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Preliminary investigation of QTLs associated with seedling resistance to ascochyta blight from *Cicer echinospermum*, a wild relative of chickpea

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Abstract Accessions from *Cicer echinospermum*, a wild relative of chickpea (*Cicer arietinum* L.), contain resistance to the fungal disease ascochyta blight, a devastating disease of chickpea. A linkage map was constructed based on an interspecific F_2 population, derived from a cross between a susceptible chickpea cultivar (Lasseter) and a resistant *C. echinospermum* accession (PI 527930). The linkage map incorporated 83 molecular markers, that included RAPD, ISSR, STMS and RGA markers; eight markers remained unlinked. The map comprised eight linkage groups and covered a map distance of 570 cM. Six out of the eight linkage groups were correlated to linkage groups from the integrated *Cicer* map using STMS markers. Quantitative trait loci (QTLs) associated with ascochyta blight resistance were detected using interval mapping and single-point analysis. The F_2 population was evaluated for seedling and stem resistance in glasshouse trials. At least two QTLs were identified for seedling resistance, both of which were located within linkage group 4. Five markers were associated with stem resistance, four of which were also associated with seedling resistance. QTLs from previous studies also mapped to LG 4, suggesting that this linkage group is an important region of the *Cicer* genome for resistance to ascochyta blight.

Keywords *Cicer arietinum* · *Cicer echinospermum* · Ascochyta blight · Linkage map · Interspecific hybridization · Disease resistance · QTLs

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Introduction

Ascochyta blight, caused by the fungal pathogen *Ascochyta rabiei* (Pass) Labr., is a major constraint to the production of chickpea (*Cicer arietinum* L.). The pathogen attacks all aerial parts of the plant at any growth stage, and may cause yield-losses up to 100% (Nene 1982). Extensive evaluation of chickpea germplasm has revealed a low proportion of resistant accessions (Singh et al. 1981; Reddy and Singh 1984). Sources of seedling resistance to an Australian isolate of *A. rabiei*, were recently identified in accessions from wild *Cicer* species (Collard et al. 2001). The identification of resistant accessions from *Cicer reticulatum* and *Cicer echinospermum* was of particular importance because fertile interspecific hybrids with chickpea can be produced (Singh and Ocampo 1993, 1997; Pundir and Mengesha 1995), therefore, new sources of resistance to ascochyta blight may be successfully transferred into chickpea.

The identification of molecular markers closely linked to or 'tagging' resistance genes is of great benefit for breeding for resistance, because it allows breeders to select on the basis of marker genotype rather than resistance phenotype. There are numerous advantages of using molecular markers in breeding, via marker-assisted selection, compared to conventional breeding methods. The advantages include the substitution of time-consuming and often unreliable field evaluations with molecular tests and the selection of multiple genes (Michelmore 1995; Young 1996). The selection of multiple resistance genes or quantitative trait loci (QTLs) may be of particular importance with respect to ascochyta blight because resistance, in some chickpea genotypes, appear to

be polygenic (Tekeoglu et al. 2000) or quantitative (Santra et al. 2000). Furthermore, markers may assist with the monitoring of introgression, and accelerate the recovery of a recurrent parent and minimize donor genetic material (Michelmore 1995).

By utilizing a linkage map as a 'framework', the number and genomic positions of genes conferring quantitative resistance may be determined using QTL analysis (Paterson 1996; Young 1996). The number and position of QTLs for resistance to many plant diseases have been determined using QTL analysis (Michelmore 1995; Young 1996; Mohan et al. 1997). Markers that are tightly linked to resistance-QTLs may then be utilized in breeding programs via marker-assisted selection.

Limited genetic variation detected within cultivated chickpea has directed most chickpea mapping studies to utilize populations derived from interspecific hybrids between chickpea and *C. reticulatum* – the presumed progenitor of chickpea (Simon and Muehlbauer 1997; Winter et al. 1999, 2000; Santra et al. 2000). To-date, populations derived from *C. arietinum* × *C. echinospermum* interspecific hybrids have not been used for identifying QTLs associated with any traits, despite *C. echinospermum* possessing resistances to ascochyta blight and several other diseases such as fusarium wilt, leaf miner and bruchids (Singh et al. 1994). A linkage map constructed on an interspecific *C. arietinum* × *C. echinospermum* population would provide useful information for identifying the genomic position of QTLs conferring ascochyta blight resistance from *C. echinospermum*. Therefore, the aims of the study were to: (1) construct a linkage map based on a F_2 population generated from an interspecific *C. arietinum* × *C. echinospermum* population; and (2) identify QTLs associated with seedling resistance to ascochyta blight.

Materials and methods

Mapping population

An interspecific cross was made between chickpea cultivar Lasseter (susceptible) and *C. echinospermum* accession PI 527930 (resistant). Accession PI 527930 was the most genetically similar *C. echinospermum* accession to Lasseter (Collard et al. 2003). A large number of F_2 seeds were produced by taking stem cuttings from a single F_1 plant following the method described by Collard et al. (2002). All plants used for seed production were grown in the glasshouse-facility at the University of Melbourne.

DNA extraction and molecular-marker analysis

DNA was obtained from parental, F_1 and F_2 plants following the method described by Taylor et al. (1995). Primers corresponding to RAPD, ISSR and STMS markers used for the construction of previous *Cicer* linkage maps, were selected for mapping analysis (Simon and Muehlbauer 1997; Santra et al. 2000; Winter et al. 2000). Degenerate RGA primers (Kanazin et al. 1996; Chen et al. 1998; Shen et al. 1998) and RAPD primers used to fingerprint wild *Cicer* accessions (Collard et al. 2003) were also used for map construction. Primers were synthesized by Life Technologies, USA. Primers were carefully selected for their reproducibility; only primers that produced reproducible and clearly resolvable bands were selected for mapping the F_2 population.

RAPD analysis was performed according to the procedure described by Simon and Muehlbauer (1997). A selection of five inter-simple sequence repeat (ISSR) primers, previously used in mapping by Santra et al. (2000) and Winter et al. (2000), was used for ISSR analysis. PCR amplification was performed following the procedure described by Ratnaparkhe et al. (1998). STMS analysis was performed following the appropriate reaction conditions and PCR amplification cycles described by Huttel et al. (1999) and Winter et al. (1999). RGA analysis was performed following the appropriate PCR protocols for the primers (Kanazin et al. 1996; Chen et al. 1998; Shen et al. 1998). RAPD, ISSR and RGA PCR amplification products were resolved by gel electrophoresis in 2% (w/v) agarose in tris-borate (TBE) buffer, and visualized by staining the gels with ethidium bromide and viewing using a UV-transilluminator. STMS PCR amplification products were separated on 2% agarose, 3.5% Metaphor agarose or 5% polyacrylamide, depending on the resolution required for the discrimination of parental alleles. Polyacrylamide gel-electrophoresis was performed by mixing PCR amplification products with a loading buffer, denatured at 90 °C for 3 min, run for 2.5 h at a constant 50 W and detected by silver staining (Promega, Australia). PCR was performed on a MJ Research 200 Thermal Cycler. Each marker was tested for either 3:1 segregation ratios for dominant markers or 1:2:1 segregation ratios for codominant markers using chi-square analysis ($P < 0.05$).

Marker nomenclature

Molecular marker loci were given two-part names consisting of the name of the primer used and the approximate size of the marker in base pairs. Primers that amplified more than one polymorphic marker were named using the primer name followed by lower-case letters (from highest to lowest molecular weight). All RAPD markers had the prefix CS (Simon and Muehlbauer 1997), UBC [University of British Columbia; Santra et al. (2000)] or OP (Operon Technologies) followed by the primer kit name (e.g. B, BA or BB). ISSR markers had the prefix UBC and numbers within the range of 801–899. STMS markers were named according to the primers described by Huttel et al. (1999) and Winter et al. (1999). RGA primers were named according to the primers described by Chen et al. (1998).

Map construction

A linkage map was constructed on the F_2 population using Mapmaker/EXP version 3B (Lincoln et al. 1993a). Linkage groups were established using the 'group' command at a LOD score of 3 and a maximum map distance of 50 cM. Marker order within linkage groups was initially determined using the 'compare' command of Mapmaker. Additional markers were added using the 'try' command. The 'ripple' command was used to scrutinize marker order. Markers that could not be placed on a linkage group at a LOD threshold above 2.0 (but appeared to be linked to the linkage group using the group command) were listed below the appropriate linkage group. Map distances were calculated using the Kosambi mapping function.

Inoculation of plants

Parental and F_2 plants were inoculated with *A. rabiei* following the method described by Collard et al. (2001), except that they were inoculated at the 10–12 leaf stage (28 days) rather than the 6–8 leaf stage (15 days). The second and third leaves from the tops of all plants were removed for DNA extraction 7 days before inoculation. The lengths of stems were measured on the day of inoculation.

Phenotypic evaluation

Parental and F_2 plants were evaluated for resistance to ascochyta blight at the seedling stage. Seedling resistance was evaluated using the nine-class scale, 14 days after inoculation (Collard et al. 2001). Stem resistance (at the seedling stage) was also assessed using the linear infection index (LII) reported by Riahi et al. (1990) at 21 days after inoculation. The LII, a quantitative score, was calculated by measuring the number of stem lesions multiplied by the average lesion length divided by the total stem length; the final value was then expressed as a percentage (Riahi et al. 1990). Due to poor-seed germination, the F_2 population was evaluated for seedling and stem resistance in five separate trials. Lasseter plants were used as controls to monitor the level of infection in each trial, and the mean disease scores and stem-infection scores were subjected to analysis of variance (ANOVA).

Detection of QTLs

Two methods were used to detect QTLs for ascochyta blight resistance: interval mapping and single-point analysis. Interval mapping was performed using the program Mapmaker/QTL ver 1.1 (Lincoln et al. 1993b). Putative QTLs were detected using a LOD threshold of 2.0 based on an unconstrained or free-genetics model. Single-point analysis was performed with one-way ANOVA and simple linear regression, using MINITAB Release 12.22 (Minitab Incorporated, State College Pa., USA). The effect of a QTL – estimated by the coefficient of determination (R^2) – was used to estimate the percentage of phenotypic variation explained from the marker. Markers with $P < 0.05$ were considered to be associated with a putative QTL for ascochyta blight resistance. Stem-infection data (based on LII) were not normally distributed after logarithmic, arcsine and square-root data transformations. Therefore, the Mann-Whitney and Kruskal-Wallis non-parametric tests were applied in order to detect markers that were associated with QTLs (for dominant and codominant markers, respectively). In order to detect epistatic interactions, two-way ANOVA with an interaction component were tested between all pairs of markers that were significantly associated with resistance in the one-way ANOVAs.

Results

Seed germination

Large seed numbers were required to establish a mapping population of 100 F_2 plants, due to 6.1% seed germination. Abnormalities for the germination of some F_2 seeds were observed. Some F_2 seeds developed a radicle but not a hypocotyl, whereas other F_2 seeds developed a hypocotyl but not a radicle. Reduced levels of seed germination were also observed for the parental accessions: 8.3% and 5.5% for Lasseter and PI 527930 respectively.

General features of the linkage map

The linkage map was composed of a total of 83 markers including 54 RAPD, 14 STMS, 9 ISSR and 6 RGA markers (Fig. 1). The map comprised eight linkage groups (LGs) covering 570 cM and the average spacing between markers was 7.2 cM. Markers from both parents were generally well represented on LGs. Unique placements within LGs could not be determined for four markers. Eight markers remained unlinked. Segregation distortion of all markers was tested using chi-square tests ($P < 0.05$). A total of 25 out of the 91 markers (27%) had segregation ratios that significantly deviated from the expected 3:1 and 1:2:1 ratios for dominant and codominant markers, respectively. Distorted markers were often linked together and present on the same LGs. Distorted markers appeared on four LGs (3a, 3b, 5 and 7) as shown in Fig. 1.

Comparison with previous *Cicer* maps

Six LGs could be correlated to the LGs from the integrated *Cicer* map by Winter et al. (2000) and were assigned numbers based on this study (Fig. 1). Two LGs could not be assigned due to a lack of STMS markers and were designated A and B. The order of STMS markers within LGs, but not distances between markers, was generally conserved between the integrated *Cicer* map (Winter et al. 2000). There were no similarities in the marker order of RAPD or ISSR markers detected when compared to orders of markers from previous studies (Simon and Muehlbauer 1997; Santra et al. 2000; Winter et al. 2000).

Phenotypic evaluation

The severity of infection was similar across the five separate trials, since there were no significant differences detected between the mean-disease scores ($P = 0.201$) or mean-stem infection scores ($P = 0.563$) for the Lasseter controls in each F_2 trial. Since there were no significant differences between trials, the phenotypic scores of individual F_2 plants were pooled for the entire population and used for QTL analysis. A total of 97 F_2 plants were evaluated for seedling and stem resistance. The mean-disease scores, standard errors, and ranges for the resistant and susceptible parents and the F_2 population are reported in Table 1.

The phenotypic distribution of disease scores for seedling resistance for the F_2 population was approxi-

Table 1 Mean disease scores, standard errors (SEs) and ranges for seedling and stem resistance for resistant and susceptible parental genotypes and F_2 population

Resistance	Resistant parent (*C. echinospermum*)		Susceptible parent (*C. arietinum*)		No. of plants	F_2 population		Range
	Mean	SE	Mean	SE		Mean	SE	
Seedling	3.9	0.2	8.5	0.1	97	6.8	0.1	3–9
Stem (%)	19.5	2.5	95.3	0.9	97	73.7	3.1	8.4–100

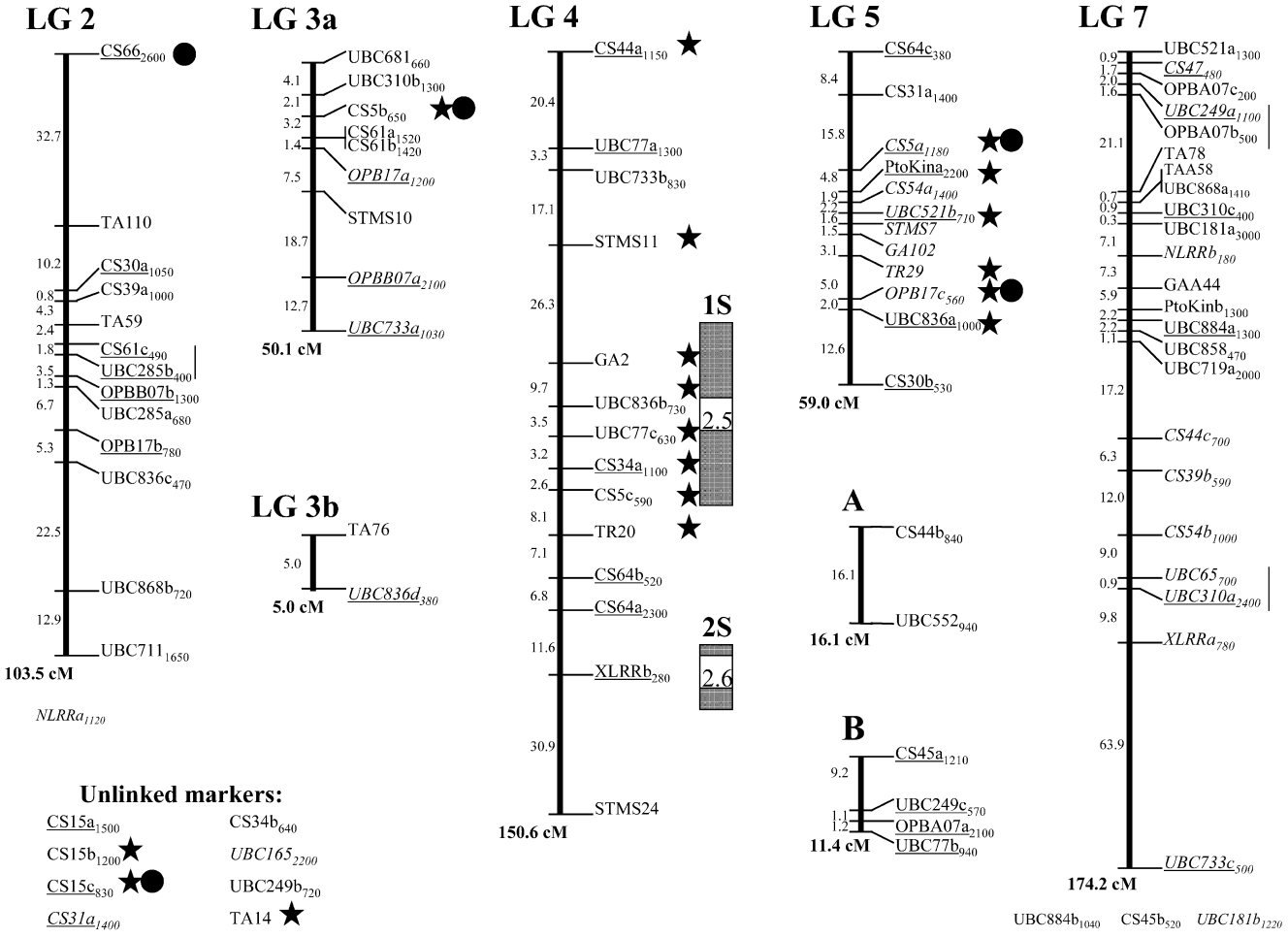

Fig. 1 Linkage map and genomic positions of QTLs for resistance to ascochyta blight. Marker alleles from *C. echinospermum* are underlined. Unique placements within linkage groups could not be determined for four markers; these markers with are listed below linkage groups. Vertical lines indicate interchangable marker orders (based on equal LOD values). Markers with distorted segregation ratios are indicated in italics. QTLs for seedling (S) detected by interval mapping are indicated by shaded rectangles. Maximum LOD scores are indicated within rectangles. Markers that were significant for seedling and stem resistance are indicated by stars and filled circles respectively

Table 2 Segregation ratios for stem resistance based on 97 F_2 individuals

Modified F_2 dihybrid model	Observed ratio[a]	Expected ratio	Chi-square value	*P* value
15:1	82 S:15 R	91 S:6 R	14.4	<0.001
13:3	82 S:15 R	79 S:18R	0.61	0.43
12:4	82 S:15 R	79 S:31 R	8.4	0.004
10:6	82 S:15 R	61 S:36 R	19.5	<0.0001
9:7	82 S:15 R	55 S:42 R	31	<0.0001
12:3:1	51 S:31 I:15 R	73 S:18 I:6 R	29.5	<0.0001
10:3:3	51 S:31 I:15 R	61 S:18 I:18 R	11.5	0.003
9:6:1	51 S:31 I:15 R	55 S:36 I:6 R	14.5	<0.001
9:4:3	51 S:31 I:15 R	55 S:24 I:18 R	2.8	0.24

[a] S, susceptible; I, intermediate; R, resistant

mately normally distributed, suggesting that seedling resistance was under polygenic control (Fig. 2a). The phenotypic distribution for stem resistance (based on the LII) for the F_2 population was trimodal, with the population being heavily skewed towards susceptibility (Fig. 2b). A strong correlation (r = 0.86) was observed between seedling and stem resistance.

The trimodal phenotypic distribution for stem resistance suggested that resistance was conferred by two loci and was consistent with the modified F_2 dihybrid models described by Hartl and Jones (1998). A modified F_2 dihybrid model was defined as a two-gene genetic model that departed from the standard 9:3:3:1 ratio for two independently segregating (i.e. unlinked) genes (Hartl and

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

Marker	Parental allele[a]	Linkage group[b]	Seedling resistance[c,d]		Stem resistance *P*
			R^2 (%)	*P*	
$CS66_{2600}$	E	2	(2.5)	(0.068)	0.049
$CS5b_{650}$	A	3a	5.9	0.017	0.037
$CS44a_{1150}$	E	4	5.9	0.017	ns
STMS11	C	4	4.4	0.040	ns
GA2	C	4	9.5	0.002	ns
$UBC836b_{730}$	A	4	7.0	0.009	ns
$UBC77c_{630}$	A	4	7.5	0.007	ns
$CS34a_{1100}$	E	4	7.3	0.007	(0.073)
$CS5c_{590}$	A	4	3.7	0.058	ns
TR20	C	4	7.8	0.008	ns
$CS64b_{520}$	E	4	6.4	0.014	ns
$XLRRb_{280}$	E	4	(3.4)	(0.073)	ns
$CS5a_{1180}$	E	5	5.3	0.023	0.041
$PtoKina_{2200}$	E	5	6.0	0.018	ns
$UBC521b_{710}$	E	5	6.3	0.013	ns
TR29	C	5	8.1	0.009	ns
$OPB17c_{560}$	A	5	4.7	0.032	0.025
$UBC836a_{1000}$	E	5	4.9	0.030	ns
$CS15b_{1200}$	A	U	7.6	0.007	ns
$CS15c_{830}$	E	U	4.6	0.038	0.029
TA14	C	U	6.8	0.02	ns

[a] Marker detected from A: *C. arietinum* parent; E: *C. echinospermum* parent; C: codominant marker
[b] U = unlinked marker
[c] Markers just above the significance threshold ($P = 0.05$) for seedling or stem resistance are listed in parentheses
[d] ns = not significant *P* value

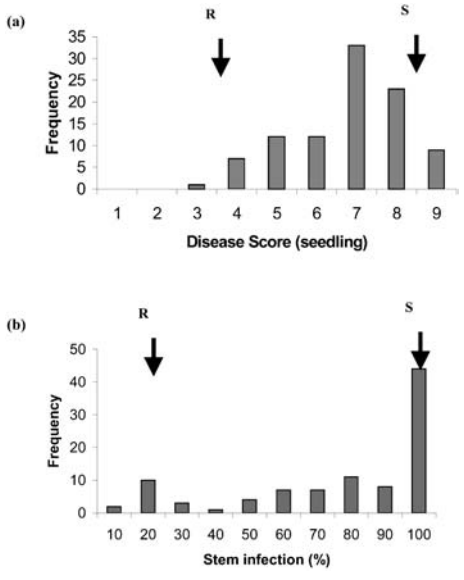

Fig. 2 Frequency distribution of disease scores for ascochyta blight resistance in the F_2 population. **a** Seedling resistance. **b** Stem resistance. The mean disease scores of the resistant and susceptible parents are indicated by the letters R and S, respectively

Jones 1998). F_2 Plants that had LII scores within two standard deviations of the resistant parent were classified as resistant; all other plants were classified as susceptible for two-category models (Table 2). Susceptible F_2 plants were classified as susceptible and intermediate for three-category models. F_2 plants that had LII scores more than two standard deviations of the resistant and susceptible parents, were classified as intermediate (Table 2). Two modified F_2 dihybrid models were consistent with the data; the 13:3 and the 9:4:3 models suggested that two loci with epistatic interactions conferred stem resistance. Both these dihybrid models are appropriate when recessive alleles mask the expression of a different unlinked gene (Hartl and Jones 1998).

QTLs for seedling resistance

Twenty markers were associated with seedling resistance using single-point analysis (Table 3). Nine of these markers were located on LG 4, eight of them in a single contiguous region (Fig. 1). Three unlinked markers – $CS15b_{1200}$, $CS15c_{830}$ and TA14 – were also significantly associated with seedling resistance (Table 3). Interval mapping detected two regions that were significantly associated with seedling resistance on LG 4 (Fig. 1). A third region, near the marker $CS44a_{1150}$, was just below the LOD threshold of significance using interval mapping (LOD = 1.9) (data not shown).

Combining the data from both interval mapping and single-point analysis, two putative QTLs for seedling resistance were deduced. Both QTLs were located on LG 4 (Fig. 1). The first QTL was detected using both interval-mapping and single-point analysis and was located between the markers STMS11 and TR20 (Fig. 1). The highest LOD score (2.5) was detected in the interval between the markers $UBC836b_{730}$ and $UBC77c_{630}$. The second QTL for seedling resistance, detected by interval mapping, was near the marker $XLRRb_{280}$. Single-point

Table 4 Genotypic means for markers associated with seedling resistance

Marker[a]	Allele	Linkage group	Genotypic mean[b]				
			AA	E_	AE	EE	A_
$CS5b_{650}$	A	3a	–	–	–	6.1	7.0
$CS44a_{1150}$	E	4	7.4	6.6	–	–	–
STMS11	C	4	7.3	–	6.6	6.5	–
GA2	C	4	7.4	–	6.7	6.2	–
$UBC836b_{730}$	A	4	–	–	–	6.1	7.0
$UBC77c_{630}$	A	4	–	–	–	6.1	7.0
$CS34a_{1100}$	E	4	7.3	6.6	–	–	–
$CS5c_{590}$	A	4	–	–	–	6.2	6.9
TR20	C	4	7.3	–	6.7	6.2	–
$CS64b_{520}$	E	4	7.4	6.6	–	–	–
$CS5a_{1180}$*	E	5	6.0	6.9	–	–	–
$PtoKina_{2200}$*	E	5	6.0	7.0	–	–	–
$UBC521b_{710}$*	E	5	5.9	6.9	–	–	–
TR29*	C	5	6.0	–	7.0	7.2	–
$OPB17c_{560}$*	A	5	–	–	–	7.2	6.6
$UBC836a_{1000}$*	E	5	6.1	6.9	–	–	–
$CS15b_{1200}$*	A	U	–	–	–	7.4	6.6
$CS15c_{830}$*	E	U	6.3	7.0	–	–	–
TA14	C	U	7.4	–	6.8	6.3	–

[a] Lasseter marker alleles contributing resistance are indicated with asterisks
[b] AA = homozygous for *C. arietinum* marker allele; A_ = homozygous and heterozygous for *C. arietinum* marker allele; EE = homozygous for *C. echinospermum* allele; E_ = homozygous and heterozygous for *C. echinospermum* marker allele; AE = heterozygous

analysis detected that the marker $XLRRb_{280}$ was just above the significance threshold (Table 3).

In general, F_2 individuals that had marker alleles from the *C. echinospermum* parent were more resistant than F_2 individuals that had marker alleles derived from Lasseter (Table 4). Comparison between the homozygote and heterozygote classes for three STMS markers indicated additive gene action for seedling resistance (e.g. GA2) because the heterozygote classes had mean disease scores that were approximately equal to the midpoint between the two homozygous classes.

All markers that were significantly associated with seedling resistance were tested for epistatic interactions. The exceptions were significant markers that were closely linked to markers with higher R^2 values; only these markers were considered to be associated with QTLs. Markers were tested for interactions with other markers associated with seedling resistance that were either unlinked or located at least 50 cM apart. No epistatic interactions were detected between markers for seedling resistance.

QTLs for stem resistance

Five markers were significantly associated with stem resistance (Table 3). Four markers were also associated for seedling resistance; marker $CS66_{2600}$ was just above the threshold for seedling resistance (Table 3). F_2 individuals that had $CS66_{2600}$ and $CS5b_{650}$ marker alleles from *C. echinospermum* were more resistant than individuals that had marker alleles from Lasseter (Table 5). However, F_2 individuals that had $B17c_{560}$, $CS5a_{1180}$ and $CS15c_{830}$ marker alleles from Lasseter were more resistant than individuals that had marker alleles from *C. echinospermum* (Table 5). No interactions were detected between the markers using two-way ANOVA.

Table 5 Genotypic means for markers associated with stem resistance

Marker[a]	Allele	Linkage group	Genotypic mean (%)[b]			
			AA	E_	EE	A_
$CS66_{2600}$	E	2	84	70	–	–
$CS5b_{650}$	A	3a	–	–	62	77
$CS5a_{1180}$*	E	5	59	76	–	–
$OPB17c_{560}$	A	5	–	–	84	68
$CS15c_{830}$*	E	U	64	78	–	–

[a] Lasseter marker alleles contributing resistance are indicated with asterisks
[b] AA = homozygous for the *C. arietinum* marker allele; A_ = homozygous and heterozygous for the *C. arietinum* marker allele; EE = homozygous for the *C. echinospermum* allele; E_ = homozygous and heterozygous for the *C. echinospermum* marker allele

Discussion

Seed germination

The reduced levels of germination in F_2 seed observed in this study may have been caused by genetic factors associated with *C. arietinum* × *C. echinospermum* populations. Previous studies utilising *C. arietinum* × *C. echinospermum* populations reported differences in the fertility of interspecific F_1 hybrids (Ladizinsky and Adler 1976; Singh and Ocampo 1993, 1997; Pundir and Mengesha 1995). Different *C. arietinum* and *C. echi-*

nospermum accessions were used for interspecific hybridization, which suggested that particular combinations of genotypes produced fertile hybrid plants. Therefore, *C. arietinum* and *C. echinospermum* accessions were carefully selected for crossing based on their genetic similarity (Collard et al. 2003), however, poor seed germination was still observed. Abnormal meiosis, reduced pollen fertility of F_1 hybrids and the observation of abnormal germination of F_2 seeds are similar to characteristics of the 'hybrid dysgenesis' phenomenon in wheat (Tsujimoto and Tsunewaki 1985, 1988).

The finding that all seed produced in the glasshouse had poor germination (i.e. not only F_2 seed) compared to seeds that were obtained from external sources, suggested that other factors also caused the reduced levels of seed germination. Seed dormancy is an important stage in the life cycle of many wild plants and has been previously reported for seed from wild *Cicer* species including *C. echinospermum* (Singh and Ocampo 1997). However, previous studies involving *C. arietinum* × *C. echinospermum* interspecific populations did not report seed dormancy for F_2 plants (Pundir and Mengesha 1995; Singh and Ocampo 1997). Therefore, the reduced levels of seed germination obtained for seed that was bulked in the glasshouse may also have been caused by unknown environmental factors during seed set and/or seed storage.

Construction of the linkage map

This is the first report of a linkage map produced from a *C. arietinum* × *C. echinospermum* interspecific population utilising molecular markers. The LOD score was reduced from 3 to 2 in order to attach unlinked markers to existing LGs and join smaller LGs together; however, these markers remained unlinked. The LGs 3a and 3b contained STMS markers that implied that they were from different segments of LG 3 from the integrated *Cicer* map (Winter et al. 2000). However, these two LGs could not be connected even at a LOD score of 2. This suggested that the region connecting LGs 3a and 3b remains unmapped, and the inclusion of additional markers would be needed to join the two LGs together. Alternatively, the inability to join these LGs may indicate the reciprocal translocation observed by Ladizinsky and Adler (1976) for interspecific hybrids generated from *C. arietinum* and *C. echinospermum*.

The two small LGs A and B were connected at LOD 2 and may also represent the same LG. Therefore at least seven, but possibly only six, chromosomes have been mapped, and at least one but possibly two chromosomes appear to be unmapped. Possible explanations to detect segregating markers from the(se) unmapped chromosome(s) include: inadequate marker coverage; selective elimination of certain gametes; abnormalities in a chromosome pairing; or a combination of factors. Abnormalities during meiosis in interspecific F_1 plants generated from *C. arietinum* × *C. echinospermum* crosses, that could prevent or cause drastically reduced recombination, have been previously reported (Ladizinsky and Adler 1976; Pundir and Mengesha 1995). Such factors could prevent the introgression of genes or QTLs located on these chromosomes from *C. echinospermum*.

Comparison with previous *Cicer* maps

The *C. arietinum* × *C. echinospermum* map incorporated evenly spaced RAPD markers from two previous maps derived from *C. arietinum* × *C. reticulatum* populations, since both maps were constructed using predominantly RAPD markers: the first published *Cicer* map (Simon and Muehlbauer 1997) and a map containing QTLs associated with resistance to ascochyta blight (Santra et al. 2000). Comparison of RAPD markers was difficult because the sizes of RAPD markers were not always specified in previous studies. However, based on the map order of RAPD markers, there were no similarities of marker order between the *C. arietinum* × *C. echinospermum* map and the previous *C. arietinum* × *C. reticulatum* maps. This result was not surprising since the majority of allele sizes and parental sources of RAPD markers between studies were different. These differences suggest that the RAPD markers represented different marker loci on the different maps. RAPD markers that were independently analysed from three different *C. arietinum* × *C. reticulatum* mapping populations, were generally found to be transferable in the study by Simon and Muehlbauer (1997). These findings suggest that the transferability of RAPD markers to other *Cicer* mapping populations may be limited to the species used for the interspecific cross, and may not be possible between wider crosses.

Comparison of ISSR markers was difficult because, generally, the sizes of ISSR markers were not specified in previous studies. Although only a small number of ISSR markers were incorporated in the *C. arietinum* × *C. echinospermum* map, there was no obvious similarity with previous *Cicer* maps containing ISSR markers (Santra et al. 2000; Winter et al. 2000). Therefore ISSR markers, like RAPD markers, appeared to be limited in their transferability to different *Cicer* mapping populations. As seems to be the case with RAPD markers, the transferability of ISSR markers to other *Cicer* mapping populations may be limited to the species used for the particular interspecific cross.

Only the order of STMS markers appeared to be conserved between the *C. arietinum* × *C. echinospermum* linkage map and previous linkage maps, although only 15 STMS markers were used. However, the distances between STMS markers were different between maps. The study by Choumanne et al. (2000) reported that STMS markers from chickpea are useful as syntenic markers within the first-crossibility group, that includes chickpea, *C. reticulatum* and *C. echinospermum*. The conservation of linkage of a small number of STMS markers in this study is consistent with this concept.

Segregation distortion

Overall, 27% of markers were distorted and most of these markers (60%) were distorted in favour of the wild parent. Segregation distortion from previous maps was also skewed towards the wild parent (Winter et al. 1999, 2000). The level of segregation distortion was much higher in previous *Cicer* maps, where distortion levels of 38% and 39% were reported for the integrated *Cicer* map and the STMS map, based on recombinant inbred populations (Winter et al. 1999, 2000, respectively). The distorted markers tended to be located in specific regions of the genome. Two LGs (3 and 5) with distorted markers in the present study were the same as the LGs with distorted markers from the STMS map (Winter et al. 1999). The most prominent example was LG 5 (equivalent to LG 3 from the STMS map) for which more than 50% of markers on this LG were distorted on both maps.

The detection of common regions containing distorted markers could be due to poor recombination at these regions in both populations. Alternatively, the detection of common regions could be attributable to the presence of genetic factors within these regions, which confer a selective advantage during pre- or post-zygotic phases of reproduction (Xu et al. 1997). Linkage group 7 contained a high proportion of distorted markers in the present study. However the corresponding LG from the STMS map (LG 5) did not contain any distorted markers. The distorted markers on LG 7 were from both *C. arietinum* and *C. echinospermum*, suggesting that recombination was not impaired. Therefore, the regions containing distorted markers on this LG may harbour genetic factors that provide a selective advantage to progeny derived exclusively from *C. arietinum* × *C. echinospermum* populations. Such genetic factors may have been associated with the reduced level of germination for F_2 seed.

Detection of QTLs

Phenotypic evaluation of the F_2 population was hampered by poor-seed germination. Five trials were required in order to have sufficient seed numbers for QTL analysis. Although no significant differences between trials were detected for the Lasseter controls, variation in environmental conditions (temperature, humidity and photoperiod) would probably have occurred between glasshouse trials. In hindsight, additional chickpea genotypes should have been included as controls to quantify the environmental variation between trials, although no significant differences between glasshouse trials were previously detected for three control genotypes (Collard et al. 2001). This 'background noise' may have caused some inaccuracies for the phenotypic scoring of F_2 and the parents, and thus affected the detection of QTLs identified in this study. For this reason, a relatively lenient significance threshold (LOD $\geq$ 2.0 for interval mapping and $P < 0.05$ for single-point analysis) for detection of QTLs was used, since it was more important to avoid rejecting a QTL based on low significance (Type-II error) rather than accept a false QTL (Type-I error). Therefore, all of the QTLs identified should be confirmed in other *C. arietinum* × *C. echinospermum* populations derived from the same parental accessions used in this study. Whenever possible, the identification of QTLs for any traits should be independently verified by utilizing larger population sizes, multiple replications and environments, and advanced generations or parallel populations (Haley and Andersson 1997; Young 1999).

QTLs for seedling resistance

Interval mapping and single-point analysis produced similar, but not identical results. The first putative QTL for seedling resistance, located between the markers STMS11 and TR20 within LG 4, was detected using both methods. The highest LOD score (2.5), detected by using interval mapping, coincided with the markers with the lowest P values determined using single-point analysis. This relatively large region, detected by both methods, may have contained more than a single QTL. This reflects the uncertainty inherent in QTL mapping, that is the inability to define the precise location of QTLs on a genetic map (Tanksley 1993; Liu 1998). Both interval-mapping and single-point analysis usually underestimate the number of genes determining quantitative traits because two or more QTLs, that are closer together than approximately 20 cM, usually appear as a single QTL in small populations (<500 individuals).

Interval mapping detected a second putative QTL for seedling resistance near the marker $XLRRb_{280}$ on LG 4. This marker was generated from a resistance gene-analogue (RGA) primer designed from the *Xa21* rice-gene conferring resistance against *Xanthomonas oryzae*, which causes leaf blight (Chen et al. 1998). The primer was designed from the leucine-rich repeat (LRR) region of the gene (Chen et al. 1998). The LRR motif is a conserved domain of many plant resistance genes, and LRR motifs have been implicated in protein-protein interactions or ligand binding in signal transduction pathways (Baker et al. 1997). The main function of the LRR is thought to be pathogen recognition but may participate in downstream signalling (Baker et al. 1997). Degenerate RGA primers designed from LRR sequences have been used to 'land' on resistance genes that also have conserved LRR regions. Therefore, the marker $XLRRb_{280}$ could represent the LRR region of an ascochyta blight-resistance gene. However, a higher LOD score might be expected for a marker that is part of a resistance gene; so, alternatively, marker $XLRRb_{280}$ could be linked to a QTL for seedling resistance, and actually would not be part of a resistance gene. The LRR region represented by marker $XLRRb_{280}$ could represent a segment from another resistance gene lying within a cluster of resistance genes (Kanazin et al. 1996). The mapping of additional markers flanking marker $XLRRb_{280}$ could provide a more-accurate position for the QTL in this region.

Single-point analysis detected that the marker $CS44a_{1150}$, at the distal end of LG 4, was significantly associated with seedling resistance but was just below the significance threshold using interval mapping (LOD = 1.9). The marker $CS44a_{1150}$ may also be linked to a QTL with a small effect. Alternatively, the QTL could lie beyond marker $CS44a_{1150}$ and, so, flanking markers could not be utilized by interval mapping. The confidence interval of detecting QTLs relative to flanking markers is influenced by the distance of the QTL from the end of the chromosome (Darvasi et al. 1993). Therefore, a third QTL for seedling resistance may reside near $CS44a_{1150}$, and the further addition of markers on both sides of this marker could be necessary to detect QTLs for seedling resistance using interval mapping.

Interval mapping did not detect any QTLs on LG 5, despite the identification of six markers that were significantly associated with seedling resistance using single-point analysis. One explanation for the failure to detect QTLs on this LG could be the presence of distorted markers. Segregation distortion increases the rate of false linkages in F_2 populations and affects the accuracy for determining the order of markers (Lorieux et al. 1995; Liu 1998). The identification of four other markers, including three unlinked markers that were associated with seedling resistance, suggests that additional QTLs could be located within other genomic regions.

QTLs for stem resistance

Five markers were associated with stem resistance; this was not consistent with the modified F_2 dihybrid models predicted from the phenotypic distribution for stem resistance. Therefore, stem resistance may be controlled by more than two genes. Santra et al. (2000) reported two major QTLs predicted from genetic models but found an additional QTL that was associated with ascochyta blight resistance using QTL analysis. Tekeoglu et al. (2000) reported that three major recessive genes controling resistance, predicted from genetic-model fitting; however, evidence for additional minor genes was also reported.

Four markers were also significant for seedling resistance. This could indicate associations with common QTLs that confer seedling and stem resistance. Finding the same QTL conferring both stem and seedling resistance is consistent with the high correlation between seedling resistance and stem resistance. Alternatively, the marker could be associated with two different QTLs located within the same region (one for seedling resistance and the other for stem resistance) since many plant-resistance genes are arranged in clusters (Kanazin et al. 1996).

Both the 13:3 and 9:4:3 modified F_2 dihybrid models, that were consistent with the phenotypic distributions for stem resistance, implied that the gene action for one gene was recessive and that there were epistatic interactions between QTLs. However, no epistatic interactions between QTLs were detected. One explanation for the failure to detect epistatic interactions may be the misclassification of the phenotype based on the marker genotype, since all markers were dominant. Dominant markers linked to recessive alleles may lead to the misclassification of phenotype. Alternatively, the size of the population used in this study was too small. Relatively few examples of epistatic interactions have previously been reported for plant disease-resistance mapping studies, which may be attributed to the small size of mapping populations typically used (Young 1996).

Epistatic interactions between ascochyta blight resistance genes have been previously reported by Dey and Singh (1993) and Tekeoglu et al. (2000). In both studies, epistasis was detected by analyzing the segregation of resistant and susceptible progenies in F_2 and backcross populations (Dey and Singh 1993) and recombinant inbred lines (RILs) (Tekeoglu et al. 2000). Santra et al. (2000) utilized one of the RILs generated by Tekeoglu et al. (2000) for mapping QTLs for ascochyta blight resistance. However, no epistatic interactions were detected between QTLs for ascochyta blight resistance and between QTLs and other markers (Santra et al. 2000).

Correlation with previous QTLs associated with ascochyta blight resistance

Previous efforts to map resistance genes for ascochyta blight have utilized sources of resistance from *C. arietinum* germplasm (Santra et al. 2000; Udupa and Baum 2002; Flandez-Galvez et al. 2003). Three QTLs associated with ascochyta blight resistance were detected in field trials using recombinant inbred line (RIL) populations derived from a *C. arietinum* × *C. reticulatum* cross (Santra et al. 2000). STMS markers were recently integrated into the map by Santra et al. (2000) and STMS markers located in the vicinity of QTLs for ascochyta blight resistance were identified (Tekeoglu et al. 2002). Three major loci were detected using RIL populations by Udupa and Baum (2002).

The position of QTL 2 from Santra et al. (2000) was deduced to be in the same region as QTL 1 for seedling resistance on LG 4 (Fig. 1). This deduction was based on the STMS markers incorporated by Tekeoglu et al. (2002) and the ISSR marker UBC836b (700 bp) from Santra et al. (2000). This marker may correspond to $UBC836b_{730}$ on LG 4 in the present study, assuming that the small size-difference between markers was due to errors in the estimation of size. Interestingly, the locus *ar2b* from Udupa and Baum (2002) and another QTL for ascochyta blight resistance identified from intraspecific chickpea populations were identified within the same region on LG 4 (Flandez-Galvez et al. 2003). However, due to the limitations of genetic mapping and QTL analysis, it cannot be determined whether this particular QTL is the same in all studies. This specific region within LG 4 may contain a cluster of resistance genes. More accurate information regarding the actual number of resistance genes within this cluster could be obtained by the use of

larger mapping populations and more common markers from saturated *Cicer* linkage maps. However, the identification of QTLs for ascochyta blight resistance within LG 4 deriving from different resistant genotypes strongly suggests that this region is important for ascochyta blight resistance in the *Cicer* genome.

This is the first report of the identification of markers associated with QTLs for resistance to ascochyta blight from *C. echinospermum*. The markers reported to be associated with ascochyta blight resistance, once confirmed in other populations, should be useful for identifying other markers that are more tightly linked to QTLs associated with resistance. These markers should also be useful for monitoring the location of QTLs for resistance to ascochyta blight in other *Cicer* mapping populations, especially those derived from wild *Cicer* species. Since several QTLs for ascochyta blight resistance from different populations appear to reside within LG 4, high-resolution mapping could be necessary for the identification of suitable markers for marker-assisted selection and the pyramiding of different resistance genes from different sources into chickpea cultivars.

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