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IDENTIFICATION OF NEW QTL CONFERRING RESISTANCE TO ANTHRACNOSE (*ASCOCHYTA RABIEI*) IN A RIL POPULATION OF CHICKPEA

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ABSTRACT

Ascochyta blight (AB) is a consistent problem affecting large growing areas of chickpea in all countries where this crop is cultivated. This disease is capable of causing large yield losses under conducive environmental conditions. To characterize the genetics of resistance to AB in chickpea, a population consisting of 77 recombinant inbred lines (RILs) derived from an inter-specific cross of *Cicer arietinum* (FLIP84-92C, resistant parent) x *Cicer reticulatum* Lad. (PI 599072, susceptible parent) was used. Each RIL and the parents were inoculated with blight spores by spraying. The RILs were scored for disease reactions under greenhouse conditions at 20 °C in a 12 h photoperiod. A linkage map was constructed using RAPD markers. Eleven linkage groups were obtained, of which three were small. The map spanned 889.1 cM with an average marker density of 10.1 cM. Two QTL were detected on linkage groups 1 and 4, which together explained 31% of the total phenotypic variation for AB resistance. These markers can improve precision of molecular breeding in this population.

Keywords: Ascochyta blight, chickpea, linkage map, QTL analysis.

INTRODUCTION

Cultivated chickpea (*Cicer arietinum* L.) is a self-pollinated diploid (2n=2x=16) annual grain legume, and an important crop for the Indian subcontinent, West Asia, North Africa, Southern Europe, and North and Central America (Santra et al., 2000). It is the third most cultivated pulse after dry beans and peas (http://faostat.fao.org).

Ascochyta blight (AB) caused by *Ascochyta rabiei* (Pass) Labr (teleomorph: *Didymella rabiei* (Kov.) v. Arx.) is the most important disease in chickpea, causing up to 100% crop loss under favorable conditions for the pathogen (Singh and Reddy, 1983). This fungus infects all aerial parts of the plant and causes necrotic lesions, which are circular on the leaflets and pods, and are elongated and irregular on the stems and petioles (Bayraktar et al., 2007). Ascospores (the sexual phase) are the primary inoculum for AB epidemics, which are ejected from pseudothecia and disperse through the wind (Barve et al., 2003). The sexual phase of the pathogen is important in disease epidemiology and pathogen diversity (Morjane et al., 1994), and the asexual phase repeatedly causes secondary disease cycles during the growing season (Jamil et al., 2000).

The character of AB resistance in chickpea was reported in many studies to be controlled by one or two dominant or recessive complementary genes (Vir et al., 1975; Eser, 1976; Singh and Reddy, 1983; Tewari and Pandey, 1986). Resistance may be inherited as quantitative character Muehlbauer and Kaiser, 1994; Millan et al., 2003). On the other hand, it has been suggested that two complementary genes (Santra et al., 2000; Kusmenoglu, 1990), one recessive and five dominant genes (Dey and Singh, 1993) control resistance in chickpea. Tekeoglu et al. (2000) demonstrated that AB resistance was controlled by two or three complementary major recessive genes and several minor modifiers.

Quantitative trait locus (QTL) mapping is an effective method for studying complex and polygenic forms of disease resistance (Young, 1996). To date, a number of QTL for resistance to AB have been identified (Santra et al., 2000; Millan et al., 2003; Tekeoglu, 2002; Flandez-Galvez et al., 2003; Udupa and Baum, 2003; Cho and Muehlbauer, 2004; Iruela et al., 2006). The primary QTL for AB resistance derived from 'FLIP84-92C' and

'ILC3279' were located on LG2 and LG4 (Santra et al., 2000; Tekeoglu, 2002; Udupa and Baum, 2003; Cho and Muehlbauer, 2004). Santra et al. (2000) conducted a study to determine the genetics of resistance to AB in chickpea and detected two QTL (QTL-1 with interval markers of UBC733b, UBC181a and QTL-2; UBC836, Dia4) on a linkage map constructed with RAPD, ISSR, and isozyme markers in a recombinant inbred line (RIL) population derived from a cross between *C. arietinum* (resistant) x *C. reticulatum* (susceptible). Using the same population, Tekeoglu et al. (2002) integrated six co-dominant sequence-tagged microsatellite site (STMS) markers to QTL regions (Gaa47 on QTL-1 and Ta72s, Ta2, Ts54, Ta146, and Ga2 on QTL-2). In addition, using the same RIL population, Rakshit et al. (2003) mapped DNA Amplification Fingerprinting (DAF) markers (OPS06-1 and OPS03-1) tightly linked to the locus where QTL-1 was located.

This study aimed to identify new QTL associated with resistance to AB in a RIL population derived from an inter-specific cross between *C. arietinum* x *C. reticulatum*.

MATERIALS and METHODS

Plant materials

The plant material was kindly supplied by Dr. Fred J. Muehlbauer from the USDA Agricultural Research Service, Washington State University, Pulman US and delivered to Ege University, Department of Bioengineering, Molecular Genetics Laboratory. The RIL (F6:7) population was generated by the single-seed descent method. Seventy-seven RILs obtained from an inter-specific cross between *C. arietinum* (FLIP 84-92C, resistant) x *C. reticulatum* (PI 599072, susceptible) were used for mapping the population.

DNA isolation and PCR assay

The miniprep method described by Doyle and Doyle (1990) was used with some modifications. Three-four young chickpea leaves were collected in a 1.5 ml microfuge tube. The leaves were ground to a fine powder in liquid nitrogen. 300 µl of ice-cold extraction buffer (25 ml nuclei lysis buffer, 10 ml 5% sarcosyl, 0.1 g sodium bisulfite), 300 µl of nuclei lyses buffer (2 g CTAB, 1.8 g EDTA, 11.6 g NaCl, 20 ml 1M Tris pH:8) and 125 µl of 5% sarcosyl was added, vortexed for 30 seconds, incubated in a 65 °C water bath for 20 min, and cooled for 5 min. 725 µl of chloroform: iso-amyl alcohol (24:1) was added, and the tubes were gently mixed for 5 min, and then centrifuged at 2200 rpm for 15 min. The aqueous phase was transferred to a fresh tube, and the DNA was precipitated with an equal volume of cold ethanol. A short centrifugation at 5,000 g for 1 min was applied to precipitate DNA to the bottom of the tube, the ethanol was removed, and 700 µl of ethanol was added to wash the DNA pellet. After the removal of ethanol, the samples were placed in a cabinet, air-dried overnight, dissolved in 500 µl of TE buffer (pH 7.5), and stored at -20 °C until use.

Random amplified polymorphic DNA (RAPD) analysis

RAPD analyses were performed as described by Paran et al. (1997). A total of 384 10-mer RAPD primers (Operon Technology Inc. Alameda CA, US) were used to survey the parents. The primers that displayed polymorphism in parents were applied to the population. RAPD analyses were repeated four times to confirm the same band pattern for the same primers. PCR analysis was carried out in 15 µl reaction buffer containing 20-40 ng of genomic DNA, 10X buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-Cl (pH 8.0, 22 °C), 1% Triton X-100, 1mg/ml BSA), 2 mM of $MgSO_4$, 5 pmol of primer, 100 µM of each dNTP, and 1 U of Taq DNA polymerase enzyme. PCR reactions were achieved in PTC-100 and PTC-225 Peltier thermal cyclers (MJ Research, Inc. Nevada, US). The amplification was started with denaturation at 94 °C for 30 s, followed by 35 cycles of 94 °C for 25 s, 35 °C for 45 s, and 72 °C for 1 min. The cycling was completed with a final extension at 72 °C for 5 min and the samples were kept at 4 °C for an indefinite time.

Inter simple sequence repeat (ISSR) analysis

For the ISSR analysis, the procedure given by Tanyolac (2003) was followed. The annealing temperatures for ISSR primers (807, 808, 809, 810, 812, 813, 814, 815, 846 and 847, University of British Columbia) varied between 46 °C and 52 °C according to the GC content. Amplification products (both RAPD and ISSR) were resolved in 2% agarose gel, stained with ethidium bromide, and visualized using Kodak EDAS 290 imaging system under UV light. The size marker Lambda DNA digested with EcoRI and HindIII (Fermentas; #SMO191) was used in every gel to determine the molecular weights of the PCR products.

Statistical analysis

Analysis of variance (ANOVA) of the resistance reaction of RILs was performed to ascertain the homogeneity of infection in the experimental plot. Segregation of each marker in the RIL population was analyzed for goodness of fit to the expected ratio of 1:1 using the chi-square test. Construction of linkage groups (LGs) was performed using MAPMAKER V3.0b (Lander et al., 1987). A LOD score threshold of 3 and a maximum recombination fraction of 0.25 were employed to establish LGs. The Kosambi mapping function was used to estimate map distances (Kosambi, 1994). Markers with a probability level of $P < 0.001$ based on LOD were considered to have a good association with a putative QTL. Putative QTL detection was carried out using single markers analysis (SMA) and interval mapping with a mapping step size of 1 cM in Qgene V.3.06 (Nelson, 1997)

Evaluation of RILs for reaction to AB

Three seeds for each RIL were planted into viols (11x20 wells) at the greenhouse of the Department of Bioengineering at Ege University on the May 25, 2006. The seedlings were transferred to pots containing a 50% soil-turf mixture. All pots were moved to the growth room (20 °C and 12 h photoperiod) in the same department on June 19, 2006. AB obtained from Gaziantep University, Turkey had previously been tested by Ozkilinc et al. (2010) in terms of aggressiveness. The spores of AB were sprayed with a spore suspension of 1 x 10^6 spore/ml on June 20, 2006. The room was pulverized with water three times a day to maintain the humidity level required by the pathogen to develop the disease. The disease symptoms of RILs were scored two weeks after inoculation. Scoring was performed according to the 1-9 scale of Singh and Reddy (1983) modified by Collard et al. (2001). The pot trial was designed as three replications for each RIL.

RESULTS and DISCUSSION

A total of 77 RILs were evaluated for AB reaction. The frequency distribution of the disease scores of RILs is shown in Figure 1. The mean disease scores of RILs and the resistance lines were 4.72 and 2.17, respectively. The disease scores of the resistant parent (FLIP 84-92C) and susceptible parent (PI 599072) were 1 and 9, respectively.

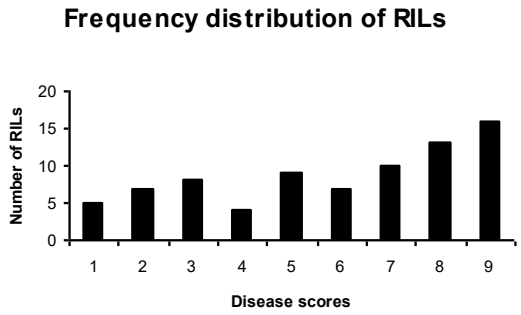

Figure 1. Frequency distribution of the disease scores.

A total of 384 decamer primers and 10 ISSR primers were surveyed to detect polymorphism among the parents. Of the primers surveyed, 136 decamers and 7 ISSRs were polymorphic (37%). The amplification of polymorphic primers in RIL DNAs revealed 90 clear and scorable bands that generated 160 polymorphic bands. The average band per polymorphic primer was 0.56. The 160 polymorphic bands were subjected to a linkage analysis, which revealed 11 LGs containing 118 molecular markers that covered 889.1 cM. A total of 42 markers were unlinked. The average marker distance was 10.1 cM. The linkage map statistics are listed in Table 1, and the molecular marker map of the chickpea genome is shown in Figure 2.

Table 1. Characteristics of the linkage map.

Linkage Groups	Length (cM)	Number of Markers	Average Distance (cM)
LG 1	5,8	3 (% 3,4)	1,9
LG 2	28,6	3 (%3,4)	9,5
LG 3	316,8	25(%28,7)	12,7
LG 4	246,5	23(%26,4)	10,7
LG 5	70,1	8 (% 9,2)	8,7
LG 6	58,2	5 (% 5,7)	11,6
LG 7	84,8	8 (% 9,2)	10,6
LG 8	14,0	2 (% 2,3)	14,0
LG 9	35,4	5 (% 5,7)	7,0
LG 10	12,1	2 (% 2,3)	12,1
LG 11	16,9	3 (% 3,4)	5,6
Total	**889,1**	**87**	**10**

SMA showed that F14a (1590), F14(700) and AD16a(677) markers on LG1, and D18(831), G07(40) and AE18a(570) markers on LG4 had a significant association with resistance to anthracnose ($P<0.001$). F14a (1590) explained 20% of total phenotypic variation alone. Two QTL were identified on LG1 and LG4 by interval mapping (Table 2). Multiple regression analysis revealed that these QTL explained 31% of total phenotypic variation together. Interval mapping of these QTL is presented in Figure 3.

It was considered that the three markers located on LG1 could be useful for marker assistant selection (MAS) studies. For this purpose, the resolution of the existing map can be enhanced or this locus can be integrated into different chickpea linkage maps.

In this study, we used a *C. arietinum* x *C. reticulatum* inter-specific RIL population. RIL populations are frequently preferred in mapping studies since they are homozygous and allow working in different environmental conditions. Another advantage of RILs is that contrary to F2 populations, dominant and co-dominant markers have similar information content, which allows the integration of dominant markers, such as RAPD, which are easy-to-use and cost-effective (Winter et al., 2000).

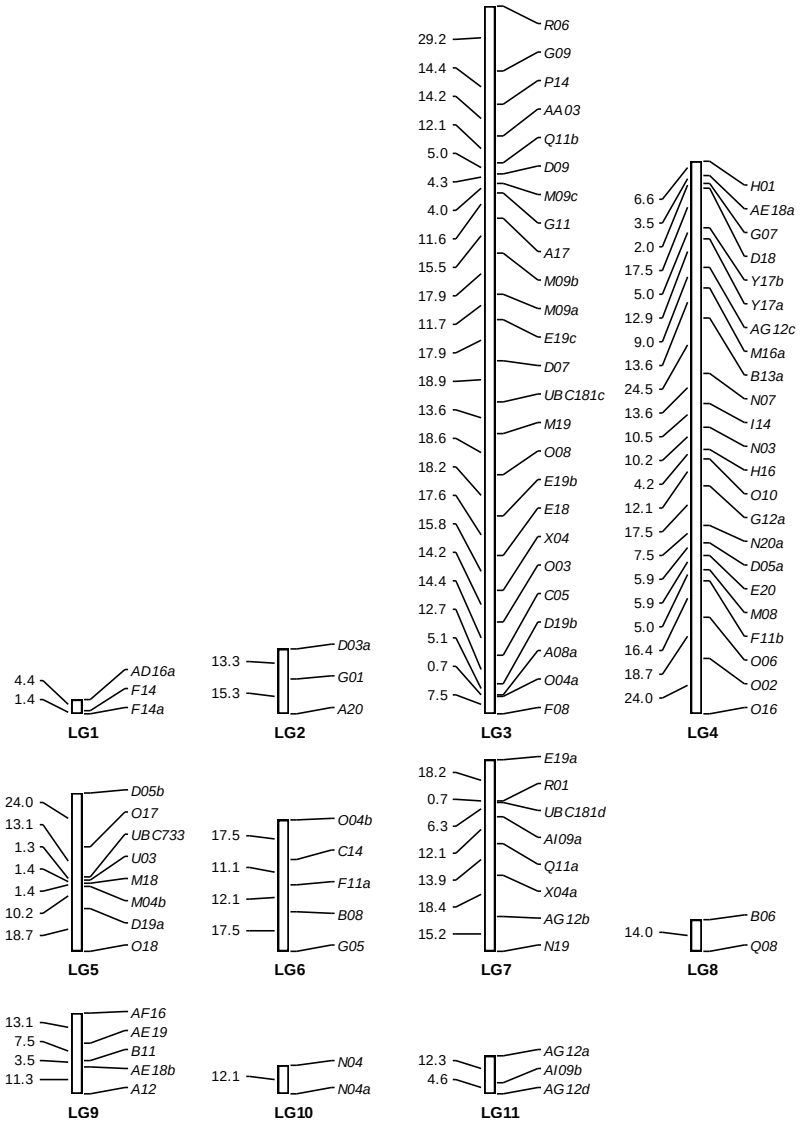

Figure 2: Interspecific map of the chickpea genome. Marker distance was set in cM by the Kosambi function, with 1 cM–1.4 Mbp. The linkage groups are numbered as LG1 to LG11.

AB-resistant chickpea cultivars have attracted primary interest in breeding programs due to severe losses caused by the disease. Virulent strains of AB always cause disease under favorable conditions even in the resistant germplasm (Cho and Muehlbauer, 2004). In the current study, the reaction of RIL to AB was investigated by inoculating the lines with spores of the disease and scoring them. Scores of 1- 3 were accepted as resistant and those over 3 as susceptible, as described by Tewari and Pandey (1986).

The population of this study consisted of 19 resistant and 58 susceptible individuals (Fig. 1). The resistance level (33%) fit the 1:3 ratio, which might be evidence that resistance is controlled by two complementary genes in the cross used. As a result of two-year experiments, Santra et al. (2000) found a similar segregation ratio of 1 resistant: 3 susceptible lines, confirming the genetic model revealing that two complementary genes conferred resistance to AB in the resistant parent (FLIP 84-92C). On the other hand, Tekeoglu et al. (2000) reported that their data fit the segregation ratio of 1 resistant: 7 susceptible lines, as expected for three recessive complementary genes conferring resistance. These different results obtained from different crosses indicate that the genetic model of resistance to AB changes according to parents used as the source of crossing.

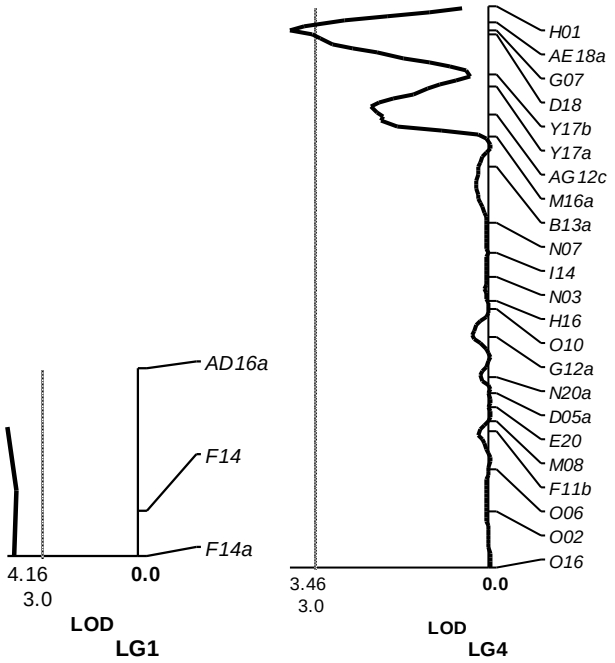

Figure 3. Interval QTL mapping analysis of resistant to AB in the chickpea RIL population derived from C. arietinum×C. reticulatum cross.

Linkage mapping and QTL analysis are the best method to detect markers strongly associated with the disease. To date, several linkage maps have been constructed for chickpea (Tekeoglu 2002; Flandez-Galvez et al. 2003; Udupa and Baum, 2003; Cho and Muehlbauer, 2004; Collard et al., 2001; Winter et al., 2000). In the current study, the linkage map created revealed 11 LGs, three of which were small (Fig 2). It appears that these LGs corresponded closer to the chromosome number of chickpea (*C. arietinum*, 2n=2x=16). The inter-specific linkage map consisted of 118 markers, which covered 889.1 cM, with an average marker density of 10.1 cM. Considering the physical size of the chickpea genome estimated as 750 Mbp (Arumuganatha and Earle, 1991), 1 cM would relate to 360 kbp on average. However, more markers need to be mapped for the current linkage map to make a correlation between LGs and chromosomes for chickpea. Similarly, Cobos et al. (2006) also generated 11 LGs using 125 markers comprising 58 RAPD decamer primers, six ISSRs, 14 TMSs, and four morphological loci. Winter et al. (2000) generated eight LGs in chickpea using 82 markers, the majority of which were STMS markers.

In the current map, most markers were consistent with those reported by Cobos et al. (2006) and Winter et al. (2000), since they used RAPD (Operon) markers. For instance, markers F14, AD16, AE18, N03, N04, and Y17 were common markers, but they were located in different LGs. Markers F14 and AD16 were in the same LG in the current study, but they were placed in different LGs in Cobos et al. (2006), who did not find a link between the two. LG1 in our study could be a part of LG1 of Cobos et al. (2006) and LG4 of Winter et al. (2000). Santra et al. (2000) also developed a chickpea linkage map using RAPD markers but we were not able to compare our map with that of Santra et al. (2000) since they used a UBC-RAPD primer set in their study.

We identified two QTL using RAPD and ISSR markers as detailed in Table 2. QTL-1 was in LG1 and consisted of markers F14, F14a and AD16a with a LOD score of 4.16 (Fig 3). QTL-2 was located in LG4 and comprised markers G07, D18 and AE18a with a LOD score of 3.46 (Fig 3). We also identified a third QTL adjacent to QTL-2 that contained markers (Y17b, Y17a and AG12) with a small effect (LOD score of 3.1). QTL-1 and QTL-2 explained 31% of the total variation for AB resistance. Iruela et al. (2006) developed SCAR markers from RAPD markers Y17 and AG12, which were strongly associated with resistance to AB and mapped the SCAR markers close to Y17 and AG12 markers at a 2.6 cM distance in the same LG as in our map. We found a second QTL region (G07, D18 and AE18a) neighbor to QTL in LG4, where the SCAR markers detected by Iruela et al. (2006) were located at a 22.9 cM distance. Santra et al. (2000) reported three QTL explaining 50.3% of the total phenotypic variation using RAPD primers (UBC sets) in LG1, LG4, and LG6. In the current study, we used three markers (UBC733, UBC681 and UBC181), which were previously used and associated with AB resistance by Santra et al. (2000), in order to detect QTL associated with AB. Among these markers, UBC733 and UBC681 were not linked according to our mapping studies. The amplification of the third marker from Santra et al. (2000), UBC181 did not show any significant association with AB resistance in the QTL analysis. Anbessa et al. (2009) found five QTL using four different mapping populations. All these QTL together explained 56, 48, 38, and 14% of

the estimated phenotypic variations in four different crosses.

Table 2. The results of single marker analysis.

Marker	LG	R2	LOD	P
F14a (159)	LG 1	0,2037	3,76	0
F14 (70)	LG 1	0,1947	3,62	0,0001
AD16a (677)	LG 1	0,1977	3,54	0,0001
G07 (40)	LG 4	0,1874	3,47	0,0001
D18 (831)	LG 4	0,1687	3,09	0,0002
AE18a (570)	LG 4	0,1476	2,67	0,0006

Marker AE18a570 in LG4 was also related with disease resistance by Millan et al. (2003), which strengthens the idea that this marker is in a QTL region. C05(861) in LG3, AG12c(1146) in LG4, and AI09b(1276) in LG11 in our study were mapped in a single LG by Millan et al. (2003), who reported that these markers were all associated with resistance to AB.

In conclusion, we detected five new markers (AD16, F14, F14a, G07, and D18) tightly linked to QTL for AB resistance. These markers can improve precision of molecular breeding in this population. This result could be a starting point to identify a candidate resistant gene for AB in chickpea. For practical use in chickpea breeding and especially MAS, the close linkage of genes for resistance to AB and several QTL markers is of great importance as it allows the use of at least one of the highly polymorphic markers for the analysis of the segregation of AB genes in a wide range of germplasm. Efforts should be made to convert RAPD markers to SCAR markers since the latter are more useful than the former, being locus-specific and identifiable in different genetic backgrounds (co-dominant), thus reducing the chance of misclassifying individuals in segregating populations (Chowdhury et al., 2001). The use of co-dominant markers, such as SCAR is desirable in MAS to detect heterozygous individuals in early generations (Iruela et al., 2006). Subsequent selection in segregating generations through tightly linked molecular markers developed in this study will allow for substantial progress in the improvement of field resistance to AB in chickpea. For future work, we suggest identifying AB-resistant genes with tightly linked co-dominant and dominant DNA markers to contribute to the pyramiding of AB genes in chickpea to develop a resistant cultivar.

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