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Construction of a linkage map based on a *Lathyrus sativus* backcross population and preliminary investigation of QTLs associated with resistance to ascochyta blight

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Abstract A linkage map of the *Lathyrus sativus* genome was constructed using 92 backcross individuals derived from a cross between an accession resistant (ATC 80878) to ascochyta blight caused by *Mycosphaerella pinodes* and a susceptible accession (ATC 80407). A total of 64 markers were mapped on the backcross population, including 47 RAPD, seven sequence-tagged microsatellite site and 13 STS/CAPS markers. The map comprised nine linkage groups, covered a map distance of 803.1 cM, and the average spacing between markers was 15.8 cM. Quantitative trait loci (QTL) associated with ascochyta blight resistance were detected using single-point analysis and simple and composite interval mapping. The backcross population was evaluated for stem resistance in temperature-controlled growth room trials. One significant QTL, QTL1, was located on linkage group 1 and explained 12% of the phenotypic variation in the backcross population. A second suggestive QTL, QTL2, was detected on linkage group 2 and accounted for 9% of the trait variation. The *L. sativus* R-QTL regions detected may be targeted for future intergenus transfer of the trait into accessions of the closely related species *Pisum sativum*.

Introduction

Ascochyta blight, primarily caused by *Mycosphaerella pinodes* (Berk. and Blox.) Vestergren in Australia is a major constraint to the production of field pea (*Pisum sativum* L.). The pathogen affects all aerial parts of the plant, causing leaf and pod spots and stem girdling on the mature plant and foot rot on seedlings (Lawyer 1984), thus contributing to a reduction of seed quality and yield. Yield losses as high as 40% have been reported in infected field pea crops (Bretag et al. 1995; Tivoli et al. 1996); however, estimated yield losses are generally in the order of 20–30% in southern Australia (Bretag et al. 1995).

Field pea cultivars vary in their reaction to infection by *M. pinodes*; however, complete resistance to infection has not been observed (Wroth 1998). Primitive *P. sativum* accessions and *P. fulvum* have been identified as sources of resistance; however, conflicting reports with regard to this resistance have been made (Clulow et al. 1991; Bretag 1991; Wroth 1996; Gurung et al. 2002). Conversely, many accessions of *Lathyrus sativus* L., commonly known as grasspea or chickling pea, were shown to be highly resistant to *M. pinodes* (Weimer 1947; Gurung et al. 2002). Since *L. sativus* is also a member of the Viciae tribe along with *Pisum*, it may serve as a potential source of resistance genes, which in the future may be incorporated into resistance breeding programs for field pea. Although intergeneric hybridisation barriers exist between *Pisum* and *Lathyrus* that prevent the transfer of resistance genes, using conventional methods (Weimer 1947), successful fusion of *P. sativum* and *L. sativus* protoplasts via chemical fusion methods was reported by Durieu and Ochatt (2000). With more advanced techniques that facilitate the transfer of foreign DNA, such as cloning of resistance genes and transformation, the transfer of ascochyta blight resistance genes from the genus *Lathyrus* to *P. sativum* has renewed potential.

Prior to initiating advanced gene transfer techniques, the effect and chromosomal location(s) of the genes

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conditioning *M. pinodes* resistance in *L. sativus* must be determined. For this, the development of a molecular genome linkage map and the localisation of major resistance loci are necessary. Quantitative trait loci (QTL) mapping of disease resistance loci facilitates the use of molecular approaches to understanding the nature of disease resistance QTLs and may eventually lead to the subsequent manipulation of resistance genes by the cloning of the underlying genes. Furthermore, identifying molecular markers closely linked to resistance genes, which may be utilised via marker-assisted selection and map-based cloning, will serve as tools for precise and speedier future breeding programs.

In Australia, resistance in *P. sativum* to *M. pinodes* was shown to be under polygenic control and therefore conditioned by QTL, which may be heavily influenced by the environment (Wroth 1999). QTL mapping was previously used to determine the chromosomal loci of mechanisms involved in multiple gene resistance to fungal pathogens in field pea (Dirlewanger et al. 1994; Timmerman-Vaughan et al. 2002) and chickpea (Santra et al. 2000; Flandez-Galvez et al. 2003). Three QTLs were shown to confer resistance to *Ascochyta pisi* race C in pea, using RFLP markers (Dirlewanger et al. 1994). Thirteen QTLs were detected for partial resistance to field epidemics of ascochyta blight in pea (Timmerman-Vaughan et al. 2002). Santra et al. (2000) detected two QTLs conferring resistance to ascochyta blight in chickpea, and at least two QTLs were also identified for seedling resistance in *Cicer echinospermum*, a wild relative of chickpea (Collard et al. 2003).

At present, little is known about the genetic factors controlling resistance to *M. pinodes* of *L. sativus*. There have been no reports of QTL studies on *L. sativus*, in particular using QTL analysis to detect genes associated with resistance to *M. pinodes*. Therefore, the aims of this study were to: (1) construct a linkage map based on a backcross population generated from an inbred resistant and a susceptible *L. sativus* accession and (2) determine the location and effect of QTLs associated with resistance to *M. pinodes*.

Materials and methods

Mapping population

The F₁ individuals were derived from a cross between one resistant plant of *L. sativus* accession (ATC 80878) and one susceptible plant of *L. sativus* accession (ATC 80407). The phenotype of these accessions was previously determined in temperature-controlled growth-room bioassays (Skiba 2003). Seeds of parental lines were obtained from the Australian Temperate Field Crop Collection (ATFCC), Horsham, VIC, Australia, and were inbred prior to crossing. A backcross mapping population was produced by crossing true F₁ individuals with resistant accession ATC 80878. All plants used for seed production were grown in the

glasshouse facility at RMIT University, Bundoora, VIC, Australia.

DNA extraction and molecular-marker analysis

DNA was extracted from young leaf tissue from the two parental plants, F₁ and backcross plants, following a modified CTAB method described by Taylor et al. (1995). RAPD and field pea-derived sequence tagged microsatellite site (STMS) primers were screened via PCR to detect polymorphisms between the two parental accessions. Sequences for the field pea STMS primers were as reported by Ford et al. (2002). STS and CAPS markers developed from defence-related expressed sequence tags (EST) sequences generated from a *L. sativus* cDNA library were also screened (Skiba et al. 2003). Only reproducible and clearly resolvable amplification products were selected for mapping in the backcross population.

All PCR reactions contained 40 ng template DNA within a total volume of 25 µl and were performed in a Thermo Hybrid PCR Express Thermal Cycler. For RAPD analysis, reaction mixtures contained PCR buffer (10 mM Tris-HCl, 50 mM KCl, 0.1 mg ml⁻¹ gelatin, pH 8.3), 2.5 mM MgCl₂, 0.24 mM each dNTP, 0.5 U *Taq* DNA polymerase (Invitrogen, Australia) and 0.24 µM primer. PCR amplification using RAPD primers was performed as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 15 s, annealing at 40°C for 40 s and elongation at 72°C for 1 min. The final extension step was held at 72°C for 5 min.

For STMS analysis, the PCR reaction mixture was identical to the RAPD method, except that 0.4 µM of both the forward and reverse primers were included in each reaction. PCR amplification was performed following the protocol of Ford et al. (2002), with minor modifications as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 30 s and elongation at 72°C for 1 min. The final extension step was held at 72°C for 5 min.

The STSs were amplified from genomic DNA using STS primer pairs developed by Skiba et al. (2003). PCR assays were carried out as described above. Standard amplification conditions were initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 41–65°C for 1 min, 72°C for 1 min and a final extension step was held at 72°C for 5 min. The optimal annealing temperature for each primer pair was based on the ability to amplify a single fragment/product from genomic DNA (Skiba et al. 2003). PCR products were digested with a range of restriction endonucleases to detect polymorphisms (Skiba et al. 2003).

All PCR amplification products were separated on 1.5% agarose gel in 1× TBE buffer, stained with ethidium bromide, visualised under UV light and recorded using the Gel Doc system (Bio-Rad, Australia). Each marker was tested for the expected 1:1 segregation ratio in the backcross population using a chi-square test

($P < 0.05$). Markers that showed distorted segregation ($P > 0.05$) were not used in the map construction.

Marker nomenclature

Each RAPD and STMS molecular marker was given a two-part name consisting of the name of the primer used and the approximate size of the marker in base pairs. For RAPD markers, the first part corresponded to the primer used (one or two letters followed by a two-digit number), followed by the approximate size of the band in base pairs. STMS markers were named according to the primers described by Ford et al. (2002), followed by the approximate band size. For markers detected using the STS primers, an abbreviation of the EST name was used (Table 1).

Map construction

A linkage map of the ATC 80878×ATC 80407 backcross population was constructed using MapManager QTX (Manly et al. 2001). Markers were assigned to linkage groups using the *Make linkage groups* command at $P = 0.001$, which was equivalent to a LOD score of 3. Map distances were calculated in centiMorgans, using the Kosambi function. The *Ripple* command was used to scrutinise marker order.

Inoculation of plants and phenotypic evaluation

Eighteen 14-day-old parental and 92 backcross plants were inoculated with a mixed spore suspension con-

taining equal spore numbers of three virulent and single-spored *M. pinodes* isolates: WAL3, T16 and 4.9 from Walpeup Victoria, Tasmania, and Tammin Western Australia, respectively. These isolates were previously found to be highly aggressive on a range of field pea accessions (Ford et al. 1999). The inoculum was prepared, and the plants were artificially inoculated following procedures described by Skiba and Pang (2003), except that plants were grown and incubated in a temperature-controlled growth room at 22°C, with a 16-h photoperiod (260 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Leaves from the tops of all plants were removed for DNA extraction 6 days before inoculation.

Disease severity was assessed on parental and backcross plants at 14 days post inoculation. The percentage stem area with lesions (%SL) was determined using a modification of the disease assessment key by James (1971), as used by Gurung et al. (2002).

QTL detection

The stem infection data (%SL) and the linkage map were used to detect QTLs for stem resistance to ascochyta blight at the seedling stage using three methods: single-point analysis, simple interval mapping and composite interval mapping. Each method was performed using MapManager QTX. The significance of each potential association between a marker and a QTL was measured by a likelihood ratio statistic (LRS). The LRS may be converted to the conventional base-10 LOD score by dividing by 4.61 (twice the natural logarithm of 10, Haley and Knott 1992). Markers were considered to be associated with a putative QTL for ascochyta blight if they exceeded an LRS threshold of 9.22 (equivalent to LOD 2).

Single-point analysis was performed on all loci on the linkage map, using the *Links report* command in MapManager QTX. The effect of a QTL was estimated by the difference between the total trait variance and the residual variance. Expressed as a percentage of the total variance, the effect was used to estimate the percentage of phenotypic variation explained by a marker locus.

For QTL detection, simple interval mapping did not control for the effects of potential other/background QTLs, whereas composite mapping did so by reducing or controlling the effects of background loci of other QTLs. All QTL scans were performed on map intervals of 1 cM. Empirical determination of experiment-wise error rates was performed with a permutation test in MapManager QTX, with 1,000 permutations of trait data. This method was used to establish the significance of the LRS generated by the interval mapping procedure, which gave ~95% confidence on the location of the QTLs based on the marker intervals (Churchill and Doerge 1994). In order to detect significant interactions between any QTLs detected, a general linear model (GLM) analysis was conducted using Minitab (Minitab, State College, Penn., USA), release 11.2.

Table 1 Abbreviation of expressed sequence tags (ESTs) used as marker names for STSs

STS primer no.	EST name/ nucleotide match	Abbreviation/ marker name
24	Disease resistance response protein DRRG49 C	DRRG49-C
59	Disease resistance response protein 39 precursor	DRRP-39
81	Pathogenesis-related protein 4A	PR-4a
159	Beta-glucan-binding protein	B-GlucBP
304	Cutinase negative acting protein	Cut_Neg
342	Glutathione peroxidase	Glut_Perox
351	Lipid transfer protein	Lipid_Trans
524	S-adenosylmethionine synthetase 2	S-ade_Syn
574	Cf-9 resistance gene cluster	Cf-9
612	Laccase-like protein	Laccase
616	Putative auxin-repressed protein	Auxin-Rep
674	Putative WD-repeat protein	WD-repeat
753	TMV resistance protein homologue	TMV
761	Polygalacturonase inhibitor protein	Polygal_In
786	Multi-resistance protein	MR-P
787	6-Phosphogluconate dehydrogenase	6-Phos_dehy
792	EREBP-4	EREBP-4
896	PR-1a precursor	PR-1a
923	Defence-related peptide 1 (PSD1)	DRP-1
1005	Chalcone reductase	Chal_Re

Results

Molecular-marker analysis on backcross population

Of the 59 RAPD markers detected, 12 (20%) segregated significantly different to the expected 1:1 ratio for dominant markers in the backcross population ($P < 0.05$, Table 2). Only one of the eight STMS markers (PSMPSA7), and five of the 20 STS/CAPS markers (25%) showed significant deviation from the expected ratio (Table 2). These markers were subsequently deleted from the dataset and were not used in the construction of the linkage map. Markers generated by STS primers 786 and 792 were difficult to score in the backcross population, as they were not clearly resolved on the 1.5% agarose gels and thus could not be used for map construction.

General features of the linkage map

A total of 67 markers, including 47 RAPD, 7 STMS and 13 STS/CAPS markers, were used to construct the linkage map. Linkage analysis revealed that the three RAPD markers W19_2500, B07_2000 and B18_300 were located at the same position on the map as markers G05_480, B18_780 and A14_900, respectively, and thus were removed from the dataset. The resultant linkage map was composed of 64 markers (Fig. 1). The map comprised nine linkage groups covering 803.1 cM, and the average spacing between markers was 15.8 cM. The longest linkage group was 246.9 cM (linkage group 1)

Table 2 Chi-square test for the distribution of molecular markers that significantly deviated from the expected 1:1 ratio in the backcross population

Primer/marker	Missing data ^a	Expected frequency ^b	Observed frequency ^b	χ^2
OPA03	2	45:45	31:59	8.71*
OPA06	5	43.5:43.5	25:62	15.74*
OPA12	2	45:45	55:35	4.44**
OPG04	1	45.5:45.5	26:65	16.71*
OPG10	0	46:46	28:64	14.09*
OPG16	0	46:46	34:58	6.26**
OPG17	2	45:45	33:57	6.40**
OPN04	2	45:45	27:63	14.40*
OPP11	3	44.5:44.5	54:35	4.06**
OPW04	1	45.5:45.5	29:62	11.97*
OPX17	2	45:45	31:59	8.71*
OPAO11	0	46:46	35:57	5.26**
PSMPSA7	1	45.5:45.5	28:63	13.46*
Chitinase (58)	3	44.5:44.5	31:58	8.19*
Laccase (612)	0	46:46	35:57	5.26**
Auxin-Rep (616)	1	45.5:45.5	29:62	11.97*
WD-Repeat (674)	2	45:45	63:27	14.40*
6-Phos-dehy (787)	0	46:46	36:59	4.35**

Significance levels: * $P < 0.01$, ** $P < 0.05$

^aNumber of backcross individuals that did not amplify any products for this primer

^bMarker present:marker absent

and the shortest was 6.8 cM (linkage group 8). Four markers (B04_400, B12_700, Polygal_In and DRRP-39) remained unlinked to any linkage group at $P = 0.001$ (LOD 3.0). In an attempt to attach these unlinked markers to existing linkage groups and join smaller linkage groups together, the LOD score was reduced to 2.0 ($P = 0.01$). Subsequently, unlinked markers B04_400 and B12_700 were attached to linkage group 5 and Polygal_In was attached to group 1. Marker DRRP-39 remained unlinked and marker W18_780 was now regarded as unlinked at the lower LOD score. Linkage groups 2, 4 and 5 were connected to linkage group 3 to make a large group consisting of 30 markers, with the remaining groups 6, 7, 8 and 9 remaining unchanged.

Of the 13 STS/CAPS markers, only two could not be mapped; disease resistance response protein 39 precursor (DRRP-39) and Polygalacturonase inhibitor protein (Polygal_In). Four STS markers were located on group 1, β -glucan-binding protein (B-GlucBP), Cutinase negative acting protein (Cut_Neg), Cf-9 resistance gene cluster (Cf-9) and Defence-related peptide 1 (DRP-1). TMV resistance protein was located on group 2, Pathogenesis-related protein 4a (PR-4a) was located on group 3; no STS markers were detected on group 4. Lipid transfer protein (Lipid_trans), Disease resistance response protein DRRG49-C (DRRG49-C), Glutathione peroxidase (Glut_Perox), Chalcone reductase (Chal_Re) and S-adenosylmethionine synthetase (S-ade_Syn) were located on groups 5, 6, 7, 8 and 9 respectively.

Phenotypic evaluation

A total of 92 backcross plants were evaluated for seedling resistance. The frequency distribution of the disease reaction for the backcross population is presented in Fig. 2. Plants of the resistant parental accession (P_1) displayed disease scores ranging from 3% to 20%, whereas plants of the susceptible parental accession (P_2) produced %SL values ranging from 20% to 50%. Only three of the 92 backcross plants assayed displayed %SL values outside the parental range, i.e. 1% and 80%, therefore indicating that the case for transgressive segregation is weak. A Kolmogorov–Smirnov normality test performed on the stem infection data from the backcross mapping population indicated that it did not fit a normal distribution; therefore, the phenotype data were transformed by \log_{10} transformation prior to QTL analysis.

QTL detection for ascochyta blight resistance

Three markers (Cf-9, B04_1100, M16_500) were identified as being associated with ascochyta blight resistance, at an LRS threshold of 9.22 by single-point analysis (Table 3). These markers were located in a single, continuous region on linkage group 1 (Fig. 1). Two farther markers, positioned together on linkage group 3,

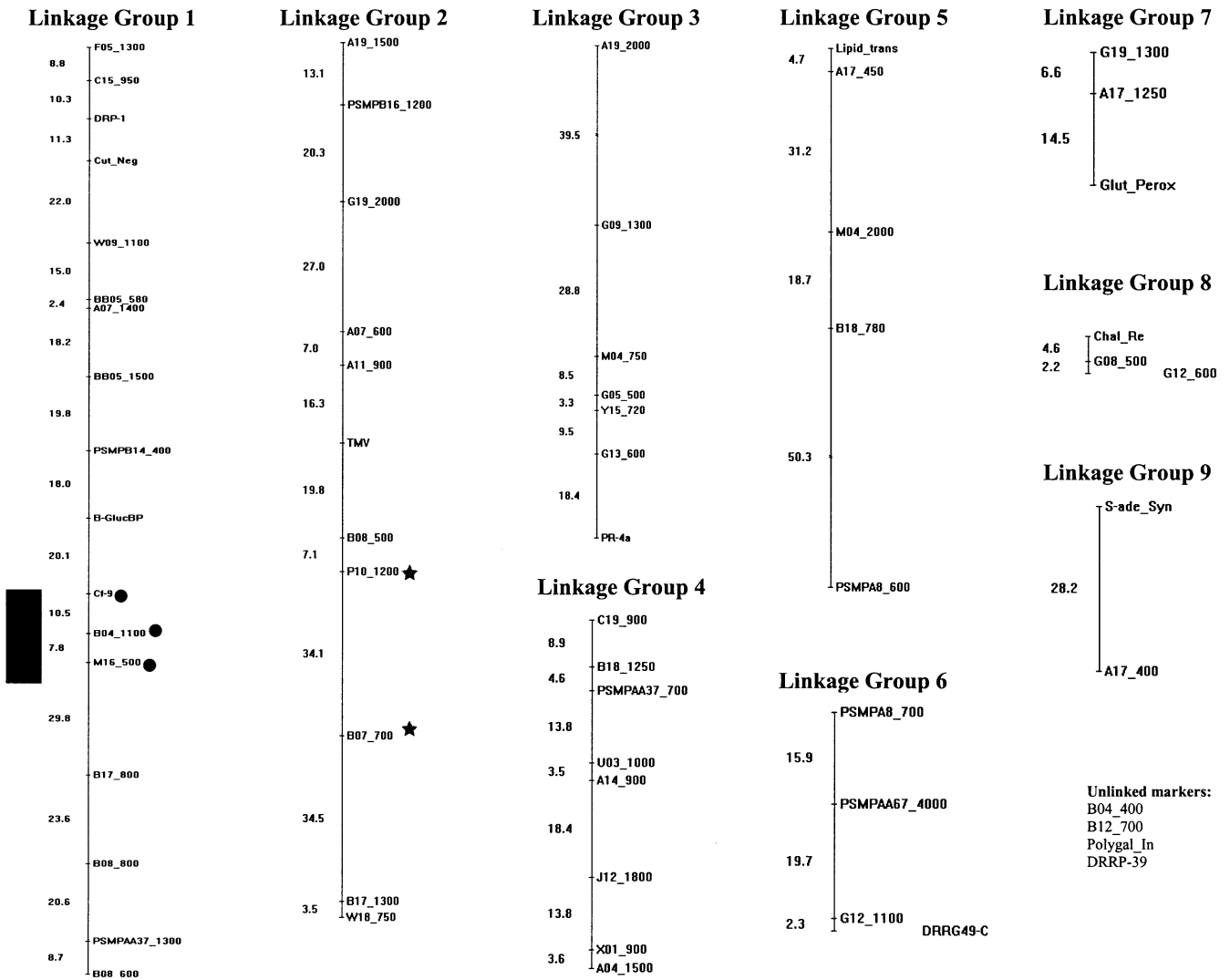


Fig. 1 Linkage map and genomic positions of quantitative trait loci (QTLs) for resistance to ascochyta blight. The QTL detected using simple and composite interval mapping is indicated with a *box*.

Markers significantly associated for seedling resistance using single-point analysis are represented by *circles*, while those markers which possessed significant *P*-values are represented by *stars*

P10_1200 and B07_1400, fell short of the LRS threshold; however, the *P*-values were <0.05 (Table 3), suggesting that these two markers may be significantly associated with a QTL (Fig. 1).

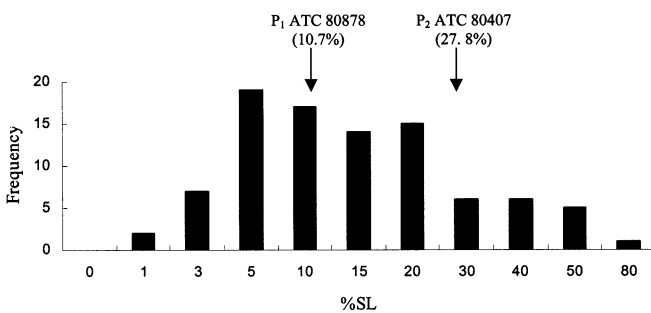


Fig. 2 Frequency distribution of disease scores [percentage stem area with lesions (%SL)] for ascochyta blight resistance in the backcross population. The mean disease score of the resistant parent (P₁: ATC 80878) was 10.7%, with a phenotypic range of 3–20%. The mean disease score of the susceptible parent (P₂: ATC 80407) was 27.8%, with a phenotypic range of 20–50%

Simple interval mapping detected one region significantly associated with resistance on linkage group 1 (QTL1), composed of the same three markers identified in the single-point analysis (Fig. 1), with the highest LRS value detected 3 cM away from marker M16_500 (LRS=11.4, Table 4). QTL1 accounted for 11% of the phenotypic variance observed in the backcross population. A second potential QTL was detected on linkage group 2 (QTL2), approximately 10 cM from marker P10_1200, which accounted for up to 8% of the phenotypic variation (Table 4). However, this region fell below the LRS threshold of significance using interval mapping (LRS=8.3, LOD=1.8, Fig. 1; Table 4).

Composite interval mapping of linkage group 1, where the effects of marker P10_1200 (QTL2) were

Table 3 Significant markers associated with ascochyta blight resistance identified using single-point analysis

Marker	Linkage group	LRS ^a	Percent ^b	<i>P</i>	Additive regression coefficient ^c
Cf-9	1	10.5	10	0.001	0.26
B04_1100	1	10.4	10	0.001	0.26
M16_500	1	10.7	10	0.001	0.26
P10_1200	2	7.3	7	0.007	-0.22
B07_1400	2	3.9	3	0.047	-0.18

^aLRS Likelihood ratio statistic for association of the trait with this locus

^bThe amount of total variance which would be explained by a QTL at this locus

^cThe additive regression coefficient for the association

controlled, increased the LRS values for markers Cf-9, B04_1100 and M16_500 (Table 4). The permutation test set a significant LRS value of 11.6, which these markers exceeded (Fig. 3). This supported the possibility that a QTL may lie within this region on linkage group 1.

Composite interval mapping of linkage group 2, where the effect of the major QTL located near Cf-9, B04_1100 and M16_500 on linkage group 1 (QTL1), was controlled also increased the LRS value for marker P10_1200 to 10.6 (Table 4). However, following permutation tests, this QTL was determined to be just below the significant LRS threshold of 12.1, therefore labelling this as a 'suggestive' QTL by MapManager QTX (Fig. 4).

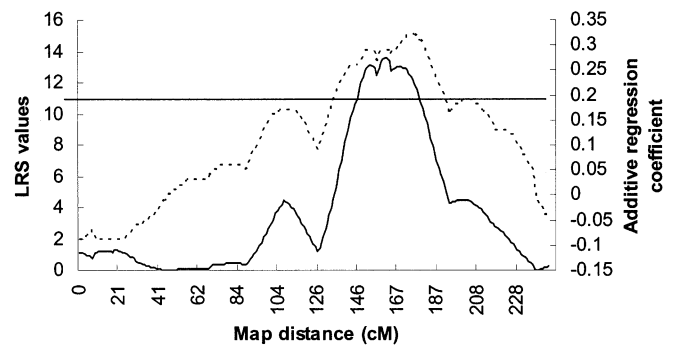
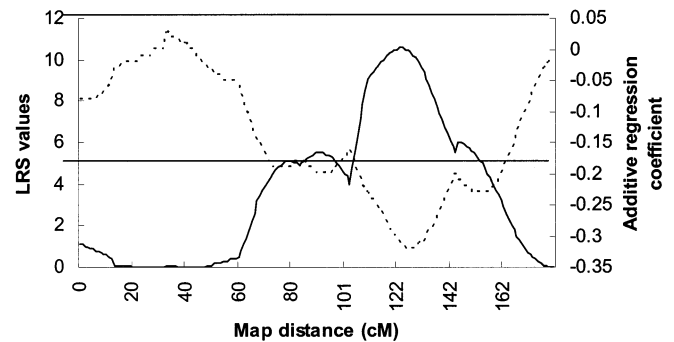
After combining the data from simple and composite interval mapping and single-point analysis, one consistent putative QTL for ascochyta blight resistance (QTL1) was deduced, located on linkage group 1. This QTL was located between markers Cf-9, B04_1100 and M16_500, with M16_500 being the closest marker to QTL1 (displaying the highest LRS value of 13.6, Table 4; Fig. 3). A second potential QTL (QTL2) was detected only by single-point analysis, which was near the markers P10_1200 and B07_1400 in linkage group 2 (Table 1). These markers fell short of the LRS threshold of 9.22; however, they possessed a significant *P*-value. Simple and composite interval mapping, followed by permutation tests revealed that P10_1200, was just below the significance threshold and could only be labelled as 'suggestive' (Table 4; Fig. 4).

Table 4 LRS values for markers located closely to potential QTLs following simple and composite interval mapping

Marker	Linkage group	Simple			Composite		
		LRS	Significant LRS threshold ^a	Percent ^b	LRS	Significant LRS threshold ^a	Percent ^b
P10_1200	2	8.3	11.3	8	10.6 ^c	12.1	7
M16_500	1	11.4	11.3	11	13.6 ^d	11.6	12
Cf-9	1	11.3	11.3	11	12.0 ^d	11.6	10
B04_1100	1	10.9	11.3	10	12.9 ^d	11.6	11

^aSignificant LRS threshold following permutation tests

^bThe amount of total variance which would be explained by a QTL at this locus

**Fig. 3** LRS score plot for ascochyta blight resistance on linkage group 1. LRS values are represented by the solid line and additive effect represented by the dotted line. Significant LRS threshold at 11.6**Fig. 4** LRS score plot for ascochyta blight resistance on linkage group 2. LRS values are represented by the solid line and additive effect represented by the dotted line. Significant LRS threshold at 12.1 and suggestive LRS threshold at 5.6

In a backcross population, the dominance effect of a QTL allele is confounded with additive effects. The constrained additive regression coefficient for markers associated with QTL1 on linkage group 1 was positive (Table 3; Fig. 3). This indicated that these markers were correlated with an increase in the disease scores (%SL) and thus may be associated with susceptibility, i.e. backcross individuals that possessed; for example, the M16_500 marker allele, were more susceptible than those that did not (Table 5). Furthermore, GLM analysis showed that significant differences existed between backcross individuals that possessed the M16_500 mar-

^cWith effects of M16_500 in background

^dWith effects of P10_1200 in background

Table 5 Mean percentage stem area with lesions (%SL) for backcross individuals possessing neither, one or both markers located close to QTL1 or QTL2 for ascochyta blight resistance

Marker ^a		Mean %SL ($\pm 1/2$ LSD) ^b
P10_1200	M16_500	
A	–	20.2 (9.7)
H	–	12.8 (9.7)
–	A	12.3 (9.7)
–	H	20.7 (9.7)
A	A	16.9 (9.7)
A	H	23.5 (9.7)
H	A	7.6 (9.7)
H	H	17.9 (9.7)

^aA Marker absent in backcross individual, H marker present in backcross individual

^bLSD based on pooled Error MS ($P=0.05$)

ker allele and those that did not (Table 6). Therefore, marker M16_500 may be linked in repulsion to resistance.

Conversely, the constrained additive regression coefficient for marker P10_1200 in linkage group 2 was negative (Table 3; Fig. 4). This indicated that the presence of P10_1200 was correlated with reduced %SL and suggested that this marker may be associated with resistance. This is illustrated in Table 5, where backcross individuals that possessed the P10_1200 marker allele were more resistant than those that did not. Again, the GLM analysis showed significant phenotype differences existed among the marker genotypes (Table 6). Therefore, marker P10_1200 may be linked in coupling with resistance.

When the mean %SL for backcross individuals possessing different combinations of the two markers M16_500 and P10_1200 were compared, the lowest mean %SL was observed for backcross individuals that possessed only the P10_1200 marker (7.6%), whereas the highest mean %SL (23.5%) was detected in individuals possessing only the M16_500 marker (Table 5). The heterozygotes produced intermediate disease scores (Table 5). From the composite interval mapping, where the effects of one marker allele was controlled, saw a slight increase in the effects of the other, one would assume that there may be an association between these two marker alleles. However, GLM analysis for the combination of the two marker alleles showed no significant interaction between these two markers (Table 6).

Discussion

Construction of the linkage map

A *L. sativus* linkage map covering 803.1 cM and comprising 64 markers distributed over nine linkage groups was developed using three classes of molecular markers, RAPD, STMS and STS/CAPS markers. A genetic linkage map of *L. sativus* was generated in a previous

Table 6 General linear model analysis summary for disease scores on markers P10_1200 and M16_500

Source of variance	Degrees of freedom	Mean square	F	P
P10_1200	1	1,245.2	6.40	0.013
M16_500	1	1,586.4	8.15	0.005
P10_1200 \times M16_500	1	72.8	0.37	0.542
Error	86	194.6		
Total	89			

$$\text{LSD} = t_{0.05} \frac{\sqrt{2 \times \text{Error MS}}}{n}$$

study from 100 F₂ individuals and comprised mainly dominant markers, i.e. 71 RAPD, three isozymes and one morphological marker (Chowdhury and Slinkard 1999). This linkage map consisted of 14 linkage groups covering 898 cM, and map distances were calculated using the Haldane function, as opposed to the present study which used the more stringent Kosambi function.

In the current and previous studies, RAPD markers were shown to be a quick and simple marker system for map construction. However, in an F₂ population, as used by Chowdhury and Slinkard (1999), they are not as informative as co-dominant markers, as they are difficult to distinguish between homozygous and heterozygous individuals in the population. The present study used a backcross population to construct the linkage map, which overcame this problem. Also when using dominant markers systems such as RAPDs, 'null alleles' are sometimes difficult to score with confidence, which may lead to misclassification and distorted marker segregation.

The majority of markers that displayed distorted segregation in the backcross population in this study possessed a higher than expected frequency of null alleles (absence of the marker in the backcross population). This applied mainly to the RAPD markers; however, one STMS and five STS markers also showed distorted segregation. Segregation distortion of markers may be caused by genetic, physiological and/or environmental factors (Xu et al. 1997), as well as the possibility of the PCR reaction not working due to insufficient or degrading DNA template, or perhaps the deterioration of PCR buffers and primers due to excessive freezing-thawing or age. Segregation distortion of markers increases the rate of false linkages in F₂ populations and may also affect the accuracy for determining the order of markers (Lorieux et al. 1995). Twelve percent of the markers used in the grasspea linkage map presented by Chowdhury and Slinkard (1999) showed distorted segregation, unlike in the present map which excluded any marker which significantly deviated from the expected 1:1 Mendelian ratio. Laucou et al. (1998) and Pilet-Nayel et al. (2002) also excluded markers which exhibited distorted segregation when creating field pea linkage maps.

The linkage map constructed in this study also showed the location of 13 STS/CAPS markers, which were from a collection of 20 tested on the backcross population. These markers were generated by primers

designed from EST sequences obtained from a *L. sativus* cDNA library (Skiba 2003). The *P. sativum* linkage map of Gilpin et al. (1997) featured 29 loci representing genes of known function (ESTs). This map linked a disease resistance response protein isolated from *P. sativum* to linkage group VI. Other defence-related ESTs previously mapped by Gilpin et al. (1997) were also identified from the *L. sativus* cDNA library (Skiba 2003). Unfortunately, these ESTs could not be converted to polymorphic STS markers to be screened on the backcross population due to close sequence conservation among the parental genotypes. As a result, they could not be mapped and comparisons could not be made with the *P. sativum* map.

One limitation of this grasspea linkage map was that certain linkage groups were not adequately saturated with markers. Linkage groups 6, 7, 8 and 9 comprise only two to four markers. This may be due to a high chromosomal homology and thus, lack of recombination between the parental genotypes. This is often common when crossing two related lines of the same species, as was demonstrated in cultivated chickpea, where minimal polymorphisms were observed for certain marker systems, thus making it difficult to construct genetic linkage maps (Hüttel et al. 1999).

A key feature of a genetic map indicating completion is that the number of linkage groups obtained is equal to the number of chromosomes in the organism. Like field pea, the *L. sativus* genome consists of seven haploid chromosomes. The present linkage map consists of nine linkage groups spanning just over 800 cM. The shortage of markers may have also been responsible for the map not coalescing into seven linkage groups, corresponding to the haploid chromosome number of *L. sativus*. A difficulty associated with obtaining an equal number of linkage groups and chromosomes is that markers are not necessarily evenly distributed over the chromosome, but may cluster in some regions and be absent in others (Paterson 1996). Additionally, the frequency of recombination is not equal along chromosomes (Young 1994), whereby in the heterochromatic regions adjacent to the centromere and at the ends of the chromosome arms (telomeres), recombination occurs less frequently (Hartl and Jones 1998). Therefore, identifying markers to these regions is more difficult. The *L. sativus* map presented here is consistent with the expected length of the field pea genome (Weeden et al. 1998) and that reported previously by Chowdhury and Slinkard (1999).

Detection of QTLs for ascochyta blight resistance

Single-point analysis and interval mapping produced similar, but not identical results. A total of two genomic regions, on two linkage groups, were associated with *M. pinodes* resistance. The first putative QTL for ascochyta blight resistance in *L. sativus* (QTL1), was located on linkage group 1, in the vicinity of markers Cf-9,

B04_1100 and M16_500, spanning approximately 30 cM. This QTL was detected using both methods and the major additive effects at this locus explained up to 12% of the trait variation based on composite interval mapping. The highest LRS score (13.6) detected with composite interval mapping, coincided with the marker with the highest LRS value (10.7) ($P=0.001$) determined using single-point analysis. Two of the three markers located near this QTL were generated from RAPD primers. The third marker mapped in the vicinity of QTL1 was generated from an STS primer pair designed from a *L. sativus* cDNA clone that showed sequence similarity to the Cf-9 resistance gene of tomato. This STS primer pair may have amplified a gene in *L. sativus* similar to the Cf-9 resistance gene; therefore, the marker Cf-9 could represent an ascochyta blight resistance gene in *L. sativus*, as previously proposed for the resistance to ascochyta blight in chickpea (Flandez-Galvez et al. 2003). However, in order to accurately determine whether this marker represents a Cf-9-like gene in the *L. sativus* genome, the amplicon for this marker needs to be cloned, sequenced and subjected to databases searches.

Single-point analysis detected a second potential QTL (QTL2) for *M. pinodes* resistance near markers P10_1200 and B07_1400 on linkage group 2, which produced significant *P*-values; however, their LRS values fell short of the 9.22 threshold. The linkage phase of these markers appeared to be in coupling with resistance. Following interval mapping, these markers were again just below the LRS threshold of significance (LRS 8.3), suggesting that the P10_1200 marker (which possessed the highest LRS value of the two markers) may be linked to QTL2 with a small effect, or a QTL may lie beyond this marker. A more precise estimate of the position and effect of this QTL could be determined by mapping additional markers on both sides of marker P10_1200.

The two QTLs detected in this study explained a relatively small percentage of phenotypic variance observed in the backcross population, only 19% of the total combined effect. Some of the remaining variation may be explained by environmental factors. Small changes in the microclimate may result in an increase in disease scores in the backcross population. Croft (1999) reported that a significant increase in stem lesions was observed in *L. sativus* plants when the duration of leaf wetness/humidity was extended. Therefore, if the inoculated backcross plants were kept at high humidity for a longer duration, and plants were scored at a later date post inoculation, the two QTLs identified may explain a higher percentage of phenotypic variance. Environmental factors may also explain the observation of three backcross plants displaying disease scores outside the parental phenotypic range.

Another explanation for the relatively small effect of these two QTLs may be that perhaps other alleles are involved in the expression of resistance in *L. sativus*, which have not been detected by the current analysis.

Young (1996) suggested that the number of QTLs detected in the literature may be an underestimate of the actual number of QTLs, since many real QTLs may be rejected on the basis of statistical significance. And often when using a small population for QTL mapping, only QTLs of large effects are likely to be detected (Beavis 1998; Haley and Andersson 1997). Therefore, the population used in this experiment may have been too small to detect QTLs with minor effects. Other smaller, non-significant peaks were observed on various positions on some of the linkage groups, and perhaps if a larger mapping population was used, these smaller/minor peaks may have been determined to be significant. However, in practice, QTLs making the largest contribution to phenotype are usually the most valuable from a plant breeder's perspective for manipulation using molecular tools in breeding programs (Tanksley 1993). A larger mapping population may also increase the power to determine any interaction between QTLs. In the current study, no significant interaction between QTL1 and QTL2 was observed based on the GLM analysis. This may have been due to the possibility that the effect of one QTL on the other was so small it could not be detected with such a small mapping population. Screening a larger backcross population may result in a significant interaction between the two markers being observed.

A QTL with a large effect is still detectable by relatively distant markers, whereas a QTL with small effects is detectable only by the most closely linked markers (Gebhardt and Valkonen 2001). Therefore, the distances between markers may be too great to detect these minor QTLs. The two QTLs detected in this study were located on linkage groups that were adequately saturated with markers, with the average spacing of markers on the whole map being 15.8 cM. This is slightly better than that of the *L. sativus* map presented by Chowdhury and Slinkard (1999), with average distance between two adjacent markers being 17.2 cM.

The *L. sativus* linkage map presented in this study may be used as a reference point for future linkage studies in grasspea. The STS/CAPS markers located on this map may be more useful for comparative mapping than RAPD markers, which are not always transferable within and between species. The mapped STSs may be used as landmarks/anchors for different populations, because they are distributed across all but one of the nine linkage groups, and their optimal PCR conditions have been defined (Skiba et al. 2003). Integration of several linkage maps is necessary to detect the common functional QTLs controlling agronomically important traits across different genetic backgrounds. The use of STS/CAPS markers will allow different linkage groups to be easily integrated.

QTL mapping is a powerful method for the analysis of allelic relationships among genes controlling ascochyta blight resistance. This is the first report of the identification of markers associated with QTLs for resistance to *M. pinodes* in *L. sativus*. This study iden-

tified two potential QTLs for *M. pinodes* resistance, with one marker linked in coupling to resistance and the other linked in repulsion. Only markers linked in coupling would be of benefit for map-based cloning of genes responsible for resistance to *M. pinodes*.

Further mapping studies are required to confirm these results and to better understand the genetics of resistance to *M. pinodes* in *L. sativus*. The QTLs detected depend on the mapping population used; therefore, further QTL analyses using different cross-combinations may be necessary to identify the full set of genes controlling this trait. Furthermore, the resistance mechanisms in *L. sativus* at different developmental stages, such as maturity and pre-flowering, should be evaluated and examined. Although seedling resistance is important for early survival against *M. pinodes* infection, the pathogen can attack at any developmental stage of the plant and may be equally threatening under favourable environmental conditions. Identification of the common loci controlling ascochyta blight resistance at different developmental stages across many genotype cross-combinations would lead to an understanding of the individual genetic factors involved in ascochyta blight resistance, for the manipulation of these genes for *L. sativus* and ultimately, *P. sativum* breeding.

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