of linkage groups was set to eight. The program outputs data detailing the linkage phase, recombination rate and LOD score between markers within each homology group. Individual linkage groups within each homology group can then be identified using the Simplex Coupling (SC) group and linkage phase data. QTL were identified with the program MapManager QTXb20 (Manly et al. 2001) with $\alpha = 0.05$ (probability type I error). The regression analysis and interval mapping functions were applied. Markers with $P < 0.01$ were used to indicate QTL that had a significant effect on the phenotype. Permutation testing generated LOD threshold levels and any marker associated with a QTL above the 95% confidence interval was considered significant. To determine if epistasis was a significant factor in the disease reaction all simplex markers were tested for digenic linear × linear interactions. A threshold of $P \leq 10^{-5}$ was used to avoid detection of spurious interactions. To determine the contribution of all the QTL detected multiple regression was used. Markers associated with positive additive effects were further analysed for marker and QTL phase. For each marker, a set of possible genotypes at the QTL were identified and probabilities were calculated. These genotypes are based on the BC used to construct the mapping population, such that any marker or allele for *S. meliloti* reaction inherited from W126 can be only simplex or duplex in the F_1 (WA647).

Results

Phenotypic characterisation for reaction to *S. meliloti*

Using a paired *t*-test no significant difference was detected between the two inoculation tests, Sm1 and Sm2 ($t = 1.39$, $P = 0.168$). The pooled phenotypic characterisation data (Smp) for reaction of the BC population ((W126 × D) × D) to *S. meliloti* are shown in Fig. 1. Plants with a rating of 3 could be considered as either moderately susceptible or moderately resistant, and it is possible they may comprise a heterogeneous mixture of these categories, giving a continuous distribution in the BC population for disease reaction. However, selfing of plants with a disease rating of 3 has been shown to lead to S_1 populations which are predominantly susceptible (J.A.G. Irwin, unpublished data) indicating that these plants are more likely to tend towards susceptibility, than towards resistance. On that basis, the

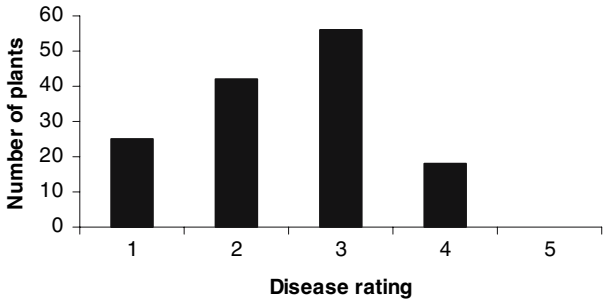

Fig. 1 Distribution of reaction to *Stagonospora meliloti* (Smp) in the backcross (BC) population (W126 × D) × D

BC population contained 47.5% resistant individuals, scoring plants with ratings of 1 and 2 as resistant, and 3, 4, and 5 as moderately to highly susceptible (Irwin et al. 2004). If W126 was simplex for a single tetrasomic gene conditioning resistance, 50% of the BC population would be expected to be resistant. A computed $X^2 = 0.35$ with $P = 0.50$–0.75 was obtained for this fit, indicating that on the basis of the observed segregations in the BC population we cannot reject the hypothesis that resistance in W126 is conditioned by a single completely dominant tetrasomic gene. However, we also cannot reject the hypothesis that disease reaction is quantitatively inherited, given the possible ambiguity surrounding the true disease rating of BC plants with a 3 score. In both inoculation tests, clone D received a rating of 5, W126 a rating of 1, and WA647 a rating of 2, as expected (Table 1).

Linkage analysis

In total, 195 simplex (168 AFLP and 27 SSR), 35 duplex AFLP and two phenotypic markers (Mackie et al. 2007) were identified from parent W126. All of the 28 AFLP primer combinations utilised were polymorphic and generated an average of 7.25 markers/primer combination. Data for these dominant segregating loci were used to construct a genetic linkage map in coupling and repulsion for the autotetraploid BC population. Only one co-dominant SSR marker was identified. Using TetraploidMap, 203 of these markers formed eight composite linkage groups, with an average of 25.4 markers/linkage group and an average distance between two markers of 3.9 cM. The total map length was 794.1 cM with 12.5% of markers unlinked (7 SSR, 10 simplex AFLP and 12 duplex AFLP).

In our autotetraploid population 14.3% of the AFLP and SSR markers did not show a typical Mendelian segregation ratio, 10.8% of the distortion was towards presence of the marker and 3.5% towards absence of the marker. The majority of the distorted markers were located on linkage groups 4, 5 and 6, and these were positioned throughout the

linkage group. Linkage groups 2, 3 and 7 contained either one or two distorted markers only. No distorted markers were located on linkage groups 1 and 8 (Fig. 2). Only seven (out of 29) of the distorted loci were skewed in the direction of the susceptible parent (D) and these were localised to linkage groups 2, 4 and 5. The majority of the distorted markers, which were skewed towards the resistant parent (W126), were located on linkage groups 2, 3, 4, 5, 6 and 7. Distortion was also observed for 44.8% of unlinked markers.

Mapping of 27 *M. truncatula* SSR (Mun et al. 2006; Gutierrez et al. 2005; http://www.medicago.org) allowed the identification of homeologous linkage groups between the two species. *M. sativa* linkage groups were named following the *M. truncatula* nomenclature. The relative orientation of linkage groups 2, 4, 5, 6, 7 and 8 between *M. sativa* and *M. truncatula* could be determined by incorporating at least two common SSR markers into each linkage group. The identification of linkage groups 1 and 3 was deduced from the addition of only one common marker. The regions to which these 27 SSR markers mapped on *M. sativa* linkage groups corresponded to their location on the respective homeologous *M. truncatula* linkage group. However, discrepancies were observed in linkage groups 2, 5 and 6 where five SSRs which had previously mapped in *M. truncatula* mapped to non-homeologous linkage groups in *M. sativa*. Markers 29 h4a, 2G11 and MtB130, which mapped to linkage group 4 in *M. truncatula*, mapped to linkage groups 5 and 6, respectively, in *M. sativa*. Markers 145p10a and MtB78 which mapped to linkage group 8 in *M. truncatula* mapped to linkage groups 2 and 6, respectively, in *M. sativa*. Furthermore these five SSR markers mapped to regions within the *M. sativa* linkage groups that do not correspond directly to their location within *M. truncatula*.

QTL analysis

Markers significantly associated with either increased resistance or susceptibility to *S. meliloti* at $P < 0.01$ are listed in Table 3. Disease reaction data from the two inoculations showed a normal distribution and QTL analyses were conducted for Sm1, Sm2 and pooled data Smp. The Ryan-Joiner Normality Test (Minitab Release 13) confirmed the data (Sm1, Sm2 and Smp) followed a normal distribution ($R = 0.9983$, $P > 0.1$; $R = 0.9984$, $P > 0.1$; $R = 0.9980$,

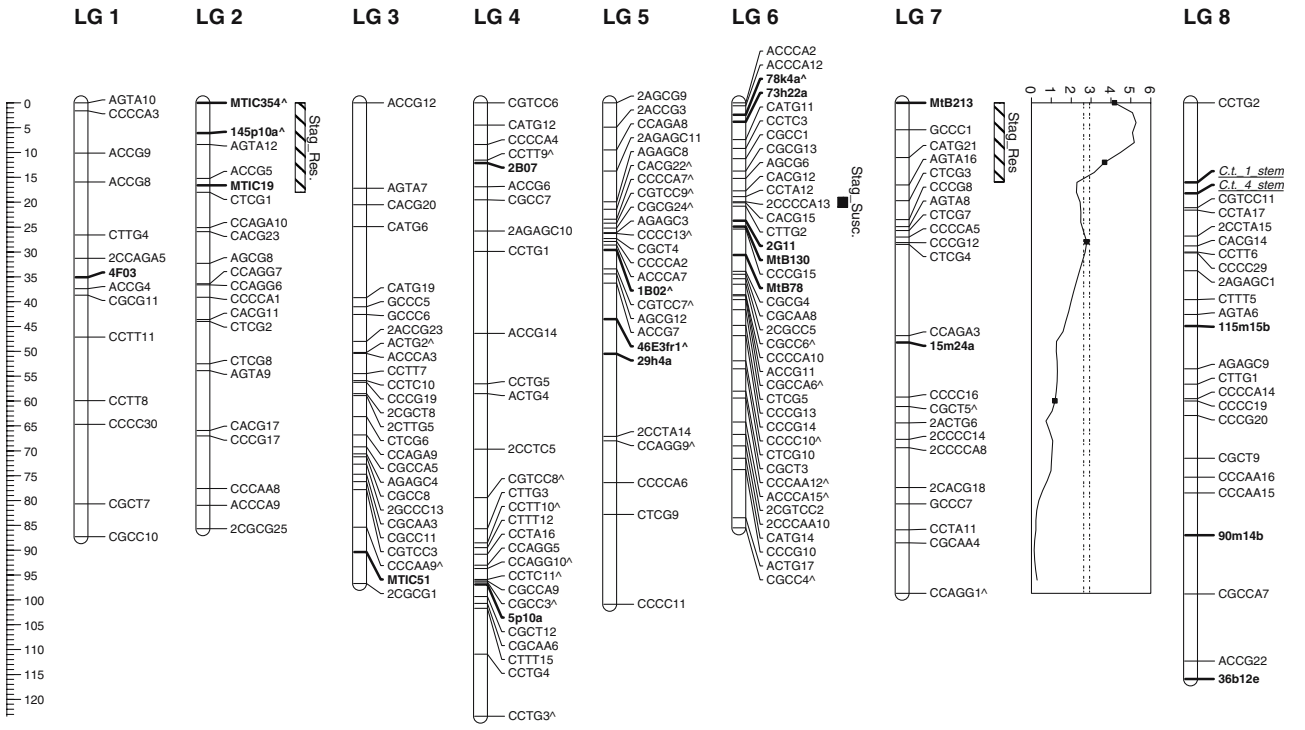

Fig. 2 An autotetraploid lucerne (*Medicago sativa*) linkage map generated from the backcross (BC) population (W126 × D) × D using amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and phenotypic markers. *Vertical bars* represent the composite linkage groups and are labelled with their corresponding *Medicago truncatula* number. *Horizontal lines* show marker positions. Genetic distances (cM) are located to the *left* of each linkage group and locus names are listed to the *right* of each linkage group. Markers with a '2' preceding the loci name are duplex markers (5:1). Marker names shown in *bold* are SSR markers. Markers at $P < 0.01$ and associated with resistance are marked by a *hatched box* and those associated with susceptibility are marked by a *black box*. Markers showing distorted segregation are indicated by a ^

Table 3 Markers for resistance and susceptibility to *Stagonospora meliloti* identified in the backcross population (W126 × D) × D at $P < 0.01$. Sm1 and Sm2 represent results from two separate inoculations conducted on all individuals. Smp is the highest rating recorded for individuals in the two tests (Sm1 and Sm2)

Test	Linkage group	Marker	% Total variance	*P*	Estimated additive effect[a]
Sm1	Unlinked	CCCAA14	8	0.00128	−0.45
	2	MTIC354[b]	7	0.00458	0.48
	2	CTCG1	7	0.00257	0.47
	6	2CCCCA13	16	0.00001	0.67
	7	MtB213	17	0.00000	−0.75
	7	CATG21	8	0.00113	−0.52
	7	CCCG12	13	0.00002	−0.67
	7	CCCC16	9	0.00054	−0.55
Sm2	Unlinked	2AGCG10	5	0.00484	−0.41
	2	MTIC354[b]	8	0.00153	0.52
	2	145p10a	5	0.00813	0.43
	2	CTCG1	8	0.00077	0.52
	6	2CCCCA13	15	0.00001	0.67
	7	MtB213	13	0.00002	−0.66
	7	CATG21	10	0.00017	−0.58
	7	CCCG12	9	0.00018	−0.58
	7	CCCC16	5	0.00526	−0.43
Smp	Unlinked	2AGCG10	5	0.00571	−0.40
	2	MTIC354[b]	7	0.00224	0.50
	2	CTCG1	7	0.00111	0.50
	6	2CCCCA13	16	0.00000	0.68
	7	MtB213	12	0.00003	−0.65
	7	CATG21	9	0.00024	−0.57
	7	CCCG12	9	0.00026	−0.57
	7	CCCC16	5	0.00685	−0.42

[a] Markers associated with a positive additive effect were further analysed to determine their phase to the disease reaction QTL

[b] These data apply to the second allele of co-dominant marker MTIC354

$P > 0.1$, respectively), where the *P*-value must be >0.05 to be considered normally distributed.

For QTL detected with interval mapping the LOD threshold was determined by a permutation test (100 permutations) and any marker over the 95% confidence interval was considered significant (Nettleton 2000). The LOD thresholds for the three different tests ranged from 2.48 to 2.92. Regression analysis revealed ten markers that were significantly associated with reaction to *S. meliloti* (Table 3). These grouped into three regions located on linkage groups 2, 6 and 7 (Fig. 2), and two unlinked markers. Interval mapping using the three sets of disease data identified the same five QTL, three that increased resistance, one that increased susceptibility and one potentially associated with a resistance allele in repulsion to the markers for susceptibility to *S. meliloti*. These explained individually from 5 to 17% of the phenotypic variation depending on the experiment. These most likely represent one QTL on each of linkage groups 2, 6 and 7, and two putative QTL associated with unlinked markers (CCCAA14 and 2AGCG10). SSR marker MtB213 on linkage group 7 explained the largest amount of variation with all three datasets, varying from 12 to 17%. The region between markers MtB213 and CCCG12 on linkage group 7 is significant at $P < 0.05$ but only markers with $P < 0.01$ are shown in Table 3. The trace graph of linkage group 7 (Fig. 2) is suggestive of two effects, although after permutation testing the second peak is not above the 95% LOD threshold. All other QTL explained between 5 and 16% of the phenotypic variation. Both positive and negative additive effects were identified as inherited from the resistant parent W126. The markers associated with positive additive effects were further analysed to determine the most likely genotype at the QTL. A simplex marker could be linked tight in coupling to an allele for susceptibility, or the marker could be linked in repulsion with an allele for resistance. In the BC population, a simplex marker in coupling with a susceptibility allele would give two genotypes and two phenotypes in a 1:1 ratio; marker present and all individuals susceptible or marker absent and all individuals resistant. A simplex marker in repulsion with an allele for resistance would give four genotypes and two phenotypes. The presence of the marker would produce two genotypes in a ratio of 1:2 :: resistant:susceptible phenotypes. The absence of the marker would produce two genotypes in a ratio of 2:1 :: resistant:susceptible phenotypes. A duplex marker linked to a simplex susceptibility allele would give four genotypes and two phenotypes. The presence of the marker would give three genotypes in a ratio of 2:3 :: resistant:susceptible phenotypes. The absence of the marker would give only a resistant phenotype. A duplex marker linked to a duplex susceptibility allele would give only two genotypes and two phenotypes. The presence of the marker would only give a susceptible phenotype, and the absence of the marker a resistant phenotype in a ratio of 5:1 with marker present and absent, respectively.

The two simplex markers with positive additive effects (145p10a and CTCG1, Table 3) each fitted the model for a simplex marker in repulsion with an allele for *S. meliloti* resistance, when the marker was present ($X^2 = 0.040–0.045$, $P = 0.75–0.90$). When the marker was absent, a significant fit to the model was only observed for 145p10a ($X^2 = 1.8$, $P = 0.2253$). These markers are located within the same region on linkage group 2 and furthermore, on the same homolog (data not shown). Each of the two alleles of the co-dominant SSR marker MTIC354 was analysed as a simplex marker. The second allele, when present, fitted the

model for a simplex marker linked in repulsion with a resistance allele ($X^2 = 1.54$, $P = 0.2733$). This allele is located in the same homolog of linkage group 2 as markers 145p10a and CTCG1 (data not shown). The first allele of co-dominant marker MTIC354 does not fit the expected ratio for a marker linked to a resistance allele. The duplex marker 2CCCCA13, located on linkage group 6, was also linked to a QTL for susceptibility. Inspection of observed genotype/phenotype ratios suggest that the marker is most likely to be linked to a simplex susceptibility allele.

The two unlinked markers, representing two putative QTL, 2AGCG10 and CCCAA14 contributed 5 and 8% of the phenotypic variation, respectively. Three out of the five QTL were consistently detected in both experiments. When the four QTL identified for each experiment were included in the multiple regression model they explained from 23 to 25% of the variation. Two significant interactions were detected: one between the QTL on linkage group 2 (marker MTIC354) and 7 (marker MtB213) and another between the same QTL on linkage group 7 (marker MtB213) and a marker on linkage group 4 (marker CGTCC8) (not previously identified as a QTL). When these were included in the model from 29 to 31% of the phenotypic variation was explained. This provides further evidence of the importance of complementary gene interactions in autotetraploid lucerne, as outlined by Bingham et al. (1994).

Discussion

The work presented here is the first report of QTL conferring reaction to *S. meliloti* in lucerne. The QTL for reaction to *S. meliloti* were located on three linkage groups, and two unlinked markers. The QTL on linkage groups 2, 6 and 7 were consistently identified for the three sets of phenotypic data (Sm1, Sm2 and Smp), indicating the presence of at least three genes conditioning reaction to *S. meliloti*. The largest QTL, contributing up to 17% of the phenotypic variation, was identified on linkage group 7 and was associated with increased resistance to *S. meliloti*. The QTL on linkage group 2 is potentially a resistance allele in repulsion to the markers for susceptibility and the QTL located on linkage group 6 was associated with increased susceptibility. These QTL contributed up to 8 and 16% of the phenotypic variation, respectively. Two unlinked markers, representing two putative QTL (2AGCG10 and CCCAA14) contributed 5 and 8% of the phenotypic variation, respectively. It is possible that additional QTL remain undetected since only a maximum of 31% of phenotypic variation has been accounted for. With the incorporation of more markers and a larger population size additional QTL may have been identified. Vales et al. (2005) conducted a study on the effect of population size on the estimation of QTL using resistance to barley stripe rust as a test and reported that the number of QTL increased as the population size increased. They also found that QTL with large effects can be detected with small populations, but it is necessary to increase the population size to be able to detect QTL with small effects. Hackett et al. (1998) demonstrated, through a simulation study into linkage analysis in tetraploids, that a population size of at least 150 individuals should be used and that around 250 individuals improved the ability to identify homologous chromosomes. They also reported that the accuracy of linkage estimates depended on the type of markers involved and that simplex–simplex coupling pairs were the most reliable, of which the majority of our markers are.

While this is the first report of detection of QTL for reaction to *S. meliloti* in lucerne, there have been extensive studies identifying major QTL for reaction to *Stagonospora nodorum* in wheat at different stages of plant growth, in different plant organs and also during varying conditions of infection (Aguilar et al. 2005; Liu et al. 2004; Schnurbusch et al. 2003). The application of these markers to selecting resistant wheat plants for use in breeding programs is also discussed in these papers. The QTL we have identified on linkage group 7 should also have application for use in marker assisted breeding for *S. meliloti* resistance in lucerne. This disease is believed to seriously limit the persistence and productivity of lucerne in southern Australia (Irwin et al. 2004), and the development of resistant cultivars should be a high priority in lucerne breeding programs.

Based solely on phenotypic data, Irwin et al. (2004) reported that resistance to *S. meliloti* in clone W116 selected from cv. Sequel was not simply inherited. Resistance was inherited as an incompletely dominant trait, with 57% of plants from an R × S cross resistant, although the backcross population had an excess of susceptible individuals. In this autotetraploid BC population derived from clone W126 from cv. Trifecta, a 1:1 segregation of R:S was obtained, fitting a model of W126 being simplex for a single dominant gene conditioning resistance. Our analysis detected one major QTL for resistance to *S. meliloti* on linkage group 7 and one QTL on linkage group 2 which is potentially a resistance allele in repulsion to the markers for susceptibility to *S. meliloti*. The trace graph of linkage group 7 (Fig. 2) is suggestive of two effects although after permutation testing the second peak is not above the 95% LOD threshold. With the addition of extra markers to the map, more markers linked to QTL for reaction to *S. meliloti* may have been identified. Further testing for validation of our markers in a larger BC population than the one we used here may assist in resolving the relative importance of these QTL to determining phenotypic response to *S. meliloti*. A further two unlinked markers (CCCAA14 and 2AGCG10) were also associated with resistance, and explained up to

8% of the phenotypic variation. Possibly with a larger population size these QTL and other QTL with smaller effects may have been consistently detected in the two tests (Sm1 and Sm2) and pooled data (Smp).

Our map contained 201 simplex and duplex DNA markers and two phenotypic simplex markers with 12.5% (29 markers) of markers unlinked; these formed eight linkage groups, which was the expected number. Duplex markers were located on every linkage group. The parent clones W126 and D, and WA647 were also screened with 517 SSR primers from *M. truncatula*. Only 27 SSR primers (6.2%) were polymorphic and suitable to genotype in our BC population. These SSRs enabled us to align our *M. sativa* linkage map with the *M. truncatula* linkage map, also resulting in putative locations for the QTL in *M. truncatula*. It is unknown whether *M. truncatula* is a host of *S. meliloti*; although the pathogen is reported to cause a leaf spot on other annual *Medicago* species (Barbetti 1988, Tivoli et al. 2006).

In our autotetraploid population 14.3% of AFLP and SSR markers do not show a typical Mendelian segregation ratio, of which 10.8% distortion was towards presence of the marker and 3.5% towards absence of the marker. This rate of distortion is between the rates Brouwer and Osborn (1999) and Julier et al. (2003) observed. Brouwer and Osborn (1999) reported 5.5% distortion in their autotetraploid map containing simplex RFLP alleles and stated that autotetraploidy could reduce the effect of deleterious alleles that cause segregation distortion. Julier et al. (2003) reported 25 and 35% distortion over the SSR and AFLP markers, respectively. The low segregation distortion rate we observed could be attributed to the large number of simplex alleles analysed (84.1%). Julier et al. (2003) also reported that lower rates of distortion are observed if a low number of duplex alleles has been studied. This could also contribute to our low rate of distortion as only 15.1% of total markers included in this study were duplex.

Medicago sativa and *M. truncatula* share highly conserved nucleotide sequences and exhibit nearly perfect synteny between the two genomes (Choi et al. 2004a; Julier et al. 2003). We mapped 27 common SSR markers which tag the eight linkage groups of the *M. sativa* genetic map. To facilitate comparison, we gave the same numbering to homeologous linkage groups. The relative orientation and nomenclature of linkage groups between *M. sativa* and *M. truncatula* could be determined due to linkage of at least two common SSR markers. The numbering of linkage groups 1 and 3 was deduced from the positioning of one common SSR marker. All these markers were similarly located in the two species except for five SSR markers in linkage groups 2, 5 and 6. Choi et al. (2004a) also reported similar observations when mapping PCT primers, the NUM1 gene and the position of the 5S rDNA locus in *M. sativa*. More common markers are needed between the two species to determine if the difference in the five SSR positions is due to translocations or the *M. truncatula* SSR markers utilised not being syntenic in *M. sativa*. Choi et al. (2004b) suggested that chromosomal rearrangements involving *Medicago* (*M. sativa* and *M. truncatula*) linkage group 6 might also be responsible for the difference in chromosome number between Medicago and pea (Kalo et al. 2004). Translocations have also been detected in other polyploid species (Moore et al. 1995).

This is the first report of QTL conditioning reaction to *S. meliloti* in lucerne. Markers linked to the QTL for resistance to *S. meliloti* will be useful for marker assisted selection of individual resistant lucerne clones, for incorporation into breeding populations, and also for comparative genomic studies in *M. truncatula*.

Acknowledgments The authors gratefully acknowledge the Cooperative Research Centre for Tropical Plant Protection, the Australian Research Council (LP0454871) and the Grains Research and Development Corporation for providing funding support for the project.

References

Aguilar V, Stamp P, Winzeler M, Winzeler H, Schachermayr G, Keller B, Zanetti S, Messmer MM (2005) Inheritance of field resistance to *Stagonospora nodorum* leaf and glume blotch and correlations with other morphological traits in hexaploid wheat (*Triticum aestivum* L.) Theor Appl Genet 111:325–336

Barbetti MJ (1988) Fungal diseases of annual medics. In: National Annual Medic Improvement Program – A workshop of the Wheat Research Council. Wheat Research Council, Melbourne, pp 71–75

Bingham ET, Groose RW, Woodfield DR, Kidwell KK (1994) Complementary gene interactions in alfalfa are greater in autotetraploids than diploids. Crop Sci 34:823–829

Brouwer DJ, Osborn TC (1999) A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.). Theor Appl Genet 99:1194–1200

Brummer EC, Bouton JH, Kochert G (1993) Development of an RFLP map in diploid alfalfa. Theor Appl Genet 86:329–332

Choi HK, Kim D, Uhm T, Limpens E, Lim H, Mun JH, Kalo P, Varma Penmetsa R, Seres A, Kulikova O, Roe BA, Bisseling T, Kiss GB, Cook DR (2004a) A sequence-based genetic map of *Medicago truncatula* and comparison of marker colinearity with *M. sativa*. Genetics 166:1463–1502

Choi HK, Mun JH, Kim DJ, Zhu H, Baek JM, Mudge J, Roe B, Ellis N, Doyle J, Kiss GB, Nevin DY, Cook DR (2004b) Estimating genome conservation between crop and model legume species. Proc Natl Acad Sci USA 101:15289–15294

Echt CS, Kidwell KK, Knapp SJ, Osborn TC, McCoy TJ (1994) Linkage mapping in diploid alfalfa (*Medicago sativa*). Genome 37:61–71

Erwin DC, Khan RA, Ribeiro OK, Lehman WF (1987) Growth, sporulation, and pathogenicity of *Stagonospora meliloti* and selection for resistance to crown rot and leaf spot in alfalfa. Plant Dis 71:181–185

Fisher RA (1947) The theory of polysomic inheritance. Philos Trans R Soc B 233:55–87

Gutierrez MV, VazPatto MC, Huguet T, Cubero JI, Moreno MT, Torres AM (2005) Cross-species amplification of *Medicago truncatula* microsatellites across three major pulse crops. Theor Appl Genet 110:1210–1217

Hackett CA, Bradshaw JE, Meyer RC, McNicol JW, Milbourne D, Waugh R (1998) Linkage analysis in tetraploid potato: a simulation study. Genet Res 71:143–154

Hackett CA, Milne I, Bradshaw JE, Luo ZW (2006) TetraploidMap for Windows, Biomathematics and Statistics Scotland. ftp://www.ftp.bioss.sari.ac.uk/pub/cah

Irwin JAG (1989) Diseases of pasture legumes in Australia. In: Marten GC, Matches AG, Barnes RF, Brougham RW, Clements RJ, Sheath GW (eds) Persistence of forage legumes. ASA Inc, Madison

Irwin JAG, Aitken KS, Mackie JM, Musial JM (2006) Lucerne genetic improvement for quantitatively inherited anthracnose (*Colletotrichum trifolii*) resistance facilitated by molecular markers. Australas Plant Path 35:573–579

Irwin JAG, Lloyd DL, Lowe KF (2001) Lucerne biology and genetic improvement—an analysis of past activities and future goals in Australia. Aust J Agric Res 52:699–712

Irwin JAG, Mackie JM, Marney TS, Musial JM, Roberts S (2004) Incidence of *Stagonospora meliloti* and *Acrocalymma medicaginis* in lucerne crowns and roots in eastern Australia, their comparative aggressiveness to lucerne and inheritance of reaction to *S. meliloti* in lucerne. Australas Plant Path 33:61–67

Jones FR, Weimer JL (1938) Stagonospora leaf spot and root rot of forage legumes. J Agric Res 57:791–812

Julier B, Fajoulot S, Barre P, Cardinet G, Santoni S, Huguet T, Huyghe C (2003) Construction of two genetic linkage maps in cultivated tetraploid alfalfa (*Medicago sativa*) using microsatellite and AFLP markers. BMC Plant Biol 3:9

Kaló P, Endre G, Zimányi L, Csanádi G, Kiss GB (2000) Construction of an improved linkage map of diploid alfalfa (*Medicago sativa*). Theor Appl Genet 100:641–657

Kalo P, Seres A, Taylor SA, Jakab J, Kevei Z, Kereszt A, Endre G, Ellis TH, Kiss GB (2004) Comparative mapping between *Medicago sativa* and *Pisum sativum*. Mol Genet Genomics 272:235–246

Kiss GB, Csanádi G, Kálmán K, Kaló P, Okrész L (1993) Construction of a basic genetic map for alfalfa using RFLP, RAPD, isozyme and morphological markers. Mol Gen Genet 238:129–137

Leath KT (1989) Diseases and forage stand persistence in the United States. In: Marten GC, Matches AG, Barnes RF, Brougham RW, Clements RF, Sheath GW (eds) Persistence of forage legumes. ASA Inc, Madison

Liu ZH, Friesen TL, Rasmussen JB, Ali S, Meinhardt SW, Faris JD (2004) Quantitative trait loci analysis and mapping of seedling resistance to *Stagonospora nodorum* leaf blotch in wheat. Phytopathology 94:1061–1067

Mackie JM, Musial JM, Armour DJ, Phan HTT, Ellwood SE, Aitken KS, Irwin JAG (2007) Identification of QTL for reaction to three races of *Colletotrichum trifolii* and further analysis of inheritance of resistance in autotetraploid lucerne. Theor Appl Genet. doi:10.1007/s00122-007-0527-z

Manglitz GR, Ratcliffe RH (1988) Insects and mites. In: Hanson AA (ed) Alfalfa and alfalfa improvement. American Society of Agronomy, Madison, pp 671–704

Manly KF, Cudmore RH, Meer JM (2001) Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome 12:930–932

Mather K (1936) Segregation and linkage in autotetraploids. J Genet 32:287–314

Mather K (1951) The measurement of linkage in heredity. Methuen and Co Ltd, London

Michaud R, Lehman WF, Rumbaugh MD (1988) World distribution and historical development. In: Alfalfa and alfalfa improvement. Agronomy Monograph 29. American Society of Agronomy: Madison, pp 25–91

Moore G, Devos KM, Wang Z, Gale MD (1995) Cereal genome evolution, grasses, line up and form a circle. Curr Biol 5:737–739

Mun JH, Kim DJ, Choi HK, Gish J, Debellé F, Mudge J, Denny R, Endre G, Saurat O, Dudez AM, Kiss GB, Roe B, Young ND, Cook DR (2006) Distribution of microsatellites in the genome of *Medicago truncatula*: a resource of genetic markers that integrate genetic and physical maps. Genetics 172:2541–2555

Musial JM, Aitken KS, Mackie JM, Irwin JAG (2005) A genetic linkage map in autotetraploid lucerne adapted to northern Australia, and use of the map to identify DNA markers linked to resistance to *Phytophthora medicaginis*. Aust J Agric Res 56:333–344

Nelson M, Phan H, Moolhuijzen P, Hane J, Williams A, Fosu-Nyarko J, Ksiazkiewicz M, Scobie M, Cakir M, Jones M, Barker S, Wolko B, Oliver R, Cowling W (2006) The first gene-based map of *Lupinus angustifolius* L.—location of domestication genes and conserved synteny with *Medicago truncatula*. Theor Appl Genet 113:225–238

Nettleton D (2000) Accounting for variability in the use of permutation testing to detect quantitative trait loci. Biometrics 56:52–58

Obert DE, Skinner DZ, Stuteville DL (2000) Association of AFLP markers with downy mildew resistance in autotetraploid alfalfa. Mol Breeding 6:287–294

Pearson CJ, Brown R, Collins WJ, Archer KA, Wood MS, Petersen C, Boothe B (1997) An Australian temperate pastures database. Aust J Agric Res 48:453–465

Schnurbusch T, Paillard S, Fossati D, Messmer M, Schachermayr G, Winzeler M, Keller B (2003) Detection of QTLs for *Stagonospora* glume blotch resistance in Swiss winter wheat. Theor Appl Genet 107:1226–1234

Stuteville DL, Erwin DC (1990) Compendium of alfalfa diseases, 2 edn. APS Press, St Paul

Tanksley SD, Young ND, Paterson AH, Bonierbale MW (1989) RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7:257–264

Tavoletti S, Veronesi F, Osborn TC (1996) RFLP linkage map of an alfalfa meiotic mutant based on a F_1 population. J Hered 87:167–170

Thoquet P, Gherardi M, Journet EP, Kereszt A, Ane JM, Prosperi JM, Huguet T (2002) The molecular genetic linkage map of the model legume *Medicago truncatula*: an essential tool for comparative legume genomics and the isolation of agronomically important genes. BMC Plant Biol 2:1

Tivoli B, Baranger A, Avila CM, Banninza S, Barbetti M, Chen W, Davidson J, Lindeck K, Kharrat M, Rubiales D, Sadiki M, Sillero JC, Sweetingham M, Muehlbauer FJ (2006) Screening techniques and sources of resistance to foliar diseases caused by major necrotrophic fungi on grain legumes. Euphytica 147:223–253

Vales MI, Schön CC, Capettini F, Chen XM, Corey AE, Mather DE, Mundt CC, Richardson KL, Sandoval-Islas JS, Utz HF, Hayes PM (2005) Effect of population size on the estimation of QTL: a test using resistance to barley stripe rust. Theor Appl Genet 111:1260–1270

Vos P, Rogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wu KK, Burnquist W, Sorrells ME, Tew TL, Moore PH, Tanksley SD (1992) The detection and estimation of linkage in polyploids using single-dose restriction fragments. Theor Appl Genet 83:294–300