Theor Appl Genet (2004) 109: 733–739
DOI 10.1007/s00122-004-1693-x

ORIGINAL PAPER

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Pathotype-specific genetic factors in chickpea (*Cicer arietinum* L.) for quantitative resistance to ascochyta blight

Received: 4 August 2003 / Accepted: 29 March 2004 / Published online: 14 May 2004

Abstract Ascochyta blight in chickpea (*Cicer arietinum* L.) is a devastating fungal disease caused by the necrotrophic pathogen, *Ascochyta rabiei* (Pass.) Lab. To elucidate the genetic mechanism of pathotype-dependent blight resistance in chickpea, F_7-derived recombinant inbred lines (RILs) from the intraspecific cross of PI 359075(1) (blight susceptible) × FLIP84-92C(2) (blight resistant) were inoculated with pathotypes I and II of *A. rabiei*. The pattern of blight resistance in the RIL population varied depending on the pathotype of *A. rabiei*. Using the same RIL population, an intraspecific genetic linkage map comprising 53 sequence-tagged microsatellite site markers was constructed. A quantitative trait locus (QTL) for resistance to pathotype II of *A. rabiei* and two QTLs for resistance to pathotype I were identified on linkage group (LG)4A and LG2+6, respectively. A putative single gene designated as *Ar19* (or *Ar21d*) could explain the majority of quantitative resistance to pathotype I. *Ar19* (or *Ar21d*) appeared to be required for resistance to both pathotypes of *A. rabiei*, and the additional QTL on LG4A conferred resistance to pathotype II of *A. rabiei*. Further molecular genetic approach is needed to identify individual qualitative blight resistance genes and their interaction for pathotype-dependent blight resistance in chickpea.

Communicated by C. Möllers

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Introduction

Ascochyta blight caused by *Ascochyta rabiei* (Pass.) Lab. in chickpea (*Cicer arietinum* L.) is an extremely devastating disease in many production areas, including the US Pacific Northwest. Since the major outbreak of ascochyta blight in the Pacific Northwest in 1987, breeding and selection for resistance has been successful in the development and release of several resistant cultivars (Muehlbauer 1996). However, new pathotypes with greater virulence—possibly due to natural recombination through the sexual cycle—now appear to cause some disease in resistant cultivars. Chemical and cultural control of ascochyta blight has proved to be expensive and inefficient, and it now appears that breeding new cultivars with enhanced resistance is needed to improve disease control.

The first genetic analysis of blight resistance in chickpea by Ahmad et al. (1952) concluded that there were two dominant genes conferring blight resistance. However, using an F_2 population from the same parental lines that were used by Ahmad et al. (1952), Hafiz and Ashraf (1953) showed that blight resistance was conferred by one dominant gene. A dominant genetic mechanism for blight resistance had been supported by many reports (Vir et al. 1975; Singh and Reddy 1983; Tewari and Pandey 1986) until Kusmenoglu et al. (1990), using a population of F_2-derived F_3 families, reported that blight resistance was conferred by two recessive genes acting additively. Tekeoglu (2000) confirmed the recessive genetic nature of blight resistance, using three recombinant inbred line (RIL) populations that were derived from crosses of resistant and susceptible germplasm lines. Santra et al. (2000) carried out a quantitative genetic study, using an interspecific genetic linkage map comprising RAPD and ISSR markers and identified three quantitative trait loci

(QTLs) for blight resistance. Tekeoglu et al. (2002) added sequence-tagged microsatellite site (STMS) markers to the same linkage map and confirmed the two QTLs for blight resistance identified by Santra et al. (2000). They postulated that the two QTLs were associated with the two recessive genes for blight resistance reported by Kusmenoglu et al. (1990). Because pathogenicity described as pathotypes is a critical factor in determining blight resistance in chickpea (Jamil et al. 2000), Udupa and Baum (2003) attempted to elucidate the genetics of pathotype-specific blight resistance in chickpea. Although they identified genomic regions for pathotype-specific blight resistance in chickpea, the genetic information was insufficient to clarify the mechanism due to the weakness of pathotype-dependent resistance patterns of their mapping population and less stringent mapping conditions to identify significant pathotype-specific resistance factors.

Therefore, our objectives were (1) to identify the genetic factors for pathotype-dependent blight resistance in chickpea using a mapping population clearly differentiating pathotype-dependent resistance and (2) to elucidate their interaction determining quantitative blight resistance.

Materials and methods

Plants

A cross of PI 359075(1) (susceptible to pathotype I and II of *A. rabiei*) × FLIP84-92C(2) (resistant to pathotype I and II of *A. rabiei*) was made at Pullman, Wash., USA in 1993, and F_2 progenies were advanced by single-seed descent to F_7 in the greenhouse during 1995–1998 (Tekeoglu et al. 2000). The resulting population of 250 RILs was evaluated for resistance to pathotypes I and II of *A. rabiei*. For genetic linkage mapping and QTL analysis for blight resistance, 133 RILs were selected from the 250 RILs to represent the distribution of disease scores of the entire population based on the report by Tekeoglu et al. (2000). The number of RILs for each resistance class was determined by the relative ratio of each class to the entire population, and selection from each resistance class was done randomly. For resistance evaluation and DNA isolation, the RILs were grown in the greenhouse for 2 weeks with 18 h of light (450 μ E m^{-2} s^{-1}) at 25°C and 6 h without light at 18°C. Relative humidity in the greenhouse varied from 30% to 70%. To achieve high relative humidity after inoculation, the RILs were grown in a growth chamber maintained at 100% relative humidity, and other growth conditions in a growth chamber were similar to those in the greenhouse.

Preparation of *A. rabiei* inoculum and resistance evaluation

Two pathotype I isolates, Ar19 and Ar21d, and ten pathotype II isolates that were randomly selected from the collections by Chen and Muehlbauer (2002) were grown on V-8 agar media prepared by the method of Tuite (1969) with the following modifications: 2 g of calcium carbonate and 14 g of agar/l were used. Cultures were grown at 20°C with 12 h light/dark for 2 weeks. Spores were harvested from agar plates and diluted in distilled water to 10^5 spores/ml. Inoculum was sprayed on chickpea leaves until leaves were completely covered. To maintain maximum relative humidity after inoculation, each pot was covered with a disposable plastic cup for 24 h (Chen and Muehlbauer 2002). Assessment of resistance was done 2 weeks after inoculation. Blight resistance of each RIL was scored based on a 1 = resistant to 9 = susceptible scale following the method of Tekeoglu et al. (2000). Field data were from the previous study by Tekeoglu et al. (2000). Statistical analysis of the data was carried out using Statistix, version 7.0 (Analytical Software, Tallahassee, Fla., USA). All experiments were repeated to verify the results.

Genetic mapping and QTL analysis

DNA samples were isolated from young leaves of the RILs using the CTAB method of Weising et al. (1995). For genetic linkage mapping, 186 STMS primer sets were screened to identify polymorphism between the two parents. PCR for STMS amplification was carried out according to Hüttel et al. (1999). Polymorphic STMS primers were used as genetic markers for linkage analysis. Each segregating marker was tested for goodness of fit to the expected 1:1 ratio by a Chi-square test ($P<0.05$). Markers with distorted distribution were rescored to confirm the segregation ratio, and all markers, including ones with distorted distribution, were used for linkage mapping. Genetic linkage mapping was carried out using MAPMAKER 3.0 (Lander et al. 1987) at an LOD score of 3.0, with a maximum distance of 25 cM between any two loci. The resulting linkage map was compared to the previous maps (Winter et al. 2000; Cho et al. 2002; Tekeoglu et al. 2002) to construct a partial consensus map based on common STMS markers in comparable linkage groups.

QTL analysis for blight resistance was carried out with the simple interval mapping function using Qgene (Nelson 1997) at an LOD score of 3.0. Single-point regression analysis was used to identify markers significantly associated with blight resistance.

Assessment of qualitative genetic effect of a major blight resistance gene contributing to quantitative blight resistance

To investigate the existence of a major gene for blight resistance and its contribution to quantitative blight resistance in chickpea, each RIL was phenotyped as resistant or susceptible by comparing its disease score to the median of the entire population, which generated a 1:1 segregation of RILs for blight resistance. Fisher's least significant difference (LSD) values were also calculated based on the variation of disease scores in the two parents of the population and the average disease score of the parental line, plus its LSD value was used as a statistical standard to phenotype RILs for resistance. Genetic linkage of blight resistance genes to STMS markers was calculated as mentioned above. QTL mapping for blight resistance was carried out without blight resistance genes on the genetic linkage map to eliminate unnecessary interaction between quantitative and qualitative data.

Results

Blight resistance of the RILs influenced by pathotypes and relative humidity

Resistance tests were carried out using isolates of pathotypes I and II of *A. rabiei* to inoculate the RIL population. The distributions of the disease scores within the RIL population were plotted using a 1 = resistant to 9 = susceptible scale (Fig. 1). When a single isolate of pathotype I, Ar19, was used to inoculate the RILs in the greenhouse, a skewed distribution of disease scores—mostly ranging from 1 to 3—was observed (Fig. 1a). The mean disease score was only 3.4 (SD=1.87). When the RILs were inoculated with one of the most virulent isolates of pathotype II in the greenhouse, CAB02-14,

distribution of disease scores followed a normal distribution (P=0.1010 by the Shapiro and Wilk test), and the mean disease score was only 5.2 (Fig. 1b). Because maximum relative humidity and optimum temperature for maximum pathogenicity of *A. rabiei* was achieved in the greenhouse by the method of Chen and Muehlbauer (2002), the pathotypes of *A. rabiei* were thought to be a major factor causing significant differences in the resistance patterns between pathotype I and II (Fig. 1a, b, respectively). These resistance patterns were significantly different from the one observed in the field (Tekeoglu et al. 2000; Fig. 1c). The mean disease score of the RILs in the field was 6.0 (SD=2.61), and only 25% of this population was classified as resistant in the field (Tekeoglu et al. 2000). When a mixture of pathotype II isolates was used to inoculate the RILs in a growth chamber maintained at 100% relative humidity, the majority of the RILs showed severe blight symptoms on leaves and stems within a week after inoculation (Fig. 1d). The distribution of disease scores was similar to that in the field with a correlation coefficient of 0.83 (P<0.001). This result showed that high relative humidity throughout the growth period was a crucial factor for severe blight symptoms in chickpea.

Genetic linkage mapping

A total of 62 out of 186 STMS primer sets showed polymorphism between the two parents, and 53 of them were mapped to eight linkage groups (LGs) composed of 11 subgroups covering 318.2 cM of chickpea genome (Fig. 2). The location of STMS markers showed a very high degree of similarity to previously reported linkage maps of Cho et al. (2002), Tekeoglu et al. (2002), and Winter et al. (1999) (Fig. 2). LG2 and LG6 that were previously reported as two separate LGs by Cho et al. (2002) and Winter et al. (1999) were combined to form one LG and named LG2B+6B. Two subgroups, LG2A and LG6A, were postulated to be linked to LG2B+6B at each end when this map was compared to previously reported interspecific and intraspecific linkage maps. Hypothetical grouping of the subgroups between LG4A and LG4B and between LG8A and LG8B were also possible by the same approach.

QTL mapping of blight resistance in chickpea

QTL analysis was carried out using two sets of disease scores collected in the greenhouse after inoculation with single isolates of pathotype I, Ar19 and Ar21d, and two sets of data collected in the field and a growth chamber after inoculation with a natural population of *A. rabiei* and a mixture of pathotype II isolates, respectively. Five QTLs for ascochyta blight resistance were identified on our linkage map (Fig. 3). Two QTLs for resistance to a pathotype I (Ar19 and Ar21d) were colocated between GA20 and GA16 on LG2A+6B, with LOD scores of 3.08 and 2.66, respectively. Because of the expected linkage between two subgroups, LG2A and LG2B+6B (Fig. 2), two QTLs for resistance to a pathotype I located on LG2A and LG2B+6B were postulated to be combined to a single QTL. Another QTL for resistance to pathotype I (Ar21d)

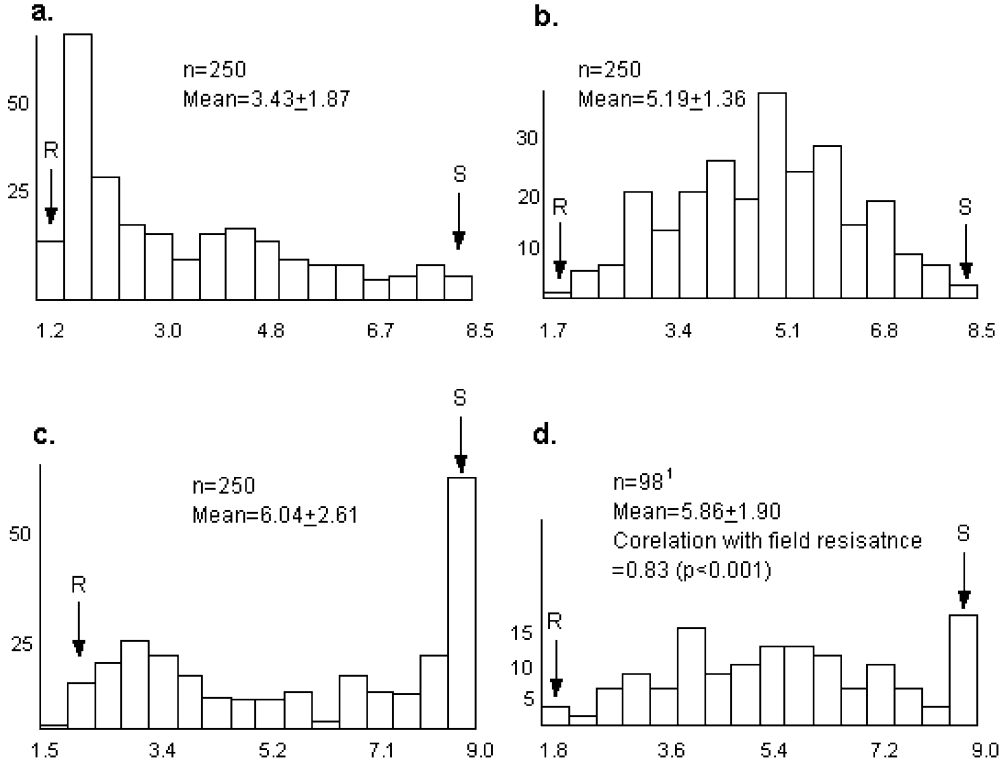

Fig. 1a–d Distribution of disease scores for two-week-old plants from a recombinant inbred line (RIL) population from the cross of PI 359075(1) (susceptible to pathotype I and II of *A. rabiei*) × FLIP84-92C(2) (resistant to pathotype I and II of *A. rabiei*) after inoculation with pathotype I and II of *A. rabiei*. **a** Inoculation with a single isolate of pathotype I, Ar19, in the greenhouse. **b** Inoculation with a single isolate of pathotype II, CAB02-14, in the greenhouse. **c** Inoculation with a mixture of natural *A. rabiei* population in the field. **d** Inoculation with a mixture of pathotype II isolates at 100% relative humidity in a growth chamber. All pots inoculated in a growth chamber and in the greenhouse were covered with plastic cups for 24 h after inoculation, following the method of Chen and Muehlbauer (2002). *R* and *S* represent the average disease scores of FLIP84-92C(2) and PI 359075(1), respectively

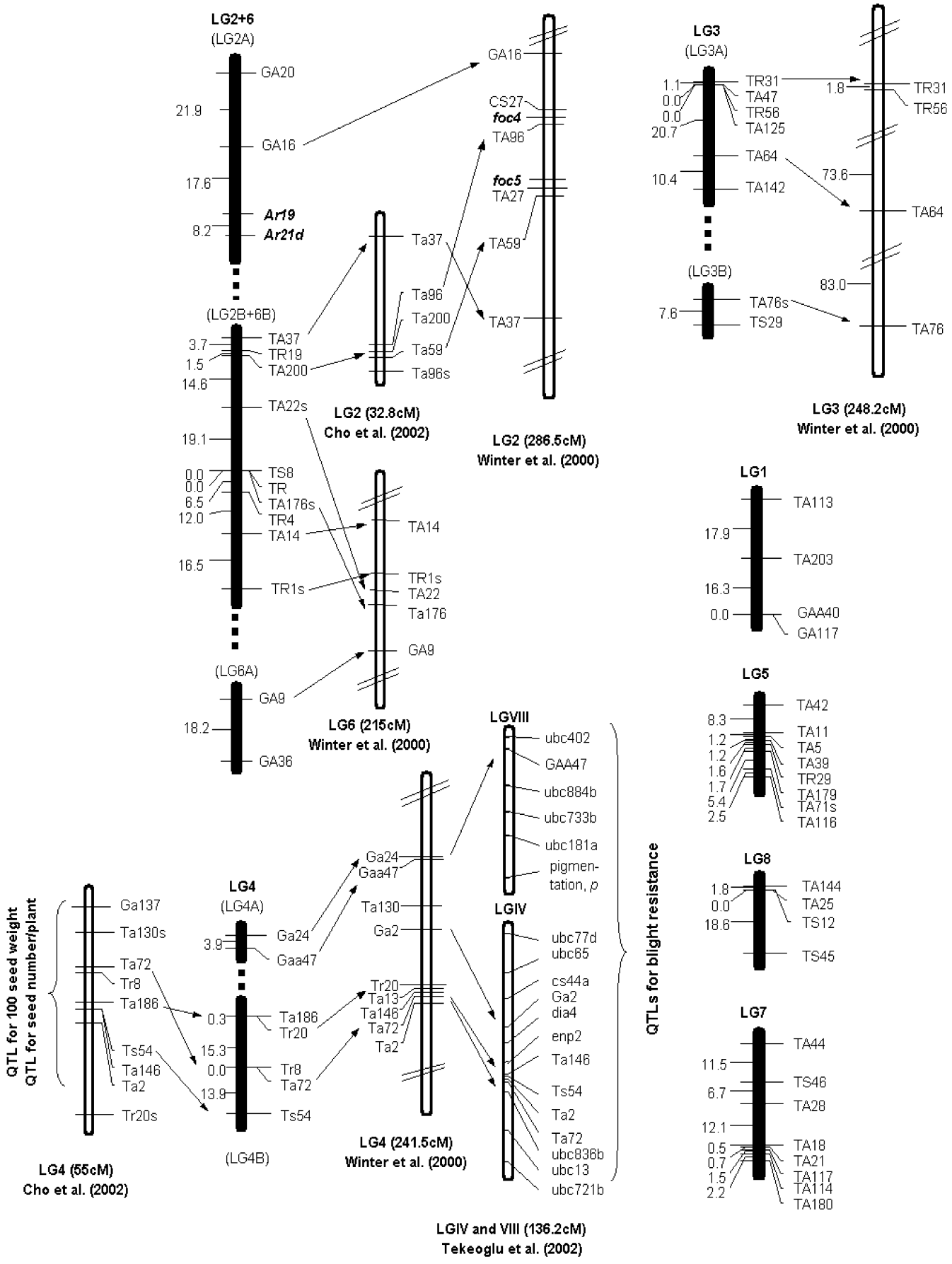

Fig. 2 Genetic linkage map of sequence-tagged microsatellite site (STMS) markers constructed using the RILs from an intraspecific cross of PI 359075(1) × FLIP84-92C(2) (*black bars*). Joint map was constructed by aligning the common STMS markers between our current map (*black bars*) and the previous maps (*white bars*) by Winter et al. (2000), Cho et al. (2002), and Tekeoglu et al. (2002)

was identified on LG2B between TA37 and TA200, with an LOD score of 3.69. One QTL for blight resistance in the field was mapped to LG4A between GA24 and GAA47, with an LOD score of 4.17. Another QTL for resistance to a mixture of pathotype II isolates in a growth chamber colocated with the QTL for blight resistance in the field, with an LOD score of 2.83. Two QTLs for blight resistance on LGIV and LGVIII of an interspecific linkage map of Tekeoglu et al. (2002) appeared to be the same as the QTLs on LG4A of our map (Figs. 2, 3).

By single-point regression analysis, Ta46, which did not show linkage to other markers, was chosen as the best single molecular marker, and explained 69.2% of the variation for resistance to Ar21d and 59.2% of the variation for resistance to a mixture of pathotype II isolates in a growth chamber. However, the STMS markers associated with the QTL for blight resistance—such as GAA47, GA24, and GA16—explained only 10.4–19.3% of the variation of resistance within the population. Low r^2 values of these STMS markers, regardless of the existence of the QTL for blight resistance, might indicate possible location of blight resistance genes between these STMS markers that could be detected only by crossing over between the flanking markers.

Two intraspecific linkage maps, including our current map and two interspecific linkage maps, were aligned parallel to each other based on their common STMS markers (Fig. 2). Two QTLs for resistance to pathotype I of *A. rabiei* on LG2A+6A of our map overlapped with the region of the recessive fusarium wilt resistance genes *foc4* and *foc5* on an interspecific map by Winter et al. (1999). Further genomic and biochemical studies are required to elucidate the significance of this genomic region for fungal resistance in chickpea.

QTLs for 100 seed weight and seed number per plant on LG4 on an intraspecific map by Cho et al. (2002) matched with the QTL for blight resistance in the field identified on LGIV on an interspecific map of Tekeoglu et al. (2002).

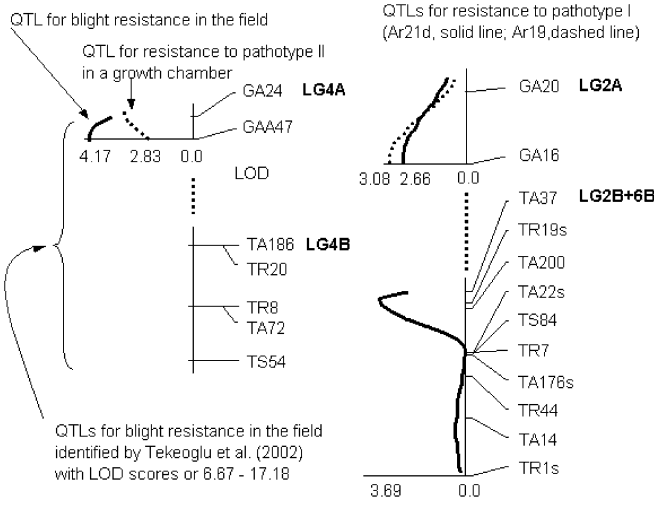

Fig. 3 Quantitative trait loci (*QTLs*) for ascochyta bight resistance in chickpea identified on an intraspecific genetic linkage map from the cross of PI 359075(1) × FLIP84-92C(2)

Even though genetic linkage between blight resistance and seed size was proposed by Singh and Reddy (1996), functional correlation between the two traits has not been proven yet, and no correlation between the two traits was observed within the RIL population used in this study (data not provided). Genetic relationship between seed size and blight resistance might be due to physical linkage between the genes for the two traits without functional correlation.

Qualitative resistance gene contributing to quantitative blight resistance

The RIL population was phenotyped for resistance to pathotype I isolates of *A. rabiei*, Ar19 and Ar21d, by comparing disease scores of each RIL to the median score for the population. Segregation ratios of RILs based on the median values of 2.92 for Ar19 and 3.0 for Ar21d were 131:119 and 61:54, respectively. Fisher's LSD (at P=0.05) values of the two parents, FLIP84-92C(2) and PI359075 (1) were 0.52 and 1.09, respectively. According to these LSD values, 1.52 [average disease score of 1.0 in FLIP84-92C(2) plus LSD of 0.52] was decided as the upper disease score limit to phenotype RILs as resistant, and 7.41 [average disease score of 8.5 in PI 359075 (1) minus LSD of 1.09] was the lower limit to phenotype RILs as susceptible. However, these LSD values could not be applicable to phenotype the RILs for blight resistance because the disease score of 1.52 was too stringent to phenotype the RILs for resistance and under this criterion, the RILs with disease scores ranging from 1.52 to 3.0 could be phenotyped as susceptible in spite of their obvious resistance. A disease score of 3.08 [average disease score of 2.0 in FLIP84-92C(2) in the field plus LSD value of 1.08] was used to classify the same population for field resistance (Tekeoglu et al. 2000), and this score was thought to be statistically feasible to phenotype RILs for blight resistance and to map a single gene for resistance. Phenotyping of resistance to pathotype II to identify a single locus was not attempted because of the multigenic nature of resistance. The RILs resistant to pathotype I segregated for resistance to pathotype II, and two or more genes segregating independently from a gene for resistance to pathotype I were required for resistance to pathotype II (data not presented). Therefore, resistance to pathotype II was thought to be determined by additive interaction between a gene for resistance to pathotype I and additional genes.

Genetic linkage mapping of the genes for resistance to pathotype I isolates of *A. rabiei*, Ar19 and Ar21d, was carried out. The gene for resistance to Ar19 named *Ar19* was linked to Ga16 on LG2+6 at the distance of 17.6 cM, and the gene for resistance to Ar21d named *Ar21d* was linked to *Ar19* at the distance of 8.2 cM (Fig. 2). In fact, these two genes were expected to be the same because the map distance between the two loci might be generated simply by experimental errors in disease scoring or minor differences in virulence between Ar19 and Ar21d. Genetic

linkage between *Ar19* (or *Ar21d*) and the STMS markers for the QTL for resistance to pathotype I indicated the possible existence of a single gene for resistance to pathotype I in this genomic region.

Discussion

Pathotype-dependent blight resistance in chickpea

Blight resistance patterns of the RILs from the cross of PI 359075(1) (susceptible to both pathotypes I and II of *A. rabiei*) × FLIP84-92C(2) (resistant to both pathotypes) varied significantly, depending on the pathogenicity of *A. rabiei* (Fig. 1) that was determined by relative humidity and pathotypes. No complete immunity of chickpea to *A. rabiei* has been identified even in highly resistant chickpea lines and pathotypes with greater virulence always had potential to cause disease regardless of the level of resistance. In that aspect, blight resistance in each RIL seemed to be genetically predetermined at a certain level that could be overcome by pathotypes with sufficient virulence.

Unlike the race-specific wilt resistance of *F. oxysporum* f. sp. *ciceri*, blight resistance did not appear to be induced by resistance mechanisms. Fusarium wilt resistance can be enhanced temporarily by inoculating susceptible lines with an avirulent race of *F. oxysporum f*. sp. *ciceri* (Hervas et al. 1995). However, isolates of pathotype II of *A. rabiei* always caused blight symptoms in the RILs regardless of preinoculation of the RILs with an isolate of lower virulence (S. Cho and F.J. Muehlbauer, unpublished data).

One of the most important factors deciding pathogenicity of *A. rabiei* was relative humidity after inoculation. Generally, humidity is considered important for pathogenicity of *A. rabiei*. Roger et al. (1999) reported that extended periods of humid conditions were required for pycnidial formation and disease development by *A. pisi* in pea. Similar to the case of ascochyta blight in pea, maximum humidity conditions dramatically enhanced pathogenicity of *A. rabiei* in chickpea. When maximum relative humidity was maintained by covering each RIL with a plastic cup following the method of Chen and Muehlbauer (2002) for more than 2 days after inoculation, most of the RILs, including resistant lines, showed severe blight symptoms (S. Cho and F.J. Muehlbauer, unpublished data). Conversely, disease symptoms on the RILs after inoculation with pathotype II isolates of *A. rabiei* were hardly observed even in a growth chamber unless the RILs were covered with plastic cups to achieve maximum humidity. Similar results were reported by Chen and Muehlbauer (2002). Severe blight symptoms in chickpea after precipitation for 3 or more days in the field during cool growing seasons might explain the outbreak of blight symptoms even in resistant chickpea lines (F.J. Muehlbauer, unpublished data,). Because changes in pathogenic and environmental conditions can significantly influence resistance patterns and eventually affect the genetic explanation of the data, careful observation of experimental conditions both in controlled and field conditions is recommended for reliable genetic tests of disease resistance.

Quantitative genetics of ascochyta blight resistance in chickpea

From significantly different resistance patterns of the RIL population, depending on pathotypes of *A. rabiei* (Fig. 1), pathotype-specific QTLs for blight resistance were identified on LG2+6 and LG4A. Because the QTL for resistance to pathotype II on LG4A colocalized with the QTL on an interspecific map by Tekeoglu et al. (2002), we concluded that the QTL on LG4A is the major locus for resistance to pathotype II of *A. rabiei* in chickpea. Two QTLs for resistance to pathotype I on LG2A and LG2B +6B were postulated to be combined to a big group based on the comparison of our current map to the map by Winter et al. (2000), and a single resistance gene named *Ar19* (or *Ar21d*) appeared to explain majority of resistance conferred by these QTL. *Ar19* (or *Ar21d*) was thought to be required for resistance to both pathotypes because the RILs resistant to pathotype I segregated for resistance to pathotype II. Therefore, resistance to pathotype II was thought to be determined by additive interaction between *Ar19* (or *Ar21d*) and the QTL on LG4A. Even though resistance to both pathotypes appeared to require *Ar19* (or *Ar21d*), a major locus providing resistance to severely virulent isolates of *A. rabiei* could be the QTL identified on LG4A. However, the inheritance pattern and the genomic location of the major gene conferring enhanced resistance to pathotype II are still not clear because the QTL for resistance to pathotype II identified on LG4A implies the unknown additive interaction between two or more genes. Further genomic study to identify individual genes for blight resistance inferred within the major QTLs is needed to elucidate the allelic or intergenic interaction among the resistance genes and eventually to enhance blight resistance in chickpea through resistance gene pyramiding.

Various mechanisms explaining blight resistance in chickpea have been proposed (Ahmad et al. 1952; Dey and Singh 1993; Tekeoglu et al. 2002). Although the quantitative nature of blight resistance in chickpea was revealed (Kusmenoglu et al. 1990; Tekeoglu et al. 2000; Flandez-Galvez et al. 2003), genetic roles of the genes in pathotype-dependent blight resistance and dominance or recessiveness of the genes could not be clarified because of dramatic changes in resistance patterns of the population depending on the pathogenic and the environmental conditions. Udupa and Baum (2003) attempted to map the genes for pathotype-specific blight resistance on an intraspecific linkage map from the cross of ILC 1272 × ILC 3279. They identified a single major gene, *ar1*, conferring resistance to pathotype I on LG2 and two QTLs for resistance to pathotype II, *ar2a* at *ar1*, on LG2 and *ar2b* on LG4. However, identification of the QTL at *ar1* on LG2 was expected to be analytical redundancy by

associating quantitative data with pre-existing resistance gene. Without *ar1* on LG2A, no QTL for resistance to pathotype II could be detected on LG2. Considering this issue, two genes, *Ar19* and *Ar21d*, were eliminated from the linkage map before QTL analysis and a major QTL was found only on LG4A. The result clearly showed that the major gene for resistance to pathotype II was different from that for resistance to pathotype I. Unlike the resistance patterns observed in our study, distribution of disease scores in the RIL population used by Udupa and Baum (2003) after inoculation with pathotype I was rather continuous and showing many RILs with disease scores lower or higher that those of two parents. This indicated that their case had too many unknown genetic factors to conduct qualitative genetic analysis of resistance to pathotype I. Using more appropriate experimental materials and methods, we could confirm genetic mechanism of pathotype-dependent blight resistance in chickpea.

In conclusion, the genetic factors for pathotype-dependent blight resistance were dissected using a RIL population showing significantly different resistance patterns to pathotypes I and II. Significant variation in blight resistance within the RIL population generated from a cross of PI 359075(1) × FLIP84-92C(2), depending on the pathogenicity of *A. rabiei* was identified. Differential resistance of RILs depending on the pathogenicity of *A. rabiei* enabled identification of pathotype-specific genetic factors on an intraspecific linkage map. *Ar19* (or *Ar21d*) on LG2+6 appeared to provide majority of quantitative resistance to pathotype I and partial resistance to pathotype II of *A. rabiei* and the QTL on LG4A was required for resistance to pathotype II of *A. rabiei*. The results presented in this report are applicable to further genomic and biochemical studies to identify individual blight resistance genes in chickpea.

Acknowledgements The authors thank to Drs. A. Kleinhofs, S.S. Jones, L.A. Hadwiger, and V.R. Franceschi for critical discussion and manuscript review.

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