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Mapping QTL for resistance to botrytis grey mould in chickpea

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Abstract Botrytis grey mould (BGM) caused by *Botrytis cinerea* Pers. ex. Fr. is the second most important foliar disease of chickpea (*Cicer arietinum* L.) after ascochyta blight. An intraspecific linkage map of chickpea consisting of 144 markers assigned on 11 linkage groups was constructed from recombinant inbred lines (RILs) of a cross that involved a moderately resistant kabuli cultivar ICCV 2 and a highly susceptible desi cultivar JG 62. The length of the map obtained was 442.8 cM with an average interval length of 3.3 cM. Three quantitative trait loci (QTL) which together accounted for 43.6% of the variation for BGM resistance were identified and mapped on two linkage groups. QTL1 explained about 12.8% of the phenotypic variation for BGM resistance and was mapped on LG 6A. It was found tightly linked to markers SA14 and TS71rts36r at a LOD score of 3.7. QTL2 and QTL3 accounted for 9.5 and 48% of the phenotypic variation for BGM resistance, respectively, and were mapped on LG 3. QTL 2 was identified at LOD 2.7 and flanked by markers TA25 and TA144, positioned at 1 cM away from marker TA25. QTL3 was a strong QTL detected at LOD 17.7 and was flanked by TA159 at 12 cM distance on one side and TA118 at 4 cM distance on the other side. This is the first report on mapping of QTL for BGM resistance in chickpea. After proper validation, these QTL will be useful in marker-assisted pyramiding of BGM resistance in chickpea.

Keywords Botrytis grey mould · Chickpea · *Cicer arietinum* L. · Linkage map · QTL mapping · Disease resistance

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Introduction

Chickpea (*Cicer arietinum* L.), also called garbanzo, is globally the third most important food legume, used mainly for human consumption and highly valued for its nutritive value, particularly as a good source of protein (17–24%), fibre, minerals

(phosphorus, calcium, magnesium, iron, zinc) and vitamins (Williams and Singh 1987). It is grown in over 50 countries and imported by over 140 countries (FAOSTAT 2008). During 2008, chickpea was grown in 11.55 million ha with production of 8.77 million metric tons and 81.2% of this area was in Southern Asia, 6.7% in Western Asia, 1.7% in South-Eastern Asia, 3.2% in Eastern Africa, 1.0% in Northern Africa, 2.6% in Oceania, 1.9% in Northern America, 1.0% in Central America and 0.4% in Europe (FAOSTAT 2008). The global average yield of chickpea is about 760 kg per ha, while a well-managed chickpea crop, which is free from abiotic and biotic stresses, yields about 3,000–3,500 kg per ha. Abiotic and biotic stresses are the major constrains to chickpea production (Gaur et al. 2008).

Botrytis grey mould (BGM) caused by *Botrytis cinerea* Pers.ex.Fr. is the second most important foliar disease of chickpea after ascochyta blight caused by *Ascochyta rabiei* (Pande et al. 2006). The incidences of BGM on chickpea has been reported in many countries, including Argentina, Australia, Bangladesh, Canada, Columbia, India, Nepal, Pakistan, Spain and USA (Nene et al. 1984; Haware and McDonald 1992, 1993; Bakr et al. 1993; Dhar et al. 1993; Karki et al. 1993; Malik et al. 1993; Haware 1998; Pande et al. 2002, 2006; Davidson et al. 2004). BGM can cause complete yield loss in years with extensive rains and high humidity (Pande et al. 2002, 2006). BGM as an epidemic form was first reported from Argentina in 1965 (Carranza 1965) and from Northern India during 1978/79 (Grewal and Laha 1983). BGM is the most serious constraint to chickpea production in Nepal and it can reach to epidemic form in wet winters. This disease is considered to be the major cause for decline in chickpea area in Nepal and Bangladesh (Bakr et al. 2002). Serious BGM epidemics were also observed in Western Australia during 1997 and 1998 (MacLeod and Sweetingham 2000).

The severity of the disease depends largely on weather conditions and inoculum levels of the pathogen (Pande et al. 2006). The disease is favored by warm humid conditions and can occur at any growth stage. Infected seed is often the primary cause of infection (Cother 1977; Burgess et al. 1997). Infected plants produce masses of spores, which may become air-borne (MacLeod and Sweetingham 2000) and spread the disease rapidly. Drooping of the affected terminal branches is a common field symptom (Haware and McDonald 1992) and branches may break off at the point of infection (Grewal et al. 1992). The flowers are most severely affected and leads to poor or no pod setting. The seeds, if formed, are generally shriveled and covered with grey fungal mat (Knights and Siddique 2002).

The limited reports available on genetics of BGM resistance suggests that the resistance is controlled by few genes. A single dominant gene 'Bor1' for resistance was identified by Tiwari et al. (1985), while two genes with dominant and recessive epistasis (13:3 ratio) were reported by Rewal and Grewal (1989) and duplicate dominant epistasis (15:1 ratio) by Chaturvedi et al. (1995).

There has been rapid advancement in development of genome map of chickpea and molecular mapping of genes/QTL controlling agronomically important traits, such as drought avoidance root traits (Chandra et al. 2004; Gaur et al. 2008), fusarium wilt resistance (Mayer et al. 1997; Ratnaparkhe et al. 1998; Tullu et al. 1998; Winter et al. 2000; Sharma et al. 2004) and ascochyta blight resistance (Santra et al. 2000; Tekeoglu et al. 2002; Collard et al. 2003; Flandez-Galvez et al. 2003; Udupa and Baum 2003; Cho et al. 2004; Lichtenzveig et al. 2005; Cobos et al. 2006; Tar'an et al. 2007; Kottapalli et al. 2008). There was no report available on mapping of QTL conferring resistance to BGM. Thus, this study was conducted to identify and map QTL for BGM resistance in chickpea.

Materials and methods

Mapping population and its phenotyping

The mapping population comprised of 126 F_{10} derived recombinant inbred lines (RILs) of a cross between a kabuli chickpea cultivar ICCV 2 which is moderately resistant to BGM and a desi chickpea cultivar JG 62 which is highly susceptible to BGM. The RILs were developed in the chickpea breeding unit of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, India following a single seed descent (SSD) method. The disease severity was scored on a 1–9 scale, where 1 = no infection on any part of the plant; 2 = minute water-soaked lesions on emerging tender leaves,

usually not seen; 3 = minute water-soaked lesions on 1–5% emerging and upper-most tender leaves, usually seen after careful examination; 4 = water-soaked lesions on 6–10% upper-most tender leaves and tender shoots; 5 = water-soaked lesions; soft rotting of 11–25% of tender leaves and shoots; 6 = water-soaked lesions and soft rotting of 26–40% of top leaves and shoots; 7 = soft rotting and fungal growth on 41–55% of the leaves and branches; 8 = soft rotting, fungal growth on 56–70% of the leaves, branches, and stems; 9 = extensive soft rotting, fungal growth on above 70% of the leaves, branches and stems. On this scale, ICCV 2 and JG 62, the two parents of the RILs, had BGM scores of 6 and 9, respectively.

The RILs were screened for BGM resistance under controlled environment conditions twice, with three replications in each screening. The RILs were planted in rows in plastic trays (30 × 20 × 5 cm) filled with sterilized sand and vermiculate (4:1) with one row of susceptible cultivar JG 62 after every nine rows of RILs. Pure culture of *B. cinerea* was multiplied on sterilized Mary gold (*Tagetus erecta*) flowers for 10 days at 20°C in Perceival incubator. Ten-day-old seedlings of test lines, along with susceptible check were inoculated with conidial suspension of *B. cinerea* @ 3×10^5 conidia ml^{-1}. Inoculated plants were incubated at 15 ± 2°C and 100% RH with a 12 h photoperiod, 2,500–3,000 lux intensity (Pande et al. 2006) till the end of the experiment. Final disease score on a 1–9 scale was recorded at 18 days after inoculation or when the disease severity on susceptible parent showed a disease score of 9.

Genomic DNA of the RILs and the parental cultivars was extracted from fresh young leaves (2 g) collected from 14-day old seedlings following the modified CTAB method described by Mace et al. (2003). In order to test the quality and quantity of DNA, the extracted genomic DNA along with the standard and undigested λ DNA in various concentrations was run on 0.8% agarose gel (containing Ethidium bromide) and was visualized on gel documentation system.

Optimization for the five major components in a PCR (concentrations of primer, template DNA, Mg^{++}, dNTP and enzyme) was carried out for every primer following a modified (5 × 5) grid (Cobb and Clarkson 1994). Optimal touch-down temperature and number of amplification cycle were also determined for each primer pair. Three different "Touch-down" PCR programs were designed with Cp 55-45, Cp 60-55, Cp 65-60 depending on the T_m value of the microsatellite primers (SSRs) (Buhariwalla et al. 2005; Kottapalli et al. 2008). PCR was setup in 5 µl reaction volume on a Gene Amp Model 9700 thermocycler (Perkin Elmer-Applied Biosystems, Germany).

The parental cultivars ICCV 2 and JG 62 were screened with SSR primers developed by Winter et al. (1999) to identify polymorphic markers. PCR reaction was setup in a 5 µl reaction volume using the appropriate optimized protocol for each primer. The PCR products were run on 1.5% agarose gel containing ethidium bromide after adding bromophenol blue dye. The amplification products were visualized in a gel documentation system. The SSR primers, which exhibited polymorphism between the parental cultivars ICCV 2 and JG 62, were used for genotyping the RILs. PCR was setup using the appropriate optimized protocol and PCR program. The total reaction volumes of 5 µl was setup in 96 and 384 well PCR plates and were amplified in a Gene Amp Model 9700 thermocycler (Applied Biosystems, Germany).

The PCR products were separated on non-denaturing PAGE (Polyacrylamide Gel Electroproresis). Generally, 6% PAGE was used for the primers whose separation was very distinct in the parents. For the primers where the polymorphic bands were closer, a higher percentage of gel 8–9% non-denaturing PAGE (Biorad and Owl sequencing gel units) was used for separation of PCR amplified products. The primers that showed polymorphic bands in the parents with only a few base pair (bp) difference were separated on 4% denaturing urea-sequencing gel (Biorad sequencing gel unit) after denaturing for 5 min at 94°C. Bands were visualized through a modified silver staining protocol (Tegelstrom 1992, Buhariwalla 2005). Gels were immersed in water for 3 min, followed by 20 min in 0.1% CTAB solution and 0.3% ammonia solution for 15 min on a mechanical gel shaker. Freshly prepared silver staining solution, consisting of 0.1% (w/v) $AgNO_3$ in 4 mM NaOH solution, to which 0.5–0.6 ml of 25% ammonia was titrated until the cloudy suspension became clear. Gels were gently agitated in the silver nitrate solution for 30 min, and developed in 1.5% (w/v) sodium carbonate and 0.02% (v/v) formamide solution until

bands appeared. The gels were rinsed in water, fixed in 1.5% glycerol solution and scanning and documentation of marker data was carried out.

QTL analysis

Join map® version 3.0 software (Van Ooijen and Voorrips 2001) was employed for linkage analysis. Genetic distances were computed using Kosambi (1944) function and LOD score of 3.0 was in construction of linkage map. Plab QTL (Utz and Melchinger 1996) and iMAS (Integrated marker-assisted selection system) were the software utilized and composite interval mapping (Jansen and Stam 1994; Zeng 1994) was used to compute 'QTL likelihood plots' covering the entire genome. A default LOD score was fixed at 2.5 to identify the QTLs on linkage groups. Estimates of R^2 value for explaining the phenotypic variance were computed from the ANOVA table using the software Plab QTL (Utz and Melchinger 1996).

Results and discussions

The frequency distribution of RILs for BGM disease incidence (recorded on 1–9 score) depicted a normal distribution indicating that resistance to BGM was quantitative in nature (Fig. 1). Analysis of variance of disease score data suggested significant variation in the genotypes for reaction to botrytis. The coefficient of variation observed was low for both the screening (0.17 and 0.19) and the correlation between scores of two screening was significant ($r = 0.56$) indicating the consistency of the disease score data. A total of 204 marker data consisting of 91 SSR, 33 RAPD, 43 DAF, 5 MP-PCR, 11 RMMFP, 17 AFLP and 4 ISSR markers and 12 morphological markers were utilized for construction of linkage map. Of these, 77.5% of the markers segregated in the ratio of 1:1 as expected for an RIL population and 22.5% of markers showed distorted segregation. The markers, which showed distorted segregation, were excluded from the map. A linkage map consisting of 144 markers was constructed comprising of 11 linkage groups with 8 major and 3 minor groups. The 8 major linkage groups (LG 1–LG 8) are in accordance with the basic chromosome number of chickpea. Among minor linkage groups, two linkage groups LG 1 and LG 6 had fragments named as LG 1B and LG 6B, respectively, and another linkage group found unlinked was designated as LG 9. The length of the map obtained was 442.8 cM with an average interval length of 3.3 cM.

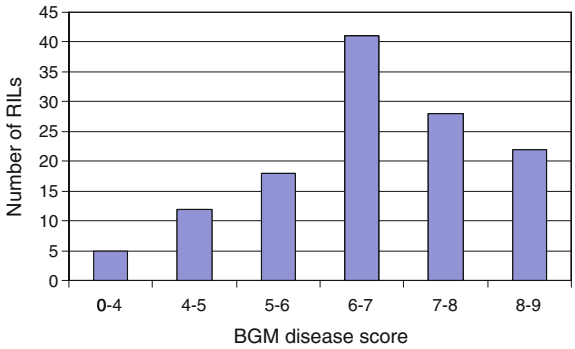

Fig. 1 Frequency distribution of BGM disease scores in ICCV 2 × JG 62 RILs

There has been a slow progress in development of a dense linkage map of chickpea because of limited number of markers available for chickpea and low level of polymorphism exhibited by the cultivated species for the available markers. As to overcome the latter constraint of lower polymorphism in the cultivated chickpea, many studies (Gaur and Slinkard 1990a, b; Kazan et al. 1993; Simon and Muehlbauer 1997; Tekeoglu et al. 2000; Winter et al. 2000; Collard et al. 2003; Pfaff and Kahl 2003; Millan et al. 2006) used interspecific mapping populations in development of a linkage map of chickpea that integrated majority of marker loci onto map (Cobos et al. 2006; Nayak et al. 2010) and enabled mapping genes/QTL for important traits. However, a majority of markers identified from interspecific mapping populations are likely to be monomorphic in intraspecific crosses and, thus, would have limited application in the applied breeding programs which largely use intraspecific crosses. Keeping this in view, several studies (Cho et al. 2002; Flandez-Galvez et al. 2003; Udupa and Baum 2003; Cho et al. 2004; Tar'an et al. 2007: Kottapalli et al. 2008) used intraspecific mapping populations for linkage mapping in chickpea. The number of markers mapped in these studies varied from 52 (Udupa and Baum 2003) to 144 (Tar'an et al. 2007). The intraspecific linkage map of 144 markers developed in this study is equivalent to the largest intraspecific linkage map of

chickpea reported so far by Tar'an et al. (2007). The present linkage map was compared with the earlier reported linkage maps (Winter et al. 2000; Cho et al. 2002; Kottapalli et al. 2008) and linkage groups 1–8 were numbered based on homologies.

Using composite interval mapping (Jansen and Stam 1994; Zeng 1994), three QTL were identified (at a minimum LOD score of 2.5) for resistance to BGM (Figs. 2 and 3a, b). QTL1 explained about 12.8% of phenotypic variation for BGM resistance and was identified on LG 6A with peak position at 36 cM. It was found to be tightly linked to markers SA14 and TS71rts36r at a LOD score of 3.74. QTL2 explained 9.5 of the phenotypic variation for BGM resistance and was found on LG 3 at LOD 2.73 having peak position at 26 cM. It was flanked by the markers TA25 and TA144 and is positioned at 1 cM away from TA25. QTL3 was the strongest QTL for BGM resistance (explained 48.0% of the phenotypic variation) and was detected on LG 3 at a LOD score of 17.74 with peak position of the QTL at a distance of 56 cM. QTL3 was flanked by TA159 at 12 cM

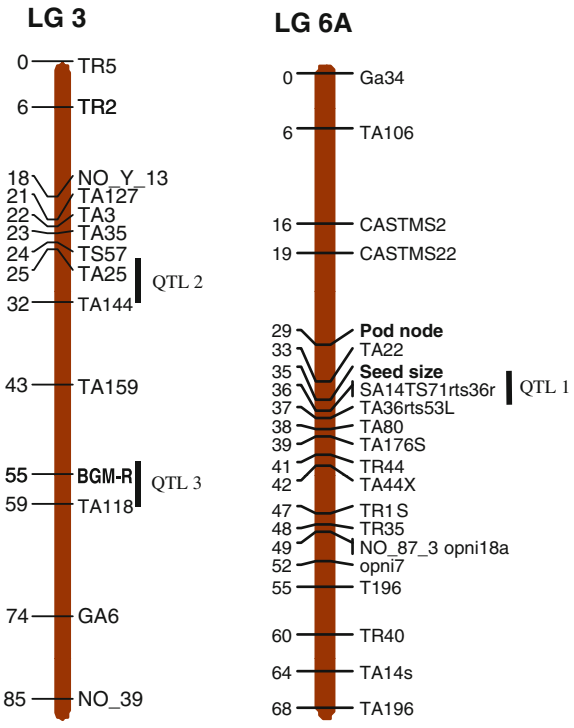

Fig. 2 Position of BGM resistance QTL on LG 3 (QTL 2 and QTL 3) and LG 6A (QTL 1) of chickpea based on the study of ICCV 2 × JG 62 RILs

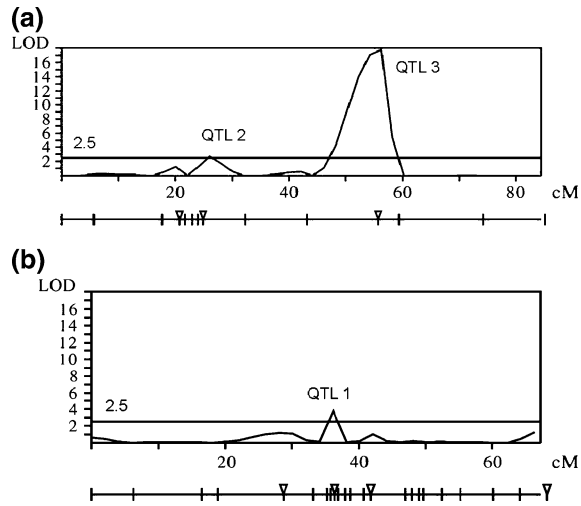

Fig. 3 **a** Graphical representation of QTL 2 (at LOD 2.73) and QTL 3 (at LOD 17.74) for BGM resistance on LG 3 of chickpea. **b** Graphical representation of QTL 1 (at LOD 3.74) for BGM resistance on LG 6A of chickpea

distance on one side and TA118 at 4 cM distance on the other side (Fig. 2). The total combined phenotypic variance explained by these three QTL was 43.6% (Table 1). It is noteworthy that the QTL were obtained at the same position irrespective of the software used in our study. To our knowledge, this is the first report on identification and mapping of QTL for BGM resistance in chickpea.

The earlier mapped disease resistance genes/QTL in chickpea included those for resistance to ascochyta blight and fusarium wilt. The largest number of genes/QTL for disease resistance has been reported on LG 2. These include six genes for resistance to different races of fusarium wilt (Mayer et al. 1997; Ratnaparkhe et al. 1998; Tullu et al. 1998; Winter et al. 2000; Sharma et al. 2004; Sharma and Muehlbauer 2007; Halila et al. 2009) and two QTL for resistance to ascochyta blight (Udupa and Baum 2003; Cho et al. 2004; Cobos et al. 2006; Iruela et al. 2007). Thus, LG 2 of chickpea has been found to be a hot spot for pathogen defense (Millan et al. 2006). One gene for fusarium wilt resistance has been mapped on LG 5 (Cobos et al. 2005; Sharma and Muehlbauer 2007) and other QTL for ascochyta blight resistance have been mapped on LG 3 (Tar'an et al. 2007; Kottapalli et al. 2008), LG 4 (Tar'an et al. 2007), LG 6 (Cho et al. 2004; Tar'an et al. 2007) and LG 8 (Flandez-Galvez et al. 2003; Lichtenzveig et al. 2005). The BGM resistance QTL identified in this

Table 1 Characteristics of QTL associated with resistance to botrytis grey mould in chickpea

Trait	QTL	Linkage group	Position of QTL on linkage group (cM)	Flanking markers	LOD	R^2 (%)	Additive
Resistance to botrytis grey mould	1	LG 6A	36	SA14-TS71rts36r	3.74	12.8	−0.63
	2	LG 3	26	TA25-TA144	2.73	9.5	−0.48
	3	LG 3	56	TA118-TA159	17.74	48.0	0.97

study and the fusarium resistance genes mapped earlier are in different linkage groups, indicating ease in combining resistance to these diseases. However, combing QTL for resistance to BGM and ascochyta blight which share the same linkage groups (LG 3 and LG 6) would require large population depending on their position.

The three QTL identified for BGM resistance together explained large phenotypic variation (43.6%) for BGM resistance. This suggests that the resistance to BGM is under control of few major genes. In earlier studies on genetics of BGM resistance, a single dominant gene 'Bor1' for resistance was identified by Tiwari et al. (1985), while two genes with epistatic interaction were reported by Rewal and Grewal (1989) and Chaturvedi et al. (1995). When we converted BGM disease score of 126 RILs into two classes—resistant (score ≤ 7) and susceptible (score ≥ 8), a ratio of 54 (resistant): 72 (susceptible) was found. The goodness-of-fit test for a 1:1 ratio (expected for a monogenic trait in RILs) gave a chi-square value of 2.29 which was non-significant at probability level of 0.1. This suggested presence of a major gene for BGM resistance. When this major gene was used in mapping it corresponded to the strongest QTL (QTL3) which was identified by treating BGM resistance as a quantitative trait and using disease score in QTL mapping. Thus, QTL3 may indeed be a major gene for BGM resistance and correspond to single gene for resistance reported by Tiwari et al. (1985).

Developing chickpea cultivars with high levels of BGM resistance has been challenging due to lack of sources of high levels of resistance in the cultivated chickpea (Pande et al. 2006). There is a need to identify diverse genes for resistance from different sources so that these can be pyramided to obtain higher levels of resistance. The markers closely linked with BGM resistance QTL identified in this study can facilitate identification of diverse genes and their pyramiding in a single genotype. Sources with higher levels of resistance, as compared to the cultivated species, are available in some wild *Cicer* species, including *C. judaicum*, *C. bijugum*, *C. echinospermum*, and *C. pinnnatifidum* (Singh et al. 1991; Haware 1998; Pande et al. 2002). Of these wild species, *C. echinospermum* is in the primary genepool and being used in transfer of BGM resistance to the cultivated species (ICRISAT 2007). As the wild species posses many undesirable traits, several cycles of backcrossing are required to recover the genome of cultivated species. The QTL mapped in this study would greatly facilitate marker-assisted backcrossing for introgression of BGM resistance from wild species and reduce number of backcrossing required, particularly when both foreground and background selections are used.

In conclusion, we constructed an intraspecific linkage map of chickpea from ICCV 2 × JG 62 RILs covering a genome length of 442.8 cM with an average interval of 3.3 cM and mapped three QTL which accounted for 43.6% of phenotypic variation for BGM resistance. These QTL, after proper validation, can be used for marker-assisted breeding for BGM resistance in chickpea.

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