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ORIGINAL PAPER

Identification of QTL for reaction to three races of *Colletotrichum trifolii* and further analysis of inheritance of resistance in autotetraploid lucerne

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Abstract Anthracnose, caused by *Colletotrichum trifolii*, is one of the most serious diseases of lucerne worldwide. The disease is managed through deployment of resistant cultivars, but new pathotypes present a challenge to the successful implementation of this strategy. This paper reports the genetic map locations of quantitative trait loci (QTL) for reaction to races 1, 2 and 4 of *C. trifolii* in a single autotetraploid lucerne clone, designated W126 from the Australian cv. Trifecta. Resistance was mapped in a backcross population of 145 individuals, and reaction was assessed both by spray and injection inoculation of stems. Resistance to injection inoculation with races 1 and 4 was incompletely dominant and closely linked (phenotypic markers 2.2 cM apart); these resistances mapped to a linkage group homologous to *Medicago truncatula* linkage group 8. When the spray inoculation data were subjected to QTL analysis, the strongest QTL for resistance was located on linkage group 8; six QTL were identified for race 1 and four for race 4. Resistance to race 2 was incompletely recessive; four QTL were identified and these include one QTL on linkage group 4 that was also identified for race 1. Modelling of the interactions between individual QTL and marker effects allowed a total of 52–63% of the phenotypic variation to be described for each of the different races. These markers will have value in breeding lucerne, carrying multiple sources of resistance to the three known races of *C. trifolii*.

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Introduction

Anthracnose, caused by *Colletotrichum trifolii*, is a serious disease of lucerne (*Medicago sativa*) and annual medics (*Medicago* spp.) (Welty 1982) in North America (Barnes et al. 1969), South Africa (Lamprecht 1986), Europe (Raynal 1977) and Australia (Irwin 1974). In warm humid climates, anthracnose stem lesions and crown rot are major limitations to lucerne persistence and productivity (Barnes et al. 1969; Irwin 1977). Management of anthracnose is largely through the use of resistant lucerne cultivars (Devine et al. 1971) and resistance to anthracnose also protects forage quality (Lenssen et al. 1991). Autotetraploid lucerne ($2n = 4x = 32$) is characterised by extreme heterozygosity and severe inbreeding depression (Busbice 1968); thus even resistant lucerne cultivars contain varying proportions of susceptible plants, with a cultivar requiring 30% resistant plants before it can be classified as resistant (Fox et al. 1991). It has been demonstrated that selection for resistance to stem anthracnose also confers resistance to *Colletotrichum* crown rot (Irwin et al. 1980), which is a major cause of plant mortality. Recurrent selection has provided an effective way of increasing anthracnose resistance levels in breeding populations of autotetraploid lucerne (Devine et al. 1971; Clements et al. 1984), and resistant cultivars are widely grown in North America, Europe and Australia.

Two pathotypes of *C. trifolii* have been described from lucerne in North America (Ostazeski et al. 1979). Race 1 is avirulent on cvs. Arc and Saranac AR, whereas race 2 is virulent on Arc but avirulent on Saranac AR. Mackie et al. (2003), using individual lucerne clones (plants), demonstrated that Australian race 1 and race 2 isolates had the same virulence patterns as the North American isolates, which were compared in the same test. Another race, designated race 4, was identified in Australia, with virulence on a clone (WA230) from the Australian cultivar UQL-1 (Mackie et al. 2003); WA230 is resistant to races 1 and 2. Race 4 has also recently been reported from Ohio, USA (Ariss and Rhodes 2006). Elgin and Ostazeski (1985) showed resistance in Arc to race 1 to be conditioned by a single dominant tetrasomic gene An_1. A second independent gene, An_2, conferred resistance in Saranac AR to races 1 and 2 (Elgin and Ostazeski 1985). Elgin and O'Neill (1988) found that in the proportion of Saranac AR plants that were resistant to race 1 only, only An_1 was present. In inheritance studies using Australian races and plants, Mackie and Irwin (1998) and Mackie et al. (2003) were unable to fit observed segregations to single tetrasomic gene models, although in some plants resistance appeared to be relatively simply inherited and incompletely dominant, whereas in others resistance was clearly quantitatively inherited and incompletely recessive (Irwin et al. 2006).

Some Australian clones were resistant following spray inoculation with *C. trifolii*, but susceptible to stem injection inoculation (Mackie and Irwin 1998), indicating the importance of the infection court in determining the final disease reaction outcome. The resistance in one such clone, W116, was quantitatively inherited, and a multi-locus region comprising three QTL was mapped to linkage group 4 of the W116 map (Irwin et al. 2006; Musial et al. 2005). However, the genetics conditioning plant response to spray (epidermal) and injection inoculation remains undetermined. Torregrosa et al. (2004) identified *M. truncatula* cv. Jemalong and F83005.5 as resistant and susceptible to *C. trifolii* race 1 respectively, and examination of disease phenotypes in F_1 and F_2 progenies of Jemalong and F83005.5 indicated that resistance was dominant and probably due to a major resistance gene, although the hypothesis of one dominant resistance gene with normal Mendelian segregation was rejected at the 1% level ($P = 0.007$). These results were obtained following both spray inoculation and inoculation of detached leaves with a spore suspension of *C. trifolii*.

Mackie et al. (2003) identified a lucerne clone, W126 from cv. Trifecta, which is resistant to races 1, 2 and 4 of *C. trifolii* following stem injection and spray inoculation. In inheritance studies with this clone (Mackie and Irwin 1998), resistance to race 1 was found to be incompletely dominant, with the majority of F_1 plants from a W126 × D (susceptible clone) cross being resistant. This paper reports research conducted to determine the genetics of resistance to *C. trifolii* races 1, 2 and 4 in clone W126, following both stem injection and spray inoculation. Using procedures described in Musial et al. (2007), a linkage map was generated in a backcross (BC) population segregating for reaction to the three *C. trifolii* races. Simple sequence repeat (SSR) markers from *M. truncatula*, which were polymorphic in the parents (W126 and D) were also mapped, allowing alignment of the autotetraploid lucerne and published *M. truncatula* maps (Thoquet et al. 2002; Choi et al. 2004).

Materials and methods

Plant materials and disease reaction types

The parental clones used in the research and their phenotypes (disease reactions) when inoculated with races 1, 2 and 4 of *C. trifolii* are shown in Table 1. Reactions of all clones for all three races were rated on a scale of 1 for highly resistant to 5 for highly susceptible (Mackie and Irwin 1998). The recurrent parent, clone D, highly susceptible (rating 5) to all *C. trifolii* races, was the same susceptible clone used to map resistance to *Phytophthora medicaginis* reported in Musial et al. (2005); it was selected from the highly winter active cultivar Demnat (Oram 1990). Clone W126, selected from cv. Trifecta, a semi-dormant cultivar (Oram 1990), was the same clone reported upon in Mackie and Irwin (1998); it is highly resistant (rating 1) to races 1 and 4, and resistant (rating 2) to race 2 of *C. trifolii*. A single resistant F_1 plant (WA647) from a W126 × D cross was identified, which had the same disease reactions to all races as W126 following spray and stem injection inoculation, except that dark brown runner lesions were manifested upon injection inoculation with race 2 compared to no macroscopic symptoms for W126 (Table 1). Clones WA647 and W126 are resistant to spray inoculation with race 2, giving ratings of 2 (necrotic flecks). A BC population of 145 individuals was generated by crossing WA647 × D, using suction emasculation.

The rating systems used to assess the disease reaction phenotypes of plants inoculated by spraying were as described by Mackie and Irwin (1998), where 1 = no macroscopic symptoms on stems, leaves or petioles, small (≤1 mm) water soaked spots on stems only; 2 = narrow, slit-like dark necrotic flecks without acervuli on stems, some petiole or leaf lesions; 3 = wide elliptical lesions with acervuli on stems, lesions usually non-girdling, petiole collapse; 4 = coalescing and girdling stem lesions with acervuli, stem collapse; 5 = plant dead. Upon hypodermic injection of stems, generally two reaction types were observed: resistant = no macroscopic symptoms; and

Table 1 Phenotypic characterisation of parental clones W126 and D, and an F_1 plant (WA647) from the cross W126 × D following spray inoculation and stem injection inoculation with *Colletotrichum trifolii* races 1, 2 and 4

Clone	Disease reaction phenotypes on stems	
	Spray inoculation	Stem injection inoculation
Race 1		
W126	Small (≤1 mm) water soaked spots (=1)[a]	No macroscopic symptoms
D	Large coalescing lesions, stems killed (=5)	Stem collapse, no dark necrosis, profuse sporulation
WA647	As for W126 (=1)	As for W126
Race 2		
W126	Small (≤1 mm) water soaked spots and very narrow necrotic flecks (=2)	No macroscopic symptoms
D	Large coalescing lesions, stems killed (=5)	Stem collapse, no dark necrosis, profuse sporulation
WA647	As for W126 (=2)	Dark brown narrow necrotic lesions 1–2 cm long, some sporulation
Race 4		
W126	Small (≤1 mm) water soaked spots (=1)	No macroscopic symptoms
D	Large coalescing lesions, stems killed (=5)	Stem collapse, no dark necrosis, profuse sporulation
WA647	As for W126 (=1)	As for W126

[a] Disease rating as described in Mackie and Irwin (1998); 1 is highly resistant and 5 is susceptible

susceptible = stem collapse and profuse sporulation without dark brown necrosis.

The F_1 individual (WA647) used to generate the BC population and each BC individual were confirmed as resulting from a cross by studying the parents and their DNA banding patterns using randomly amplified polymorphic DNA (RAPDs). All individual plants [parents, F_1 individual (WA647) and BC individuals] were clonally propagated from stem cuttings, so different clonal propagules of the same individual were used in the different tests.

Phenotypic characterisation for reaction to *C. trifolii* races 1, 2 and 4

Spray inoculation tests

Methods employed for spray inoculation of plants involved atomising to run-off with a 1×10^6 spore ml^{-1} suspension of each isolate, incubating in a humidity chamber for 48 h at 24°C in a naturally illuminated growth chamber, then assessing after a further 7 days at 24°C and natural light conditions. The following *C. trifolii* isolates were used in the experiments: race 1 (BRIP 46151), race 2 (BRIP 46149) and race 4 (BRIP 46150), the accession details were reported in Mackie et al. (2003).

The BC population was assessed as above by the inoculation of 5–7-day-old regrowth of clonal propagules of each BC individual with the above-mentioned isolates of each race, and the clonal propagules of D, W126 and WA647 were always included as controls (Test A). Another complete set of the entire BC population (different clonal propagules to those used in the first test) was inoculated as described above (Test B). The final rating assigned to each individual was the highest score recorded in the two tests (pooled).

Disease reactions of plants were assessed using the previously described 1–5 rating system.

Stem injection inoculation tests

Inoculation by stem injection, as described by Ostazeski and Elgin (1982) and Mackie et al. (2003), was performed on the 7-day-old regrowth of cuttings of the BC population. At least two stems were tested on each BC individual for each isolate of races 1, 2 and 4, with at least four injection sites per stem, spaced in the centre of the inter-nodal regions over the length of the stem (Mackie and Irwin 1998). These tests were repeated four times, on different propagules, for each race/BC individual combination. The parents (W126 and D) and WA647 were included as controls in all the tests. In all the cases, the plants gave either a resistant (no macroscopic symptoms) or susceptible (stem collapse and profuse sporulation) reaction, allowing the data for the BC population to be treated as a binary marker in map construction.

To test for linkage between reactions to the different races, Chi-square tests of association were performed.

Mapping and QTL analysis

DNA extraction, amplified fragment length polymorphism (AFLP) and SSR analysis were as reported in Musial et al.

(2007) where this same BC population was also mapped for resistance to *Stagonospora meliloti*. The mapping population consisted of W126, D, WA647 and 145 BC individuals as used in the phenotyping experiments. DNA marker analysis, segregation analysis, map construction and QTL analysis were also as reported in Musial et al. (2007). DNA markers, which were present as a single band in W126 and WA647, but absent from D, were identified and genotyped in the BC population. Stem injection inoculation data, which was generated by rating BC plants as either resistant or susceptible to each race, was also used in map construction, as for DNA markers.

Results

Segregation analysis of phenotypic data for reaction to races 1, 2 and 4 of *C. trifolii*

Spray inoculation tests

The majority of the BC population was resistant to spray inoculation with races 1 and 4 (73 and 72% respectively), while only 37% of the same population was resistant to race 2 (Table 2). Further analysis of the reaction of individual BC plants to each of the three races (Fig. 1) indicated linkage between reaction to races 1 and 4 (69 plants resistant to both races) ($X^2 = 31.9$, $P < 0.0001$), whereas only 34 and 27 individual BC plants were resistant to races 1 and 2, and races 2 and 4, respectively; no further linkages were detected.

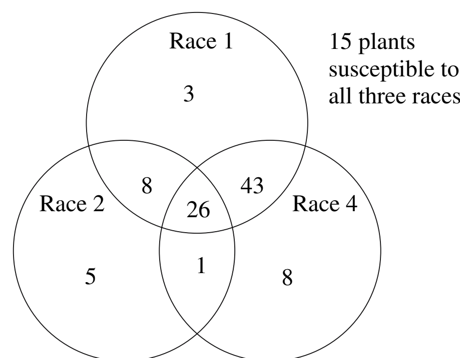

Fig. 1 A visual representation of the relationship between resistant and susceptible reactions of each plant to spray inoculation with each of the three races of *Colletotrichum trifolii* in an autotetraploid lucerne (*Medicago sativa*) backcross (*BC*) population (W126 × D) × D ($n = 109$). Numbers contained within *a circle* indicate resistant plants to that race

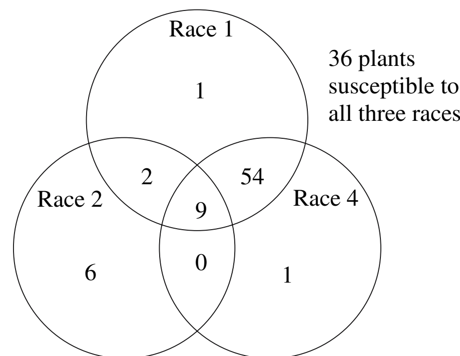

Fig. 2 A visual representation of the relationship between resistant and susceptible reactions of each plant to stem injection inoculation with each of the three races of *C. trifolii* in an autotetraploid lucerne (*M. sativa*) BC population (W126 × D) × D ($n = 109$). Numbers contained within a *circle* indicate resistant plants to that race

Stem injection inoculation tests

The same BC individuals (but different clonal propagules) that were assessed by spray inoculation were also assessed by stem injection inoculation (Fig. 2). A majority of the BC individuals were resistant to races 1 and 4, and susceptible to race 2, as observed for the spray inoculation (Fig. 1), which indicated a linkage between reactions to races 1 and 4 ($X^2 = 93.16$, $P < 0.0001$). No linkage was detected between reactions to races 1 and 2 or races 2 and 4.

Stem injection inoculation data for race 1 and race 4 approximated a 1:1 (R:S) distribution and were included in the map as phenotypic markers (C.t. 1 stem and C.t. 4 stem). Stem injection inoculation data for race 2 did not fit either a 1:1 or a 5:1 distribution, so could not be included in the generation of the map.

Table 2 Segregation for reaction to *C. trifolii* races 1, 2 and 4 following spray inoculation of a backcross (BC) population [(W126 × D) = WA647] × D, where WA647 is a single resistant F_1 plant from the cross W126 × D

Race	Number of plants in each disease class[a]				
	1	2	3	4	5
1	28	52	18	11	0
2	3	37	24	35	10
4	38	40	13	16	2

[a] Classes 1 and 2 are resistant, classes 3–5 are susceptible

Comparison of spray versus injection inoculation response on the backcross mapping population

The reaction to spray and injection inoculation of 109 individual BC plants was determined for each of races 1, 2 and 4, and 66 out of 109 individuals gave a consistent response (resistant or susceptible) to both inoculation methods across the three races (data not shown). A total of 14 plants gave non-agreement between spray and stem injection with race

1, and 25 and 16 plants gave non-agreement between spray and stem injection with races 2 and 4, respectively. In all of these cases except two, these plants reacted to give a resistant response to spray inoculation and a susceptible response to stem injection of a given race. Overall, more plants were resistant to spray inoculation than stem injection across the three races (spray: stem, race 1, 80:66; race 2, 40:17; race 4, 78:64) (Figs. 1, 2).

QTL analysis

Significant QTL ($P < 0.01$), from among the 232 DNA and stem injection inoculation phenotypic markers, for the pooled spray inoculation disease reaction data for each race are listed in Table 3. Disease reaction distributions for the BC population, from the repeat spray inoculations with each race of *C. trifolii*, were not significantly different from normal; this was confirmed using the Ryan–Joiner Normality test (data not shown) (Minitab Release 13) and indicates that *C. trifolii* reaction to spray inoculation is a quantitative trait. Data from the repeat inoculations were also subject to correlation analyses and in all cases they were greater than 0.973 ($P = 0.00$), demonstrating the consistency between the inoculation tests. The number of BC individuals for each test varied from 121 to 143. The number of plants in each test varied depending on how many clones had suitable regrowth at the time of inoculation.

Disease reaction data from the two inoculations were analysed separately for QTL analysis for the two tests (test A and test B) and also for pooled data for each race. Regression analysis ($P < 0.01$) and interval mapping using the three sets of disease data (test A, test B and pooled) for each race revealed 42 markers (not including C.t. 1 stem and C.t. 4 stem) that were significantly associated with spray reaction to *C. trifolii* (data not shown), with 30 markers detected for the pooled data sets (Table 3). The pooled data detected the same regions as that of the individual tests for each race, and only this data is presented. Three major QTL were identified on linkage groups 4, 6 and 8 (Fig. 3). Not considering the phenotypic markers, marker CCTA17 on linkage group 8 explained the largest amount of variation, ranging from 32 to 40% for races 1 and 4, respectively, which is consistent with the linkage observed for the phenotypic reaction to these two races. In addition, the QTL identified on linkage group 6 explained 7% of the variation and was again detected for both race 1 and race 4. The QTL identified on linkage group 4 explained the largest proportion of the variation for the reaction to race 2 (28%), and this marker also explained 8% of the phenotypic variation for reaction to race 1, but was not identified for race 4. The unlinked marker, CGCAA5, was consistently identified for races 1 and 4. The remaining markers explained 5–21% of the phenotypic variation to the three races, and both positive and negative additive effects were identified as inherited from the resistant parent W126. Permutation testing allowed the confirmation of highly significant ($P < 0.001$) QTL that described large effects (>11% phenotypic variation); the threshold ranged from LOD 2.67 to 3.07 for the different races. The remaining small effect QTL were identified at the suggestive level.

The markers associated with positive additive effects were analysed to determine the most likely genotype at the QTL. The simplex markers 29h4a, CACG14, MtB130 and CCCC10 did not fit any of the phenotype/genotype ratios expected for a simplex marker linked in coupling with a susceptibility allele, or a simplex marker linked in repulsion with a resistance allele. In no instance did the genotype correspond completely with the phenotype (marker present; all susceptible and marker absent (assumed), all resistant to the relevant *C. trifolii* race). Instead, the presence of the marker was associated with an increased proportion of susceptible individuals (data not shown). The proposed marker–QTL phase for markers MtB130, CCCC10 (linkage group 6, race 4) and CACG14 (linkage group 8, race 1) fits the phase of markers associated with QTL for resistance; one homologue of the linkage group contains these listed markers linked to QTL for susceptibility and the other homologue contains markers linked to QTL for resistance. Marker 29h4a is located in linkage group 5, which has no other QTL for *C. trifolii* race 1 reaction.

The simplex marker ACTG15, associated with a positive additive effect for race 2 resistance, has genotype/phenotype ratios that best fit a model of the resistance allele in repulsion to the marker ($X^2 = 2.45$, $P = 0.1463$ for marker presence). This marker is unlinked, which limits further analysis of the marker—QTL phase for the race 4 resistance, also associated with this marker. The simplex marker CCAGA3 also best fits the resistance allele in the repulsion model ($X^2 = 3.36$, $P = 0.0949$ for marker absence). It is located on linkage group 7, which has no other QTL for *C. trifolii* reaction.

The duplex marker 2CGCC5 is closest to fitting a model for a duplex marker linked to a simplex susceptibility allele. The alternate model, a duplex marker linked to a duplex susceptibility allele is farthest from fitting the gentoype/phentoype ratios observed (data not shown). This model of a duplex marker linked to a simplex susceptibility allele fits the marker—QTL phase for *C. trifolii* race 4 reaction observed on linkage group 6, where one homologue contains markers linked to resistance QTL and the other homologue contains markers linked to susceptibility QTL (MtB130 and CCCC10, identified above).

For race 1, six QTL were identified, which explained 5–41% of the phenotypic variation (Table 3). Most of the QTL identified increased resistance, except for 29h4a and CACG14, which increased susceptibility. The strongest

Table 3 Markers for resistance and susceptibility to *C. trifolii* identified in the backcross mapping population (W126 × D) × D at $P < 0.01$ following spray inoculation

Test[a]	Linkage group	Marker	Estimated additive effect[b]	Percentage of total variance	*P*
Race 1	Unlinked	CGCAA5	−0.78	19	0
	3	MTIC51	−0.39	6	0.00483
	4	CATG12	−0.49	8	0.00085
	4	CCCCA4	−0.42	6	0.00685
	4	CCTG3	−0.44	6	0.00377
	5	29h4a	0.4	5	0.00612
	6	CCCG13	−0.44	7	0.00253
	8	C.t. 1 stem	−1.1	41	0
	8	C.t. 4 stem	−1.01	35	0
	8	CGTCC11	−0.96	30	0
	8	CCTA17	−0.98	32	0
	8	CACG14	0.42	6	0.00646
	8	115m15b	−0.64	14	0.00001
	8	CTTG1	−0.44	6	0.00293
	8	CCCG20	−0.44	7	0.00262
Race 2	Unlinked	ACTG15	0.38	5	0.00861
	Unlinked	4E04	−0.5	6	0.00344
	4	CGTCC6	−0.83	15	0
	4	CATG12	−1.11	28	0
	4	CCCCA4	−1.05	25	0
	4	CCTT9	−0.8	14	0.00001
	4	ACCG6	−0.77	14	0.00001
	4	CGCC7	−0.82	16	0
	4	2AGAGC10	−0.61	8	0.00046
	4	2CCTC5	−0.72	12	0.00002
	4	CGTCC8	−0.86	15	0
	4	CTTT12	−0.59	8	0.00066
	4	CCAGG5	−0.7	11	0.00019
	7	CCAGA3	0.5	5	0.00726
Race 4	Unlinked	ACTG15	−0.74	16	0
	Unlinked	CGCAA5	−1.03	21	0
	6	MtB130	0.51	5	0.00732
	6	2CGCC5	0.71	7	0.00145
	6	CCCG13	−0.56	6	0.00258
	6	CCCC10	0.5	5	0.00936
	8	C.t. 1 stem	−1.37	39	0
	8	C.t. 4 stem	−1.4	40	0
	8	CGTCC11	−1.29	34	0
	8	CCTA17	−1.39	40	0
	8	2CCTA15	−0.79	11	0.00008
	8	CTTT5	−0.68	9	0.00028
	8	115m15b	−0.75	12	0.00004
	8	CTTG1	−0.65	9	0.00045
	8	CCCG20	−0.6	7	0.00125

[a] Pooled data set shown

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

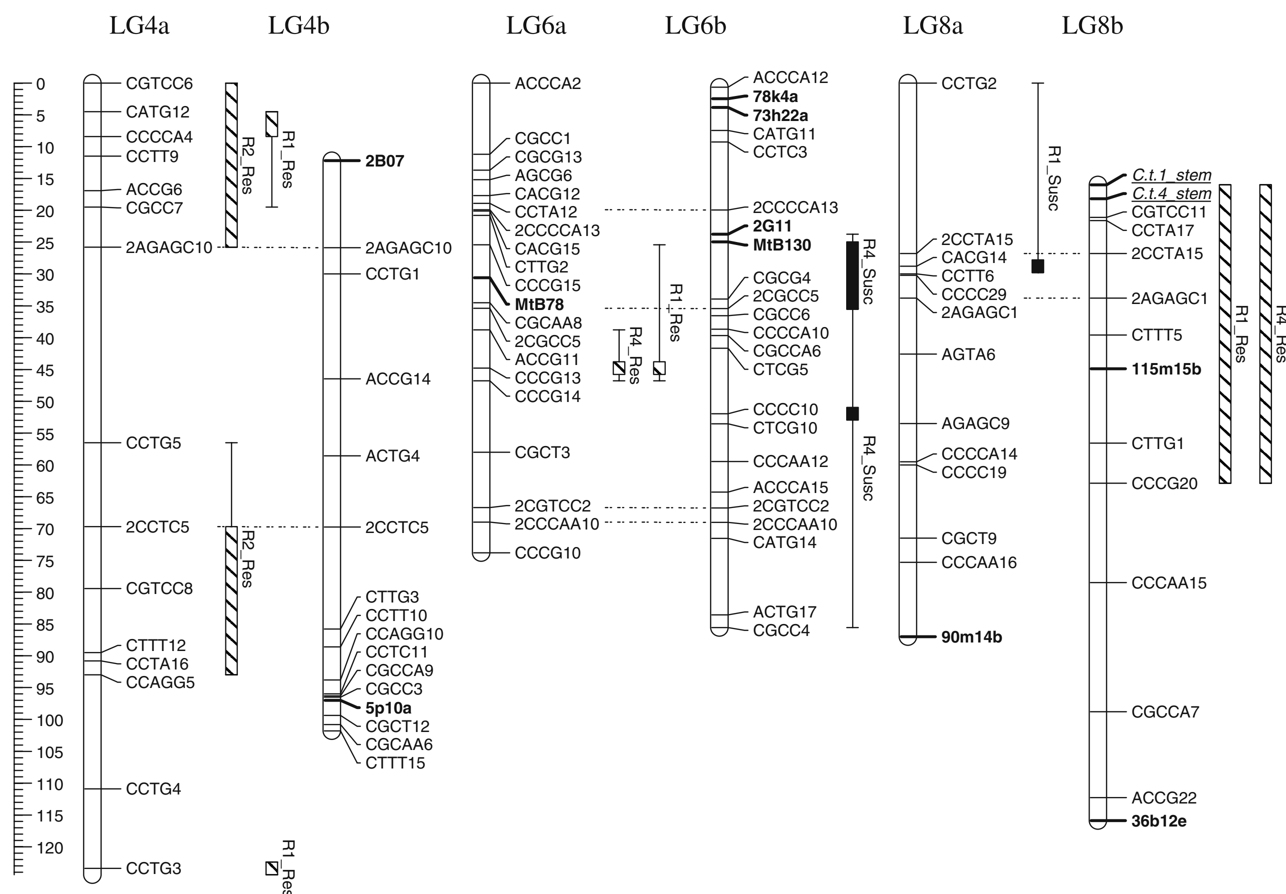

Fig. 3 Location of QTL associated with reaction to *C. trifolii* in a tetraploid lucerne (*M. sativa*) linkage map generated from the BC population, (W126 × D) × D, using amplified fragment length polymorphism (*AFLP*), simple sequence repeat (*SSR*) and phenotypic markers. *Vertical bars* represent the linkage groups, and homologues are together with their corresponding *M. truncatula* linkage group number. *Horizontal lines* show marker positions. Genetic distances (cM) are located on the left of the figure and locus names are listed to the right of each homologue. Markers with a '2' preceding the loci name are duplex markers (5:1) and linkage between homologues is indicated by a *dotted line*. 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*, further markers up to $P < 0.05$ are indicated by *whisker*. Races are indicated next to the QTL

QTL for increased resistance was located on linkage group 8 and was closely linked (QTL peak at C.t. 1 stem) with the two phenotypic markers (C.t. 1 stem and C.t. 4 stem). When these six QTL were combined in a multiple regression model, they explained 57% of the phenotypic variation. The markers were tested for interaction effects and a significant interaction was detected between markers on linkage groups 4 (CATG12) and 8 (C.t. 4 stem). When this effect was added to the model, a total of 63% of the phenotypic variation was explained.

For race 2, four QTL were identified, which explained 5–28% of the phenotypic variation (Table 3). These included one of the QTL on linkage group 4, which was also identified with race 1. Two of the QTL identified were associated with resistance in repulsion; these were located on linkage group 7 and an unlinked marker (ACTG15). When all QTL were included in the model, 46% of the phenotypic variation was explained. Again, a significant interaction was detected between markers on linkage group 3 (AGTA7) (not previously identified as a QTL) and the QTL located on linkage group 4 (CATG12), and when this was included in the model, a total of 55% of the phenotypic variation was explained.

For race 4, four QTL were identified, which individually explained 5–40% of the phenotypic variation, and when combined in a model explained 48% of the phenotypic variation. Similarly as for race 1, the strongest QTL for increased resistance was located on linkage group 8 and was closely linked (QTL peak 1.8 cM from C.t. 4 stem) to the two phenotypic markers (C.t. 1 stem and C.t. 4 stem). A significant interaction was detected between markers on linkage groups 3 (CGCC8) (not previously identified as a QTL) and 8 (C.t. 4 stem), and when this was included in the model 52% of the variation was explained.

Discussion

This is the first report on QTL for reaction to all three known races of *C. trifolii* in *M. sativa*. Past research was based on the phenotype alone, where authors could only infer the genotype. Segregation ratios for disease reactions in the BC population that we have studied suggest that resistance to *C. trifolii* races 1 and 4 in clone W126 is incompletely dominant and controlled by more than one gene. Three QTL on linkage groups 6 and 8 and an unlinked marker were contributing to resistance to both races 1 and 4, which concurred with the phenotypic data, indicating linkage between resistances to races 1 and 4. This was in contrast to resistance to race 2 in W126, which was incompletely recessive and independent of resistance to races 1 and 4. Interestingly, the unlinked marker ACTG15 was associated with reaction to both races 2 and 4, but was linked to QTL in opposite phases. The presence of this marker was linked in repulsion to resistance to race 2, but was linked in coupling to resistance to race 4. Significant interactions between markers on different linkage groups were identified for resistances to all three races, providing further evidence of the importance of complementary gene interactions in autotetraploid lucerne, as outlined by Bingham et al. (1994). These findings indicate that resistance in the clone under study was controlled by more complex genetic mechanisms than the completely dominant An_1 and An_2 genes identified by Elgin and Ostazeski (1985) in Arc and Saranac AR in the USA, An_1 conferring resistance to race 1 and An_2 conferring resistance to races 1 and 2. Also, in contrast to An_2, plants resistant to race 2 in our study were not always resistant to race 1.

In addition to the previously described An_1 and An_2 resistance genes, several authors have suggested the presence of other genetic mechanisms controlling resistance in lucerne to *C. trifolii*. Collins (1974) found anthracnose reaction to be inherited as an incompletely dominant trait, but resistance could not be explained by the segregation of a single tetrasomic gene. This is a similar situation to our observations of inheritance of reactions to races 1 and 4 by W126. Guy (1976) reported that the inheritance of resistance to *C. trifolii* in 20 lucerne varieties of diverse origin could be explained by one incompletely dominant gene, which had variable phenotypic expression. Elgin and Ostazeski (1985) consistently identified low levels of resistant plants that they did not expect in inoculations with either race 1 or race 2, based on their proposed single completely dominant gene model. Grau et al. (1989) suggested that an adult plant resistance mechanism was acting in some varieties, which were resistant in the field, but susceptible when inoculated as seedlings. This latter resistance appears to have many similarities to the quantitatively expressed incompletely recessive resistance we have identified in clone W116 (Irwin et al. 2006). O'Neill (1996) postulated that the variation they observed in disease response of diverse germplasm to isolates of race 2 suggested that a more complex resistance mechanism may be involved than the An_1 and An_2 genes.

The QTL data indicates that while races 1 and 4 share three putative regions, which contribute to resistance, race 1 and 2 only share one region. An unlinked marker, ACTG15, was linked in repulsion to resistance to races 2 and 4. Based on our results, the QTL identified for resistance to race 1 and race 4, on linkage group 8, could represent the previously described An_1 gene. The QTL for resistance to race 1 and race 2, on linkage group 4, could represent An_2. The additional QTL that we have identified help to explain the different modes of inheritance of resistance to the three races of *C. trifolii* seen here in the clone W126 from cv. Trifecta. Development of markers closely linked to QTL for resistance into sequence characterised amplified region (SCAR) markers will allow them to be used in Australian lucerne breeding. The use of marker-assisted selection (MAS) is currently proceeding for anthracnose resistance in lupins, where a molecular marker 2.3 cM away from a resistance gene has been implemented in the national breeding programme (You et al. 2005).

The mapping of fungal pathogen resistance specificities to linked loci has been observed in the interaction between *Phaseolus vulgaris* and *C. lindemuthianum*, which is closely related to *C. trifolii* (Sherriff et al. 1994). *C. lindemuthianum* resistance gene specificities mapped to one end of bean linkage group B4, and subsequent mapping of QTL for resistance, nucleotide binding site-leucine rich repeat (NBS-LRR) resistance gene analogues (RGAs) and expressed NBS-LRR resistance gene candidates (RGCs) to the same region, confirmed the presence of a resistance gene cluster (Geffroy et al. 2000; Ferrier-Cana et al. 2003). Phylogenetic studies on *M. sativa* RGAs found a number of putative RGAs that clustered with a candidate anthracnose resistance gene from common bean (Cordero and Skinner 2002). We expect that further analysis, including fine structural mapping, will reveal a similar cluster of resistance genes co-localising with the major, race-specific, *C. trifolii* resistance QTL on *M. sativa* linkage group 8. The co-localisation of other major resistance QTL to *C. trifolii* races 1 and 2 on linkage group 4 suggests the presence of a second resistance gene cluster involved in *C. trifolii* resistance in *M. sativa*. Numerous, small effect QTL for resistance were also identified on other *P. vulgaris* linkage groups and several of these mapped to genomic regions containing functional genes involved in the defence response (Geffroy et al. 2000). It is possible that the QTL we have identified for resistance to *C. trifolii* races 1 and 4 on linkage group 6, and race 2 on linkage group 4, have similarly mapped to genomic regions containing genes involved in the defence response; further mapping is likely to reveal any co-

localisations. Another genomic region that these QTL may identify is the additional resistance gene cluster, contributing to *C. trifolii* reaction. The phylogeny and genome organisation of NBS-LRR sequences in *M. truncatula* have been recently described (Zhu et al. 2002). Clusters of genomic and expressed sequence tag- (EST) derived NBS-LRR sequences were identified on most *M. truncatula* linkage groups, including those homologous to *M. sativa* linkage groups identified in our map as containing QTL for *C. trifolii* reaction. Comparison of homologous linkage groups aligned with common SSR markers suggests that there are RGA clusters in similar genomic regions on *M. truncatula* as our QTL for *C. trifolii* reaction in *M. sativa*.

This study also allowed a direct comparison of the reaction of individual BC plants to spray and injection inoculation with races 1, 2 and 4. When the injection inoculation results were mapped as a binary marker (C.t. 1 stem and C.t. 4 stem), they explained approximately 40% of the phenotypic variation in response to spray inoculation, mapping to a linkage group homologous to *M. truncatula* linkage group 8. Stem injection inoculation is a qualitative reaction and could be due to specific virulence/avirulence (Dickman et al. 2003) without the interacting responses from other genes associated with pre-penetration and penetration events, which occur on and in epidermal cells. Based on a study of six clones, Ostazeski and Elgin (1982) reported almost complete agreement between a clone's response to spray and injection inoculation with either race 1 or race 2 of *C. trifolii*, which contrasts with our findings. The differences may be due to the different genetic backgrounds (dormant vs. non-dormant) of the clones researched in the two studies and their different inheritance patterns, as discussed above. There are obvious differences between the infection courts utilised in spray and stem injection inoculation with pre- and post-penetration events, which occur on and in the epidermal cells being by-passed with injection inoculation. Churchill et al. (1988) showed that the expression of resistance to *C. trifolii* occurs near the time of epidermal cell wall penetration following penetration of the cuticle and epidermal cell by the penetration peg, originating from an appressorium; this sequence of infection processes does not differ for races of *C. trifolii* (Mould and Robb 1992). With stem injection inoculation, the barriers presented by the cuticle and epidermal cells are by-passed, and depending on the mechanism(s) by which resistance is manifested, it is conceivable that a plant may respond differently to the two inoculation methods. In our experiments, more plants were resistant to spray inoculation than stem injection, across the three races, indicating QTL expressed in epidermal cells may have an important role in conditioning field resistance. Work is needed to further elucidate the different mechanisms that are acting in response to the two inoculation methods and three races that we have employed in our research.

In *M. truncatula*, resistance to spray inoculation of detached leaves with race 1 *C. trifolii* has been described as dominant, although the observed segregations were not consistent with those of a single completely dominant Mendelian gene (Torregrosa et al. 2004). O'Neill and Bauchan (2000) studied the histopathology of *M. truncatula* response to race 1 and found that resistant reactions were similar to those found in *M. sativa*. Detailed genetic mapping of these resistances will be required to unequivocally resolve these inheritances, including those reported here for reaction to races 1, 2 and 4 in clone W126. The use of additional *M. truncatula* molecular markers will allow further study of these major QTL for resistance in the model system, perhaps assisting in the search for resistance genes. However, no resistance gene to any *Colletotrichum* sp. has yet been cloned (Torregrosa et al. 2004).

The QTL reported in this paper are for plant response to spray inoculation, which should also have application in the selection of clones with field resistance. This could be expected to be controlled by more than one mechanism. The molecular basis of resistance to *C. trifolii* is undetermined. Dickman et al. (2003) have isolated a lipid-induced protein kinase (LIPK) from *C. trifolii*, which is induced specifically by plant cutin. Gene replacement of LIPK yielded *C. trifolii* strains unable to develop appressoria, and unable to infect intact host tissue, although they were pathogenic following application to wounded tissue.

Although we have done our mapping in an autotetraploid, where QTL detection is more difficult than in diploids, we were able to identify large QTL, which individually explain 28–40% of the phenotypic variation for reaction to each of races 1, 2 and 4 of *C. trifolii*. It is likely that there are further undetected QTL, as the maximum phenotypic variation explained was 63% (race 1). There are however limits to detection of QTL with small effects in the sample size used (Gallais 2003). Additional fine structural and comparative mapping around the major QTL identified will further clarify the genetic control of anthracnose reaction in lucerne clone W126. These markers will have value in breeding lucerne carrying multiple sources of resistance to the three known races of *C. trifolii*.

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