Theor Appl Genet (2009) 118:671–682
DOI 10.1007/s00122-008-0928-7

ORIGINAL PAPER

Integration of new CAPS and dCAPS-RGA markers into a composite chickpea genetic map and their association with disease resistance

Carmen Palomino · M. D. Fernández-Romero · J. Rubio · A. Torres · M. T. Moreno · T. Millán

Received: 1 July 2008 / Accepted: 4 November 2008 / Published online: 26 November 2008

Abstract A composite linkage map was constructed based on two interspecific recombinant inbred line populations derived from crosses between *Cicer arietinum* (ILC72 and ICCL81001) and *Cicer reticulatum* (Cr5-10 or Cr5-9). These mapping populations segregate for resistance to ascochyta blight (caused by *Ascochyta rabiei*), fusarium wilt (caused by *Fusarium oxysporum* f. sp. *ciceris*) and rust (caused by *Uromyces ciceris-arietini*). The presence of single nucleotide polymorphisms in ten resistance gene analogs (RGAs) previously isolated and characterized was exploited. Six out of the ten RGAs were novel sequences. In addition, classes RGA05, RGA06, RGA07, RGA08, RGA09 and RGA10 were considerate putatively functional since they matched with several legume expressed sequences tags (ESTs) obtained under infection conditions. Seven RGA PCR-based markers (5 CAPS and 2 dCAPS) were developed and successfully genotyped in the two progenies. Six of them have been mapped in different linkage groups where major quantitative trait loci conferring resistance to ascochyta blight and fusarium wilt have been reported. Genomic locations of RGAs were compared with those of known *Cicer* R-genes and previously mapped RGAs. Association was detected between RGA05 and genes controlling resistance to fusarium wilt caused by races 0 and 5.

Communicated by M. Xu.

C. Palomino (✉) · J. Rubio · A. Torres · M. T. Moreno
Área de Mejora y Biotecnología, IFAPA,
Centro 'Alameda del Obispo', Apdo. 3092,
14080 Córdoba, Spain
e-mail: mariac.palomino@juntadeandalucia.es

M. D. Fernández-Romero · T. Millán
Dpto Genética, Universidad de Córdoba,
Campus de Rabanales Edificio C5 2a planta,
14071 Córdoba, Spain

Introduction

Productivity of chickpea (*Cicer arietinum* L.) is currently low (world average 772.23 kg/ha) compared with faba bean (*Vicia faba* L.) (1,754 kg/ha) and pea (*Pisum sativum* L.) (1569.72 kg/ha), the two most cultivated grain legumes worldwide together with chickpea (FAOSTAT 2008). Biotic stresses are important factors limiting yield and yield stability in this crop, with special incidence of two diseases, ascochyta blight and fusarium wilt. Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labrousse, infects the aerial part of the plant at any growth stage and at present affects most chickpea growing areas (Pande et al. 2005). Fusarium wilt is an important vascular disease caused by the soilborne fungus *Fusarium oxysporum* Schlechtend.: *Fr*. f. sp. *Ciceris* (Padwick) Matuo & K. Sato, that can cause up to 100% yield loss. Eight physiological races of the pathogen have been reported so far (0, 1A, 1B/C, 2, 3, 4, 5 and 6) but the number of races is likely to increase in the future (Sharma and Muehlbauer 2007). Another disease affecting chickpea production at the local level is chickpea rust caused by *Uromyces ciceris-arietini* (Gregnon) Jacs., which is widespread in the Mediterranean basin, Southeastern Europe, South Asia, East Africa and Mexico (Díaz-Franco and Pérez-García 1995).

Interspecific crosses with the related wild species *C. reticulatum* Ladiz. have been used to develop chickpea genetic maps and tagging quantitative trait loci (QTLs) or genes controlling resistance to these diseases is an important task in modern breeding programs. Two important QTLs located in chickpea LG4, and one located in LG2, all

related to ascochyta blight resistance have been widely validated (Iruela et al. 2006, 2007). LGs 3, 6 and 8 might be also implicated in ascochyta blight resistance (Flandez-Galvez et al. 2003a; Tar'an et al. 2007).

Genes controlling resistance to fusarium wilt races 1, 2, 3, 4 and 5 have been located in LG2 forming a cluster of genes in a narrow genomic area (Sharma and Muehlbauer 2007). Two genes controlling resistance to fusarium wilt race 0 ($Foc0_1$ and $Foc0_2$) have been respectively located in LG5 (Cobos et al. 2005) and LG2 (Halila et al. 2008). The latter was closely linked to the previously cited cluster of resistance genes. Recently a major gene controlling resistance to rust (*Uca1/uca1*) has been identified and mapped in the LG7 (Madrid et al. 2008).

Even though at the present time there some makers closely linked to resistance genes ready to use in marker assisted selection (MAS), it is still necessary to saturate different genomic areas in order to more accurately locate the genes or QTLs involved in ascochyta blight, fusarium wilt and rust resistance (Millán et al. 2006). Markers should also be of great help in understanding the resistance mechanisms and identifying genes directly involved in these resistance reactions.

The majority of cloned R genes contain a nucleotide-binding site (NBS) followed by a leucine-rich repeat (LRR) domain and are thus termed NBS-LRR R genes. The NBS sequence of NBS-LRR genes is characterized by the presence of several conserved motifs such as P-loop, kinase 1, kinase 2a and GLPL (Meyers et al. 1999). This conservation of protein sequence among R genes has facilitated the cloning of resistance gene analogs (RGAs) from many plant species using degenerate oligonucleotide primers designed from these conserved domains (e.g. Zhang et al. 2002; Yaish et al. 2004; Palomino et al. 2006). The RGAs can be considered as fragments of R genes based on: (1) the high sequence identities with known R genes and RGAs from other species, (2) the presence of that conserved motifs characteristic of NBS-LRR R genes and (3) the uninterrupted open reading frames (ORFs) (Noir et al. 2001).

Resistance gene analog sequences can be transformed into molecular markers for use in MAS or even lead to the cloning of the full-length functional R genes (Kuhn et al. 2003; Quint et al. 2003). However, the detection of polymorphisms using the cloned RGAs is not an easy task. Single nucleotide polymorphisms (SNPs) whose abundance, ubiquity and interspersed nature make them ideal candidates for marker-assisted plant breeding (Gupta et al. 2001). Cleaved amplified polymorphism sequence (CAPS) (Konieczny and Ausubel 1993) has been one of the most used techniques to detect single base changes by the loss or gain of a restriction enzyme recognition site. Although SNPs are one of the most common classes of DNA polymorphism in many organisms (review Gupta et al. 2001), the majority of single-base changes do not generate a restriction site difference and thus are useless for developing CAPS markers. However, studies in *Arabidopsis thaliana* have demonstrated that SNPs can be also used for the development of PCR-based markers by the dCAPS method (derived CAPS) (Neff et al. 1998; Komori and Nitta 2005). In dCAPS analysis, a restriction enzyme recognition site which includes the SNP is introduced into the PCR product by a primer containing one or more mismatches to the template DNA.

Disease resistance genes tend to be clustered in plant genomes, and RGAs identified using this approach are frequently located close to previously identified resistance loci or QTLs (Radwan et al. 2003; Rossi et al. 2003; McIntyre et al. 2005). In the case of chickpea, RGA markers have been previously mapped in LGs 2, 3 and 5 (Huettel et al. 2002) and one in LG3 (Tekeoglu et al. 2002), but no co-segregation with resistance to ascochyta blight or fusarium wilt was found. Flandez-Galvez et al. (2003b) also located RGA markers mainly clustered in LGs 2, 3 and 4 adjacent to several QTLs for *A. rabiei*.

In this work we have included new RGAs in chickpea RIL populations that segregate for these diseases in order to tag areas of interest. Two RIL populations derived from interspecific crosses and evaluated for resistance to ascochyta blight, fusarium wilt races 0 and 5 (Cobos et al. 2006, 2008) and rust (Madrid et al. 2008) were used. The aims of this study were: (1) to develop a composite map from these two RIL populations (2) to transform the RGAs previously isolated and characterized (Palomino et al. 2006) into PCR-based markers and (3) to identify new RGAs located in the vicinity of genes or QTL conferring different disease resistances.

Materials and methods

Plant material

Two $F_{6:7}$ RIL populations derived from the interspecific crosses, *C. arietinum* (ICCL81001) × *C. reticulatum* (Cr5–9) and *C. arietinum* (ILC72) × *C. reticulatum* (Cr5–10) that comprised 88 and 104 lines, respectively were studied. ICCL81001 is an earlier flowering and fusarium wilt race 0 and 5 resistant kabuli line from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) (Kumar and Haware 1983) and Cr5–9 is a susceptible wild genotype. ILC72 is an ascochyta blight resistant kabuli line from the former Soviet Union [maintained by the International Center for Agricultural Research in the Dry Areas (ICARDA) Aleppo, Syria] while Cr5–10 is a wild genotype susceptible to *A. rabiei* and resistant to *Uromyces ciceris-arietini.*

Both RILs were previously used to construct two independent chickpea genetic maps (Cobos et al. 2006, 2008; Madrid et al. 2008) that contained QTLs for resistance to ascochyta blight and fusarium wilt race 5 and 0. In the present work, a composite genetic map from both crosses has been constructed, including four new sequenced tagged microsatellite markers (STMS) (TA18, TA25, TA80 and TS12) developed by Winter et al. (1999) and seven RGA markers developed in this study (see below).

Development of PCR-based markers

Sequenced tagged microsatellite markers amplification were established according to Winter et al. (1999) in 15 µl reactions containing 30 ng of plant genomic DNA, buffer (75 mM Tris–HCl pH 9, 50 mM KCl and 20 mM $(NH_4)_2SO_4$), 1.5 mM $MgCl_2$, 250 µM dNTP, 2 µM of primer and 0.375 U of *Taq* DNA polymerase (Biotools). After denaturing the DNA for 2 min at 96°C the reaction mixture was subjected to 35 cycles of the following temperature profile: 96°C for 20 s, 55°C for 50 s and 60°C for 50 s and a final extension at 60°C for 5 min. Amplification products were electrophoreses in 2.5 gels composed of a mixture of 1:1 D-1 LOW EEO and LM-SIEVE agarose (Pronadisa, Spain) in 1× TBE buffer pH 8.5.

To develop PCR-based markers for the genetic mapping of RGAs, the consensus nucleotide sequences of ten RGAs classes previously published (Palomino et al. 2006) were obtained. RGA classes 1, 6, 8, and 9 were comprised solely by clones isolated from faba bean whereas classes 2, 3, 4, 5, and 7 included only chickpea clones. RGA10, showing 99% of within-class identity, was the only class consisting of both faba bean and chickpea clones.

The regions that were divergent among classes were identified by multiple-sequence alignment using the MEGA v. 4.0 (Tamura et al. 2007) and subsequently used for designing specific primers. Computer software Oligo 4.0 was employed to identify sequence segments with desirable internal stability curves as priming sites and to avoid potential 3′ dimer or hairpin formation (Rychlik 1995). The length of specific primers (Operon Technologies) ranged from 18 to 23 bases (Table 1). PCR products were first subjected to digestions with a set of 20 enzymes (*Alu*I, *BamH*I, *Bgl*II, *Bsp120*I, *Cau*II, *Eco*RI, *Eco*RV, *Hae*III, *Hha*I, *Hind*III, *Hinf*I, *Hpa*II, *Mse*I, *Nla*III, *Rsa*I, *Sal*I, *Sau961*, *ScrF*I, *Taq*I, *Xba*I), mainly four-base recognition sites, to detect restriction site polymorphism (CAPS markers). When no polymorphism was apparent, the dCAPS method was employed to identify allele-specific SNPs within the parental RGAs sequences. In these cases the unique PCR product amplified in each parental line were purified for direct sequencing using the QIAquick PCR purification Kit (Qiagen GmbH, Hilden, Germany). PCR products were sequenced in both directions using a BigDye terminator cycle sequencing version 3.1 kit (PE Biosystems, Foster City, CA) on an ABI Prism 3100 Genetic Analyzer (Applied Biosystem) at the SCAI (Central Service) in the University of Córdoba (Spain). Alignment of nucleotide sequences from the parental lines was carried out using the MEGA v. 4.0 (Tamura et al. 2007) in order to detect single-base differences. The dCAPS primers design (Table 2) was performed using the program "dCAPS Finder" introducing a single nucleotide mismatch adjacent to the SNP position that created a restriction site in the amplified PCR product of one parent but not in the other (Neff et al. 1998). In order to obtain similar melting temperatures between primer pairs, the length of the dCAPS primers ranged from 19 to 21 nucleotides. Reverse primers were designed approximately 69–79 bp apart from the forward primers in order to generate short fragments that could be easily detected on methaphor gels after digestion.

Table 1 Sequences of specific primers based on ten RGA consensus sequences, size of the expected products, restriction enzyme used and type of marker obtained

RGAs classes	Forward primer (5′–3′)	Reverse primer (5′–3′)	PCR product sizes (pb)	Restriction enzyme	Marker type
RGA01	ACCCTTGCACAACTTGTTTAC	GCAATCCTCCACACTTTCTTG	511	*Rsa*I	CAPS
RGA02	ACGACCCTAGCTCAAATTGTT	CACTTTTTGGCAATCTTCCTG	482	*Taq*I	CAPS
RGA03	GTACAACCATGACACTATAA	TTTCTAACAATCTCTTTGCC	456	*Hinf*I	CAPS
RGA04	GATAGTAGAATTGCTAACCATTT	CAATCCTTCACATTTCACCAC	462	*Taq* I	CAPS
RGA05	GCGATTTTGAATGTAGGAG	GGCAATCCCCCAGAATAC	486	*Hae*III	dCAPS
RGA06	ATTGGAAGCAAGTTTGAGGG	CCGGAATAAGCAATCACATC	472	–	
RGA07	GCGACCGTCTTGTATGAC	GGCCTTGAGTGTATTCTAGT	478	*Nla*III	CAPS
RGA08	GCCTTGTATGCTAGAATCTC	GGCCACGGACATAGTTTAG	466	–	
RGA09	ATGACCGAATCTCACAACAA	GTCATTAACCAACCATTCG	428	–	
RGA10	CAATTTCCTGTGTACTGCC	CCATTGGCATACCTTAGTA	440	*Rsa*I	dCAPS

– No reproducible amplification

Table 2 Target sequences, SNP mutations (bold) and dCAPS primers designed to detect the introduced mismatch in *Cicer arietinum* populations

RGA classes		Target sequence and dCAPS primers sequence[a] (5′→ 3′)	Ta[b] (ºC)	Size[c] (bp)	Enzyme	Parental lines[d]
RGA05	Forward dF5	CCTGAATCAATACTTTGTATCTTGGT**T**/CGTC TCAATACTTTGTATCTTGGC	52	109	*Hae*III	ILC72, ICCL81001
	Reverse dR5	TTGCAAATGTCAGGGAAGTT				
RGA10	Forward dF10	TGGCCTTTTCACGACATAGCCTA**T**/CTTT CCTTTTCACGACATAGCGTA	55	99	*Rsa*I	Cr5–10, Cr5–9
	Reverse dR10	AGCAAATTCTACTTCAAACTC				

[a] Base mismatch introduced is shadowed

[b] Ta: optimum annealing temperature

[c] Predicted PCR product size

[d] Parental lines used to design the dCAPS primers

Reaction mixtures of 25 µl contained 50 ng of genomic DNA, 1× PCR buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 0.25 µM of each specific primer and 0.75 U of *Taq* DNA polymerase (Biotools) were used for RGAs amplification. For some RGA classes 1.5 mM $MgCl_2$ were also assayed. PCR reactions were performed in a T Gradient PCR (Biometra), using the following conditions: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55 or 52°C for 1 or 2 min and 72°C for 2 min with a final extension step at 72°C for 10 min before cooling to 4°C. The same procedure was carried out when using the dCAPS primers. For restrictions assays 5–10 µl of the PCR products were incubated with 2 or 5 U, respectively of restriction enzymes in a final volume of 25 µl. CAPS products were separated as described for STMS markers while for dCAPS markers, 3% Metaphor agarose gels were used.

Sequence analysis of RGA classes

Sequence editing and analysis was conducted with the BioEdit ver. 7.0.1 software program (Hall 1999). In all the cases multiple alignments of nucleotide and deduced protein sequences were implemented using the MEGA v. 4.0 (Tamura et al. 2007). This software package was also used to calculate *P* distances between sequences at amino-acid level with the RGAs developed in this study and *Cicer* RGAs previously published (RGA-D2: AJ307996, RGA-D: AJ307989, RGA-DS: AJ307994, RGA-D0: AJ307995, RGA-H: AJ307993, RGA-A: AJ307986, RGA-E: AJ307990, RGA-C: AJ307988, RGA-G: AJ307992, RGA-F: AJ307991, RGA-B: AJ307987; Huettel et al. 2002).

The Gen Bank non-redundant database (NR) of nucleotide sequences as well as the expressed sequence tag (EST) database (dbEST) at the National Center for Biotechnology Information (NCBI) were screened with each consensus RGA class sequence using the BLASTN algorithm (Altschul et al. 1997).

Composite map

Segregation of the new markers (STMS and RGAs) to be included in the composite map was tested for goodness of fit to the expected Mendelian ratio of 1:1 using χ^2 analysis ($P < 0.05$). Markers with distorted segregation were also used for linkage analysis. Linkage analysis was performed using JoinMap ver. 4 (van Ooijen 2006), a minimum LOD score threshold of 3 and a maximum recombination fraction of 0.20 were employed as general linkage criteria to establish linkage groups. Kosambi's function was applied to estimate the map unit distance. *Combine groups for map integration* function were used to combine the data from the separate populations. Heterogeneity of recombination rate between common markers in the two populations was tested using χ^2 tests as implemented in JoinMap 4.

Quantitative trait loci analysis was performed using MAPQTL 5 software (van Ooijen 2004). Interval mapping (IM) (Lander and Botstein 1989; van Ooijen 1992) was used with a mapping step size of 1 cM. Significance of QTLs was empirically determined with the permutation test consisting of 1,000 replications (Churchill and Doerge 1994). The coefficient of determination (R^2) for the marker that was most closely linked to a QTL was used to estimate the percentage of the total phenotypic variation explained by the QTL.

Results

Marker development

To facilitate genetic mapping of the ten RGA classes previously obtained, the consensus RGA sequences for each class were aligned and specific primer pairs discriminating between them were designed. When genomic DNA of the four parental lines was amplified with the ten primer pairs (Table 1), no reproducible amplification was obtained for classes 6, 8 and 9 whose primers were designed from

clones isolated from faba bean, and these classes were discarded from further analysis. The remaining primer pairs amplified a single band of the expected size in all parental lines, therefore a panel of restriction enzymes was used to digest the PCR products. Polymorphisms were detected in five RGAs classes (RGA01, 2, 3, 4 and 7), allowing the development of CAPS markers (Table 1).

In classes RGA05 and RGA10 where no polymorphism was apparent with restriction enzymes, PCR products were sequenced to identify allele-specific SNPs within the parental RGA sequences. Sequence comparisons among the chickpea parental lines with these two classes revealed one and three SNPs, respectively. The SNPs detected were subsequently used to design dCAPS markers in which mismatched primers created a restriction endonuclease recognition site in one of the parental lines. In both classes the design of only one forward primer containing a mismatch at the 3′ end (C in the first dF5 base and G in third dF10 base) was sufficient to reveal a polymorphism with the corresponding enzyme (Table 2). The reverse primers were located 69 and 78 bp apart, respectively. The mismatches generated a *Hae*III (GGCC) restriction site in ILC72 and ICCL81001 for RGA05 and a *Rsa*I (GTAC) restriction site in CR5–9 and Cr5–10 for RGA10. Figure 1 shows the RGA05 region amplified, containing one SNP and the expected restriction pattern with *Hae*III.

For class RGA05 the SNPs were synonymous and the nucleotide change was not predicted to produce an amino acid change in the deduced protein sequence. By contrast, in RGA10 the SNP used to detect polymorphism produced an amino acid change in the protein sequence (Serine to Asparagine). Both single base changes consisted in a transition between parental DNA fragments.

Sequence analysis of the RGA classes

To resolve whether or not these RGAs were unique, the consensus RGA sequence of each class was used as query to screen the GenBank nucleotide sequence database by means of the BLASTN algorithm (Table 3). Only the most

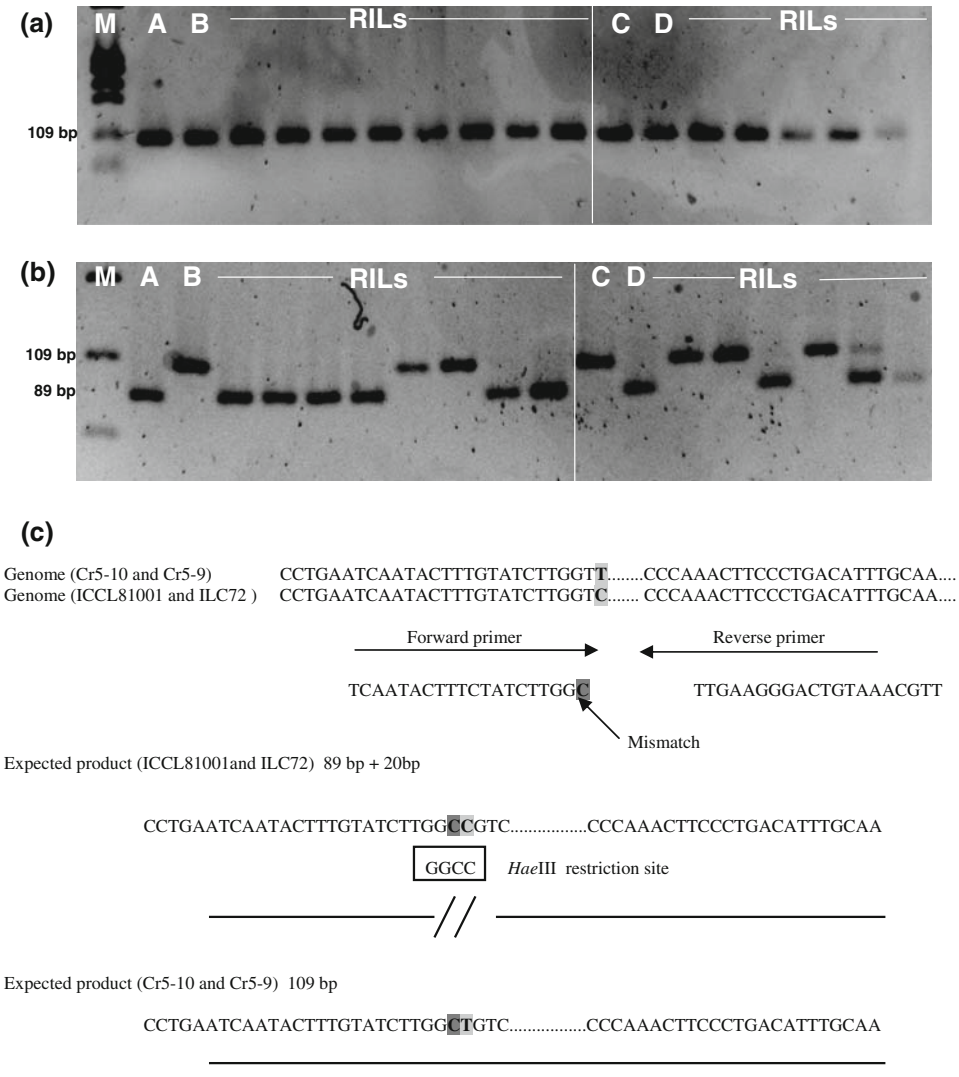

Fig. 1 **a** PCR products from dF5/dR5 dCAPS primers with parental lines A (ILC72), B (Cr5–10), C (Cr5–9) and D (ICCL81001), and several RILs. **b** Restriction pattern with *Hae*III in populations ILC72 × Cr5–10 and ICCL81001 × Cr5–9. *M* DNA size marker (ØX174) and **c** schematic illustration for dCAPS detection using primers dF5 and dR5

Table 3 Closest homologs (*R*-genes, RGAs and ESTs) of the ten consensus RGA classes used in this study, detected by BLASTN in the NCBI database

RGAs	Non-redundant database			EST database		
	Accession no.	*E* value	RGA or R-gen/organism	Accession no.	*E* value	Origin
RGA01	AF123695	$2e^{-125}$	(RGA1.1) *P. sativum*	BE326035	$8e^{-137}$	Developing stem *M. truncatula*
	AF123702	$7e^{-106}$	(RGA2.159) *P. sativum*	BG645702	$1e^{-40}$	KV3 *M. truncatula* cDNA clone
	AJ307987	$1e^{-64}$	(RGA-B) *C. arietinum*			
	AF123696	$8e^{-29}$	(RGA1.5) *P. sativum*			
	DQ205966	$3e^{-16}$	(I2) *L. esculentum*			
RGA02	AF123702	$3e^{-174}$	(RGA2.159) *P. sativum*	AW688464	$8e^{-105}$	Developing stem *M. truncatula*
	AF123695	$2e^{-87}$	(RGA1.1) *P. sativum*	BG453139	$2e^{-101}$	Developing leaf *M. truncatula*
	AJ307987	$2e^{-61}$	(RGA-B) *C. arietinum*	BG645702	$2e^{-24}$	KV3 *M. truncatula* cDNA clone
	AF123696	$1e^{-38}$	(RGA1.5) *P. sativum*			
	DQ206019	$3e^{-28}$	(I2) *C. annum*			
RGA03	CR931742	$3e^{-148}$	Clone nth2-28i20 *M. truncatula*	BF643456	$2e^{-119}$	Elicited cell culture *M. truncatula*
	AY963292	$4e^{-64}$	(Rps1-k-1) *G. max*	BF324908	$1e^{-07}$	Putative resistance protein *G. max*
	AY518519	$2e^{-16}$	(6gG9) *G. max*	Bf324859	$1e^{-07}$	Putative resistance protein *G. max*
	AF478170	$3e^{-15}$	(RGA2) *P. vulgaris*			
	AF306504	$5e^{-12}$	(B11) *P. vulgaris*			
RGA04	CR955005	$9e^{-161}$	Clon mte1-58c24 *M. truncatula*			
	AP006712	$2e^{-111}$	Clon TM0627 *L. japonicus*			
RGA05	AF186624	0	**(CP2 clone) *C. arietinum***	CX530376	$2e^{-49}$	Methyl jasmonate-elicited root cell suspension *M. truncatula*
	AF186625	$8e^{-149}$	(CP3 clone) *C. arietinum*			
	AF123699	$3e^{-135}$	(RGA2.65) *P. sativum*			
	AJ516076	$2e^{-106}$	(19C1clone) *L. culinaris*	BM526692	$3e^{-28}$	Disease resistance protein homolog *G. max*
	AJ307989	$7e^{-93}$	(RGA-D) *C. arietinum*			
	AJ307996	$6e^{-75}$	(RGA-D2) *C. reticulatum*			
	AJ307995	$3e^{-59}$	(RGA-D0) *C. reticulatum*			
RGA06	AJ516074	$3e^{-161}$	(9C1clone) *L. culinaris*	CX530376	$2e^{-88}$	Methyl jasmonate-elicited root cell suspension *M. truncatula*
	AF123699	$2e^{-87}$	(RGA2.65) *P. sativum*			
	AF186626	$2e^{-86}$	(CP4 clone) *C. arietinum*	BM526692	$8e^{-29}$	Disease resistance protein homolog *G. max*
	AJ307989	$1e^{-63}$	(RGA-D) *C. arietinum*			
	AJ307993	$6e^{-43}$	(RGA-H) *C. arietinum*			
RGA07	AY747343	$6e^{-87}$	(PLTR) *A. hypogaea*	AW774607	$1e^{-135}$	KV3 *M. truncatula* clon
	AJ516062	$5e^{-25}$	(2Kl) *L. culinaris*	AI974519	$6e^{-31}$	KV0 *M. truncatula* clon
	AF123703	$3e^{-22}$	(RGA-G3A) *P. sativum*			
	AJ307992	$2e^{-18}$	(RGA-G) *C. arietinum*			
RGA08	AF123703	0	**(RGA-G3A) *P. sativum***	CX533869	$1e^{-58}$	Methyl jasmonate-elicited root cell suspension *M. truncatula*
	AF230827	$2e^{-169}$	(RGA) *M. sativa*			
	AJ307992	$3e^{-78}$	(RGA-G) *C. arietinum*	CX533824	$2e^{-56}$	Methyl jasmonate-elicited root cell suspension *M. truncatula*
	AJ516062	$7e^{-74}$	(2Kl) *L. culinaris*			
RGA09	AJ516062	0	**(2Kl) *L. culinaris***	BG582688	$3e^{-104}$	GVN *M. truncatula* clon
	AJ516060	0	**(3K3) *L. culinaris***	CX524245	$2e^{-88}$	Aphid-infected shoot *M. truncatula*
	AF487952	$1e^{-134}$	(RGA) *M. sativa*	CX533869	$3e^{-65}$	Methyl jasmonate-elicited root cell suspension *M. truncatula*
	AJ307992	$1e^{-108}$	(RGA-G) *C. arietinum*			
RGA10	AJ307992	0	**RGA-G) *C. arietinum***	BG582688	$8e^{-99}$	GVN *M. truncatula* clon
	AF487949	0	**(RGA) *M. sativa***	CX533869	$2e^{-94}$	Methyl jasmonate-elicited root cell
	AC135160	0	**clon mth2-20m5 *M. truncatula***			Suspension *M. truncatula*
	AJ516062	$1e^{-127}$	(2Kl) *L. culinaris*	CX524245	$4e^{-64}$	Aphid-infected shoot *M. truncatula*
	AJ516060	$1e^{-121}$	(3K3) *L. culinaris*			

100% percentage of identity are in bold

significant matches obtained with other legume RGAs or resistance genes were considered. RGA04 was found unique since it did not detect any significant hit with other RGAs. The remaining RGA classes revealed significant hits with RGAs from other legume species, with e-values ranging from $5e^{-12}$ to $3e^{-174}$. Two RGA sequences were 100% identical to previously reported *C. arietinum* and *Medicago sativa* RGAs (Meyers et al. 1999; Huettel et al. 2002; Campbell 2003): RGA05 to AF186624 (CP2) and RGA10 to AJ307992 (RGA-G) and AF487949. Another two RGAs were 100% identical to previously published *Pisum sativum* and *Lens culinaris* RGAs (Timmerman-vaughan et al. 2000; Yaish et al. 2004): RGA08 to AF123703 (RGA-G3A) and RGA09 to AJ516062 (2K1) and AJ516060 (3K3). Thus, six out of ten RGA classes used in this study (RGA01, RGA02, RGA03, RGA04, RGA06 and RGA07) were novel sequences.

To determine if any of the ten RGA classes in the study were expressed, we conducted a BLASTN search of the dbEST database (Table 3). RGA04 did not reveal a significant match with any legume EST. RGA03 was similar to *Glycine max* ESTs (although with poor e-values), while RGA01, RGA02 exhibited high similarity to several *Medicago truncatula* ESTs. Interestingly, classes RGA05 to RGA10 showed similarity with *M. truncatula* and *G. max* ESTs that are expressed under pathogenic infections or induced by methyl jasmonate.

To determine the sequence similarity between the ten nucleotide consensus sequences of RGAs used in this study and the *Cicer* RGA sequences reported by Huettel et al. (2002), a pair-wise comparison among the deduced amino-acid sequences was performed (Table 4). The sequence identity between RGAs ranged from 20.1 to 100%, confirming the results obtained with the BLASTN search (Table 3). High homology was detected between RGA05 and the *C. arietinum* or *C. reticulatum* RGA-D0, RGA-D2, RGA-D (70.8%, 71.9 and 81.1% of amino acid identities, respectively). The highest percentage of identity (100%) was with RGA-Ds, a *C. reticulatum* sequence homologous to the *C. arietinum* RGA-D sequence (Huettel et al. 2002). RGA09 and RGA10 had 69.7 and 100% of identity with RGA-G, respectively, whereas a 76.1% of identity was detected between sequences RGA01 and RGA-B.

Integration of RGA markers into the composite map

A total of 55 common markers, 6 STMS, 41 RAPD (Random Amplified Polymorphic DNA), 3 ISSR (Inter Simple Sequence Repeats), the flower color locus *B/b* and 4 RGAs, located on 8 LGs, allowed us to integrate the two individual maps obtained for each population. Joint segregation analysis produced an integrated map with 169 markers, in which 6 RGAs and 4 new STMS were incorporated, covering 751 cM (Fig. 2). Linkage groups were numbered according to the map of Winter et al. (2000) using STMS as anchor markers. Clusters of marker-rich regions were observed in LGs 2, 3, 4 and 5. All distorted markers (75) in one or both populations were also integrated into the composite map and appeared to be clustered in LGs 4, 7 and 8. RGA04 and three out the four new STMS markers (TA18, TA25, TS12) revealed distorted segregation ($P < 0.001$) and mapped in LGs 7 and 8. No tight clustering of the different RGA classes were observed since RGA03, RGA05, RGA07, RGA01, RGA10 and RGA04, mapped in different linkage groups: LGs 1, 2, 3, 5, 6 and 8, respectively (Fig. 2). No RGAs were ascribed to LG7 where a new rust resistance gen (*Uca1/uca1*) has recently been located (Madrid et al. 2008).

RGA03, RGA10 and RGA04 mapped distant from other markers in LGs 1, 6 and 8, respectively. On the contrary, the remaining RGAs (RGA01, RGA05 and RGA07) mapped in highly saturated regions bearing known genes or QTLs controlling disease resistance and in the vicinity of previously reported RGAs.

Quantitative trait loci analysis revealed significant association between the resistance reaction against fusarium wilt races 0 and 5 and RGA05, explaining 20.1 and 14.2% of the phenotypic variation for *Foc0* and *Foc5*, respectively (LOD-scores 4.02 and 2.88) (Fig. 3).

Discussion

In the present study we have used NBS-LRR resistance gene candidates previously isolated and characterized from faba bean and chickpea parental lines (Palomino et al. 2006). A previous nucleotide sequence comparison of the ten RGA classes showed that the identified sequences were members of diverse groups of RGAs. The most divergent RGAs (RGA01 and RGA10) shared only 21.77% amino-acid identity while the most related RGAs (RGA01 and RGA02) were 99.42% identical (Palomino et al. 2006), therefore they represent a good source of markers for resistance gene tagging. The consensus sequence for each class allowed designing specific primers to distinguish between classes, and to develop a co-dominant and robust system (CAPS and dCAPS markers) to genotype SNPs within these RGA sequences. The specific primers designed for classes comprising only clones isolated from faba bean (RGA06, RGA08 and RGA09) did not amplify in the chickpea genome while classes RGA01 and RGA10 amplified a single band in both *C. arietinum* and *C. reticulatum*. Likewise, classes RGA02, RGA03, RGA04, RGA05 and RGA07 containing only chickpea clones amplified a single band in the chickpea parental lines.

Seven RGA classes allowed detecting variation or SNPs present within the NBS region at the inter-specific level. A

Table 4 Percent amino-acid identities of the 10 RGAs classes when compared with each other and the RGAs previously identified by Huettel et al. (2002)

	RGA10	RGA09	RGA08	RGA07	RGA06	RGA05	RGA04	RGA03	RGA02	RGA01	RGA-A	RGA-B	RGA-C	RGA-D	RGA-D0	RGA-D2	RGA-Ds	RGA-E	RGA-F	RGA-G	RGA-H
RGA10																					
RGA09	69.9																				
RGA08	61.8	63.9																			
RGA07	48.8	49.4	52.9																		
RGA06	32	34.3	39.1	37.3																	
RGA05	34.1	35.3	40.6	40.6	64.1																
RGA04	25.1	27.7	26.3	29.6	28.7	31.5															
RGA03	25.2	22	22.7	22.4	25.2	28	36.3														
RGA02	22.6	24.8	23.8	28.3	26.8	28.5	36.3	47.4													
RGA01	20.6	22.2	21.2	25.6	24.1	26.3	34.5	50.3	68.8												
RGA-A	36.1	40	44.4	38.9	44.4	47.7	27.4	27.5	32	27.5											
RGA-B	22.1	28.2	26	26.4	29.8	30.5	42.1	51.4	68.9	**76.1**	34										
RGA-C	36.8	37.3	39.6	47.2	36.2	40.6	29.1	25.3	28	23.6	39.6	26.5									
RGA-D	30.9	33.6	35.5	36.4	**72.7**	**81.1**	32.4	33.7	33.3	27.9	47.7	30.8	39.8								
RGA-D0	25	27.1	31.8	31.8	**65.9**	**70.8**	25.6	23.2	22.9	19.1	34.5	21.7	30.6	**73**							
RGA-D2	21.6	22.4	27.3	25	**62.5**	**71.9**	24.4	22	20.5	18	33.3	20.5	28.2	**76.4**	**69.7**						
RGA-Ds	30	32.5	42.5	35	**65**	**100**	32.5	32.5	27.5	30	42.5	27.5	31.6	**77.5**	**72.5**	**75**					
RGA-E	33	33	38.5	37.6	47.7	46.4	29	29.1	32.7	26.4	68.5	31.8	39.3	49.1	38.6	35.2	40				
RGA-F	21	23.8	21.6	24.9	27.5	28	39.1	46.2	55.9	50.8	33.9	57.8	29.2	33.3	20.2	20.2	25	28.2			
RGA-G	**100**	**69.7**	61.5	48.5	31.5	33.7	24.7	24.7	22.1	20.1	36.1	22.1	36.8	30.9	25	21.6	30	33	21		
RGA-H	27.3	34.6	34.5	33.6	63.6	61.3	31.5	26.9	25.7	26.1	45	29	34.3	62.8	59.6	58.4	70	46.4	28.8	27.3	

Highest percentages of identity are in bold

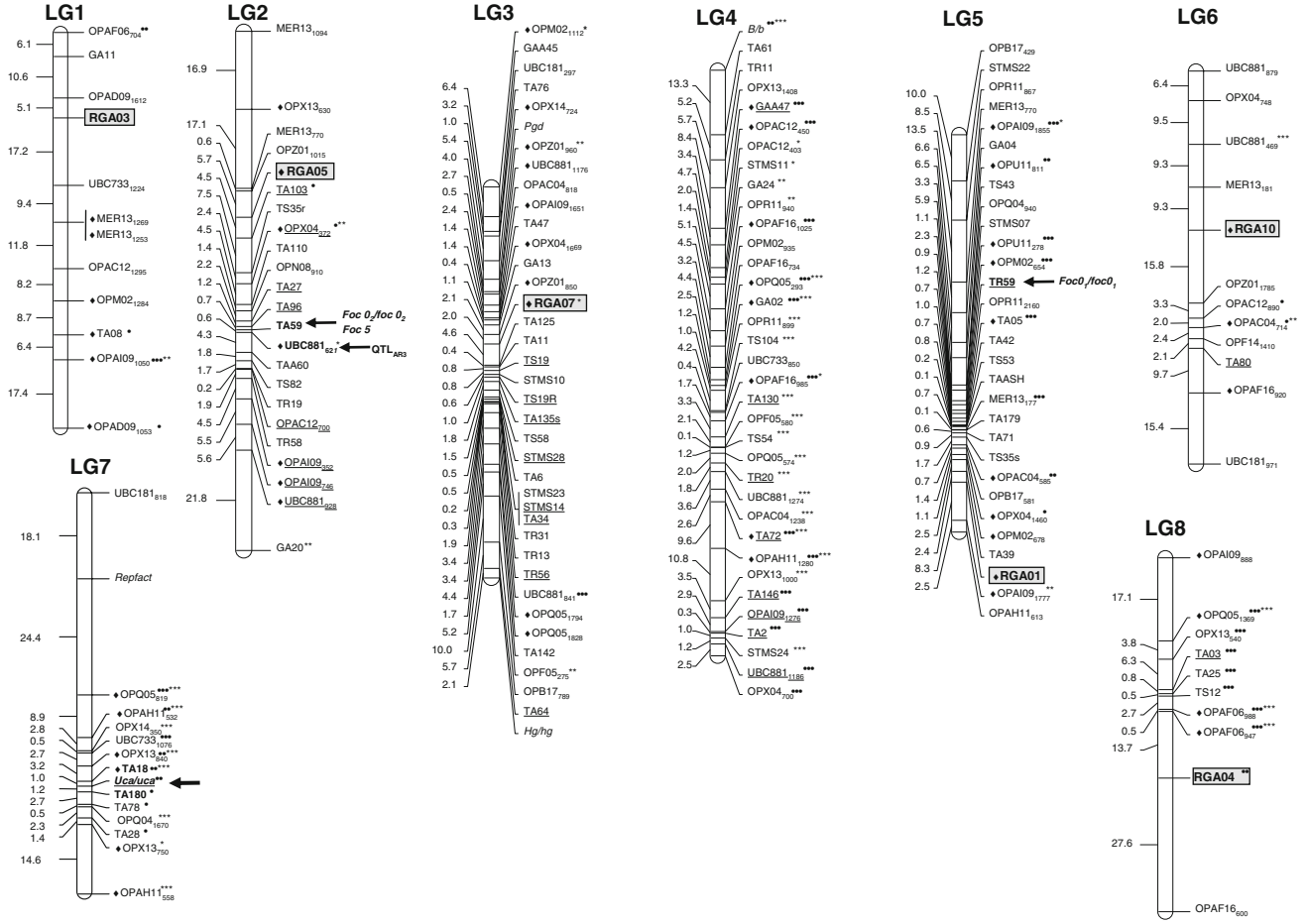

Fig. 2 Joint map of two chickpea RIL populations from crosses ILC72 × *C. reticulatum* and ICCL81001 × *C. reticulatum*. RGAs developed in this study are included in *boxes*. Indicative markers associated to disease resistance genes or QTLs previously detected using populations under study are in *bold* and indicated by *arrows*: $Foc0_2$/$foc0_2$ (Halila et al. 2008), *Foc5* (Cobos et al. 2008), $Foc0_1$/$foc0_1$ (Cobos et al. 2005), *Uca1*/*uca1* (Madrid et al. 2008), QTL_{AR3} (Iruela et al. 2007). Significant markers linked to fusarium genes or ascochyta QTLs reported in other studies are *underlined*. Distorted markers are indicated by *filled circles* (population ILC72 × Cr5–10) or *asterisks* (population ICCL81001 × Cr5–9). Shared markers are indicated by *filled diamond*

similar level of variation in NBS sites was reported by Deng et al. (2000) in intergeneric *Citrus* × *Poncirus* hybrids, since polymorphism was identified for 9 out of 11 RGAs, using CAPS markers. In our case, five CAPS and two dCAPS markers were obtained and integrated into a *C. arietinum* × *C. reticulatum* composite map developed in this study using two mapping populations.

BLASTN searches with the consensus sequences for all the RGAs classes revealed that the majority of the RGAs reported here (RGA01, RGA02, RGA03, RGA04, RGA06 and RGA07) were clearly distinct from those previously identified and should be considered novel sequences (Table 3). Some RGAs have been reported to be pseudogenes with no functional specificity (Michelmore and Meyers 1998; Rossi et al. 2003; Wicker et al. 2007). However, the continuous ORFs of the RGAs investigated in this study indicate that they may be part of functional sequences. In support of this idea, a BLASTN search of the dbEST database identified ESTs with significant homology to six of the RGAs used in this study. RGA05, RGA06, RGA07, RGA08, RGA09 and RGA10 revealed a noticeable similarity with *M. truncatula* or *G. max*. ESTs, suggesting that these RGAs are putative candidates involved in resistance mechanisms of *Cicer*.

In the composite map develop in this study, a high number of distorted markers (44.4%) has been detected as previously reported in maps derived from chickpea interspecific crosses (Winter et al. 2000; Tekeoglu et al. 2002; Cobos et al. 2006). Distortion could affect to the position and distances among markers, however linkages here obtained, are in agreement with maps reported using intraspecific populations where distorted segregation are not detected (Udupa and Baum 2003; Radhika et al. 2007; Tar'an et al. 2007). Linkage analysis allowed to include six out of ten RGAs in different LG of the composite map, confirming that these classes belong to diverse groups. The

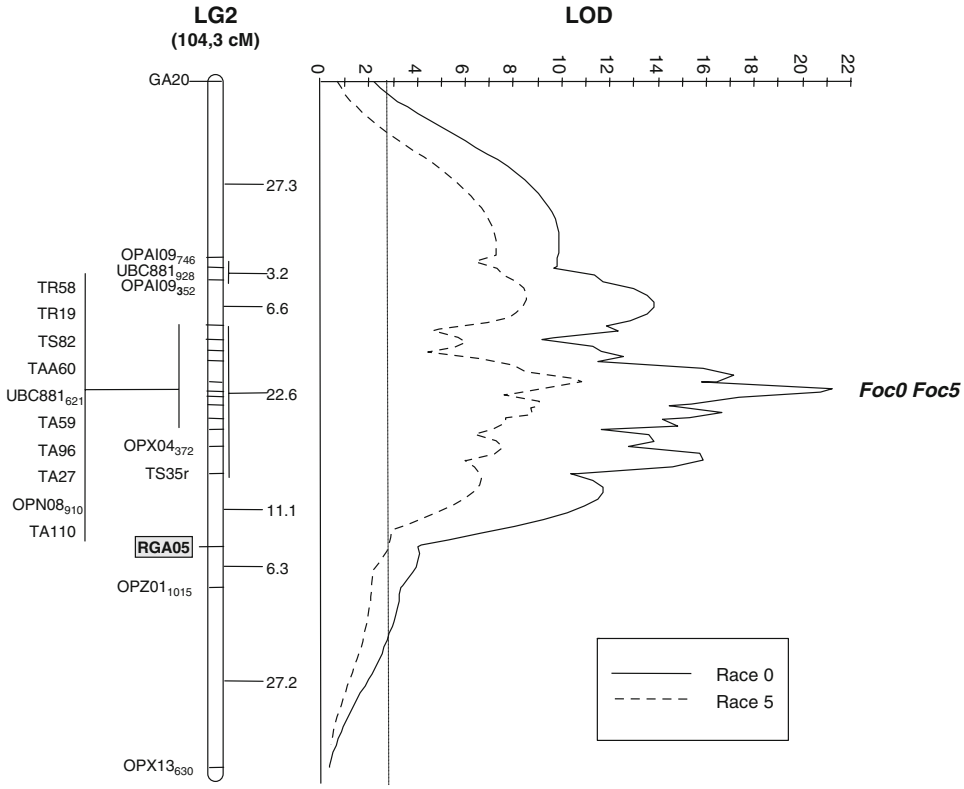

Fig. 3 Interval QTL mapping analysis for fusarium wilt resistance to races 0 and 5 located on LG2 of the *Cicer* genome using the composite map developed in this study

wide distribution of RGAs in the *Cicer* genome will facilitate future resistance gene or QTL tagging. Based on the position of STMS, resistance genes (R) genes and previously mapped RGAs, comparisons with the genomic areas containing these new RGA markers was carried out.

RGA10 and RGA4 were ascribed to LGs 6 and 8, respectively (Fig. 2), where other authors located QTLs controlling ascochyta blight resistance (Flandez-Galvez et al. 2003a; Tar'an et al. 2007). The presence of different *A. rabiei* pathotypes in our geographic area or the use of different chickpea parental lines may have prevented the detection of these QTLs in our map and of the putative association with RGA10 and RGA4.

RGA07 mapped in LG3, in close proximity to STM28 and TS19, flanking markers of a QTL for ascochyta (Flandez-Galvez et al. 2003a; Tar'an et al. 2007), together with two RGAs reported by Huettel et al. (2002) (RGA-A) and Tekeoglu et al. (2002). RGA01 mapped on LG5 where RGA-C and RGA-B were also found (Huettel et al. 2002) as well as the fusarium resistance gene *Foc0*$_1$ (Cobos et al. 2005). However, no association between these RGAs and disease resistance had been found. Similar results were obtained with RGA-A, RGA-B and RGA-C previously mapped in these LGs by Huettel et al. (2002).

On the contrary, RGA05 mapped in LG2 where a fusarium wilt resistance gene cluster (Sharma and Muehlbauer 2007) and a QTL controlling ascochyta blight (Udupa and Baum 2003; Cobos et al. 2006; Iruela et al. 2007) had been localized, together with a number of RGAs (XLRRb$_{566}$ or XLRRinv$_{665}$ Flandez-Galvez et al. 2003b; and RGA-D and RGA-Ds Huettel et al. 2002). Interestingly, in LG2 a gene controlling resistance to fusarium wilt race 0 (*Foc0*$_2$) closely linked to the resistance gene for race 5 (*Foc5*) have been detected by our group (Halila et al. 2008; Cobos et al. 2008).

A relationship between this marker and ascochyta resistance was not apparent, however, significant association between RGA05 and the two genes controlling resistance to fusarium wilt races 0 and 5 was observed (Fig. 3). Even though this marker did not explain a high percentage of phenotypic variation compared with STMS TA59 reported by Cobos et al. (2008) (race 0 $R^2 = 69.1$; race 5 $R^2 = 43.5$), significant association with fusarium wilt race 0 ($R^2 = 20.1$) and race 5 ($R^2 = 14.2$) was detected indicating that RGA05 is close to the cluster of fusarium wilt resistance genes.

Pair-wise comparison of the deduced amino acid sequences revealed a high level of similarity between RGA05 and RGA-D, RGA-D2, RGA-Ds and RGA-D0 previously obtained by Huettel et al. (2002). These authors detected closely linked multicopy RGA families in *Cicer* representing a cluster of tightly linked NBS-LRR genes. A similar pattern of genomic organization was described in species such as soybean, pea, rice or barley, with some loci tightly linked to the trait of interest (Leister et al. 1998). These results suggest that RGA05 may belong to the same NBS-LRR cluster, and opens the possibility to detect new members of the same family by tagging the genomic area.

Moreover, RGA05 showed high similarity with RGA2.65 from *Pisum sativum* which mapped in a region known to contain the disease resistance gene *Fw*, a dominant gene for resistance to *F. oxysporum* race 1 (Timmerman-vaughan et al. 2000). It would be of great interest to integrate a set of anchor markers tightly linked to *Fw* in the chickpea map in order to determine if this genomic region is conserved in both species.

The association between RGAs and resistance genes in chickpea is limited by the low number of resistance or defense reactions mapped so far in this crop. This linkage will also depend on the segregating populations and the pathotypes or races evaluated. In this study, six RGAs have been mapped in different chickpea linkage groups where major QTLs conferring resistance to ascochyta blight and fusarium wilt have been reported. Significant association between RGA05 and a QTL controlling fusarium wilt race 0 and race 5 was detected. The remaining RGAs, located in different LGs, provide a set of candidate *R* genes for further associations with additional resistance genes. Analysis of new progenies together with targeting at the more variable LRR-coding region of the resistance genes should provide ample scope for future co-segregation studies.

Acknowledgments This study was supported by the European Community project FP6-2002-FOOD-1-506223 GRAIN LEGUMES Integrated Project, CICYT project AGL2005-07497-CO2-1 and Junta de Andalucía Excelencia project AGR 00433.

References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Liman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389–3402

Campbell TA (2003) Investigation of variations in NBS motifs in alfalfa (*M. sativa*), *M. edgeworthii* and *M. ruthenica*. Can J Plant Sci 83:371–376

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963–971

Cobos MJ, Fernández MJ, Rubio J, Kharrat M, Moreno MT, Gil J, Millán T (2005) A linkage map in chickpea (*Cicer arietinum* L) in two populations from Kabuli × Desi crosses: location of a resistance gene for fusarium wilt race 0. Theor Appl Genet 110:1347–1353

Cobos MJ, Rubio J, Strange RN, Moreno MT, Gil J, Millán T (2006) A new QTL for Ascochyta blight resistance in a RIL population derived from a interspecific cross in chickpea. Euphytica 149:105–111

Cobos MJ, Winter P, Kharrat M, Cubero JI, Gil J, Millan T, Rubio J (2008) Genetic analysis of agronomic traits in a wide cross of chickpea. Field Crop Res (in press)

Deng Z, Huang S, Ling P, Chen C, Yu C, Weber CA, Moore GA, Gmiter FGJR (2000) Cloning and characterization of NBS-LRR class resistance-gene candidate sequences in citrus. Theor Appl Genet 101:814–822

Díaz-Franco A, Pérez-García P (1995) Control químico de la roya y la rabia del garbanzo y su influencia en el rendimiento de grano. Revista Mexicana de Fitopatología 13:123–125

FAOSTAT (2008) http://faostat.fao.org. Last update June 2008

Flandez-Galvez H, Ades PK, Ford R, Pang ECK, Taylor PWJ (2003a) QTL analysis for ascochyta blight resistance in an intraspecific population of chickpea (*Cicer arietinum* L.). Theor Appl Genet 107:1257–1265

Flandez-Galvez H, Ford R, Pang ECK, Taylor PWJ (2003b) An intraspecific linkage map of the chickpea (*Cicer arietinum* L.) genome based on sequence tagged microsatellite site and resistance gene analog markers. Theor Appl Genet 106:1447–1456

Gupta PK, Roy JK, Prasad M (2001) Single nucleotide polymorphisms: a new paradigm for molecular marker technology and DNA polymorphism detection with emphasis on their use in plants. Curr Sci 80:524–535

Halila I, Cobos MJ, Rubio J, Millán T, Kharrat M, Marrakchi M, J Gil (2008) Tagging and mapping a second resistance gene for Fusarium wilt race 0 in chickpea. Eur J Plant Pathol. doi:10.1007/s10658-008-9395-x

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–98

Huettel B, Santra D, Muehlbauer FJ, Kahl G (2002) Resistance gene analogues of chickpea (*Cicer arietinum* L.): isolation, genetic mapping and association with a Fussarium resistance gene cluster. Theor Appl Genet 105:479–490

Iruela M, Rubio J, Barro F, Cubero JI, Millán T, Gil J (2006) Detection of two quantitative trait loci for resistance to ascochyta blight in an intra-specific cross of chickpea (*Cicer arietinum* L.): development of SCAR markers associated with resistance. Theor Appl Genet 112:278–287

Iruela M, Castro P, Rubio J, Cubero JI, Jacinto C, Millán T, Gil J (2007) Validation of a QTL for resistance to ascochyta blight linked to resistance to fusarium wilt race 5 in chickpea (*Cicer arietinum* L.). Eur J Plant Pathol 119:20–37

Komori T, Nitta N (2005) Utilization of the CAPS/dCAPS method to convert rice SNPs into PCR-based markers. Breeding Sci 55:93–98

Konieczny A, Ausubel FM (1993) A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J 4:403–410

Kuhn Dn, Heath M, Wisser RJ, Meerow A, Brow JS, Lopes U, Schnell RJ (2003) Resistance gene homologues in *Theobroma cacao* as useful genetic markers. Theor Appl Genet 107:191–202

Kumar J, Haware MP (1983) Wilt-resistant Kabuli strains developed at ICRISAT. Int Chickpea Newsl 8:7–8

Lander ES, Botstein D (1989) Mapping Mendelian factors underlying quantitative traits using RFLP linkage map. Genetics 121:185–199

Leister D, Kurth J, Laurie DA, Yano M, Sasaki T, Devos K, Graner A, Schulze-Lefert P (1998) Rapid reorganization of resistance gene homologues in cereal genomes. Proc Natl Acad Sci USA 95:370–375

Madrid E, Rubiales D, Moral A, Moreno MT, Millán T, Gil J, Rubio J (2008) Mechanism and molecular markers associated with rust resistance in a chickpea interspecific cross (*Cicer arietinum* × *Cicer reticulatum*). Eur J Plant Pathol 121:43–53

McIntyre CL, Casu RE, Drenth J, Knight D, Whan VA, Croft BJ, Jordan DR, Manners JM (2005) Resistance gene analogues in sugarcane and sorghum and their association with quantitative trait loci for rust resistance. Genome 48:391–400

Meyers BC, Dickerman AW, Michelmore RW, Sivaramakrishnan S, Sobral BW, Young ND (1999) Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J 20:317–332

Michelmore RW, Meyers BC (1998) Clusters of resistance genes in plants evolve by divergent selection and a birth-and-death process. Genome Res 8:1113–1130

Millán T, Clarke HJ, Siddique KHM, Buhariwalla HK, Gaur PM, Kumar J, Gil J, Kahl G, Winter P (2006) Chickpea molecular breeding: new tools and concepts. Euphytica 147:81–103

Neff MM, Joseph JD, Chory J, Pepper AE (1998) dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. Plant J 14:387–392

Noir S, Combes MC, Anthony F, Lashermes P (2001) Origin, diversity and evolution of NBS-type disease-resistance gene homologues in coffee trees (*Coffea* L.). Mol Genet Genomics 265:654–662

Palomino C, Satovic Z, Cubero JI, Torres AM (2006) Identification and characterization of NBS-LRR class resistance gene analogs in faba bean (*Vicia faba* L.) and chickpea (*Cicer arietinum* L.). Genome 49:1227–1237

Pande S, Siddique KHM, Kishore GK, Bayaa B, Gaur PM, Gowda CLL, Bretag TW, Crouch JH (2005) Ascochyta blight of chickpea (*Cicer arietinum* L.): a review of biology, pathogenicity and disease management. Aust J Agric Res 56:317–332

Quint M, Dussle CM, Mechinger AE, Luebberstedt T (2003) Identification of genetically linked RGAs by BAC screening in maize and implications for gene cloning, mapping and MAS. Theor Appl Genet 106:1171–1177

Radhika P, Gowda SJM, Kadoo NY, Mhase LB, Jamadagni BM, Sainani MN, Chandra S, Gupta VS (2007) Development of an integrated intraspecific map of chickpea (*Cicer arietinum* L.) using two recombinant inbred line populations. Theor Appl Genet 115:209–216

Radwan O, Bouzidi MF, Vear F, Philippon J, Tourvieille de Labrouche D, Nicolas P, Mouzeyar S (2003) Identification of non TIR-NBS-LRR markers linked to the PI5/PI8 locus for resistance to downy mildew in sunflower. Theor Appl Genet 106:1438–1446

Rossi M, Araujo PG, Paulet F, Garsmeur O, Dias VM, Chen H, Van Sluys MA, D'Hony A (2003) Genomic distribution and characterization of Est-derived resistance gene analogues (RGAs) in sugarcane. Mol Genet Genomics 269:406–419

Rychlik W (1995) Selection of primers for polymerase chain reaction. Mol Biotechnol 3:129–134

Sharma KD, Muehlbauer FJ (2007) Fusarium wilt of chickpea: physiological specialization, genetics of resistance and resistance gene tagging. Euphytica 157:1–14

Tamura K, Dudley J, Nei M, Kumar S (2007) Genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Tar'an B, Warkentin TD, Tullu A, Vandenberg A (2007) Genetic mapping of ascochyta blight resistance in chickpea (*Cicer arietinum* L.) using a simple sequence repeat linkage map. Genome 50:26–34

Tekeoglu M, Rajesh PN, Muehlbauer FJ (2002) Integration of sequenced tagged microsatellite sites to the chickpea genetic map. Theor Appl Genet 105:847–854

Timmerman-vaughan GM, Frew TJ, Weeden NF (2000) Characterization and linkage mapping of R-gene analogous DNA sequences in pea (*Pisum sativum* L). Theor Appl Genet 101:241–247

Udupa SM, Baum M (2003) Genetic dissection of pathotype-specific resistance to ascochyta blight resistance in chickpea (*Cicer arietinum* L.) using microsatellite markers. Theor Appl Genet 106:1196–1202

van Ooijen JW (1992) Accuracy of mapping quantitative trait loci in autogamous species. Theor Appl Genet 84:803–811

van Ooijen JW (2004) MapQTL ver.5. Software for the mapping of quantitative trait loci in experimental populations. Kyazma BV, Wageningen

van Ooijen JW (2006) JoinMap ver.4. Software for the calculation of genetic linkage maps in experimental populations. Kyazma BV, Wageningen

Wicker T, Yahiaoui N, Keller B (2007) Illegitimate recombination is a major evolutionary mechanism for initiating size variation in plant resistance genes. Plant J 51:631–641

Winter P, Benko-Iseppon AM, Huettel B, Ratnaparkhe M, Tullu A, Sonnante G, Pfaff T, Tekeoglu M, Santra D, Sant VJ, Rajesh PN, Kahl G, Muehlbauer FJ (2000) A linkage map of the chickpea (*Cicer arietinum* L.) genome based on recombinant inbred lines from a *C. arietinum* × *C. reticulatum* cross: localization of resistance genes for fusarium wilt races 4 and 5. Theor Appl Genet 101:1155–1163

Winter P, Pfaff T, Udupa SM, Huettel B, Sharma PC, Sahi S, Arreguin-Espinoza R, Weingand F, Muehlbauer FJ, Kahl G (1999) Characterization and mapping sequence-tagged microsatellite sites in the chickpea (*Cicer arietinum* L.) genome. Mol Gen Genet 262:90–101

Yaish MWF, Sáenz de Miera LE, Pérez de la Vega M (2004) Isolation of a family of resistance gene analogue sequences of the nucleotide binding site (NBS) type from Lens species. Genome 47:650–659

Zhang LP, Khan A, Niño-Liu D, Foolad MR (2002) A molecular linkage map of tomato displaying chromosomal locations of resistance gene analogs based on a *Lycopersicon esculentum* × *Lycopersicon hirsutum* cross. Genome 45:133–146