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Resistance gene analogues of chickpea (*Cicer arietinum* L.): isolation, genetic mapping and association with a *Fusarium* resistance gene cluster

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Abstract Resistance gene analogues (RGAs) of *Cicer* were isolated by different PCR approaches and mapped in an inter-specific cross segregating for fusarium wilt by RFLP and CAPS analysis. Initially, two pairs of degenerate primers targeting sequences encoded at nucleotide-binding sites (NBS), which are conserved in plant disease resistance genes such as *RPS2*, *L6* and *N*, were selected for amplification. Cloning and sequence analysis of amplified products from *C. arietinum* DNA revealed eight different RGAs. Additionally, five RGAs were identified after characterisation of the presumptive RGA alleles from *C. reticulatum*. Therefore, a total of 13 different RGAs were isolated from *Cicer* and classified through pair-wise comparison into nine distinct classes with sequence similarities below a 68% amino acid identity threshold. Sequence comparison of seven RGA alleles of *C. arietinum* and *C. reticulatum* revealed polymorphisms in four RGAs with identical numbers of synonymous and non-synonymous substitutions. An *Nla*III site, unique in the RGA-A allele of *C. arietinum*, was exploited for CAPS analysis. Genomic organisation and map position of the NBS-LRR candidate resistance genes was probed by RFLP analysis. Both single-copy as well as multi-copy sequence families were present for the selected RGAs, which represented eight different classes. Five RGAs were mapped in an inter-specific population segregating for three race-specific *Fusarium* resistances. All RGAs mapped to four of the previously established eight linkage groups for chickpea. Two NBS-LRR clusters were identified that could not be resolved in our mapping population. One of these clusters, which is characterised by RFLP probe CaRGA-D, mapped to the linkage group harbouring two of three *Fusarium* resistance genes characterised in the inter-specific population. Our study provides a starting point for the characterisation and genetic mapping of candidate resistance genes in *Cicer* that is useful for marker-assisted selection and as a pool for resistance genes of *Cicer*.

Keywords Chickpea · Candidate gene approach · NBS-LRR genes · Genetic mapping · *Fusarium* resistance

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Resistance gene analogue sequences were submitted to EMBL database with accession nos. AJ307986–AJ307993 for *C. arietinum* and AJ307994 – AJ307998 for *C. reticulatum* sequences.

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Introduction

In recent years, a series of plant disease resistance (R) genes have been cloned by transposon tagging or map-based cloning from several crops and model species such as *Arabidopsis thaliana* (Baker et al. 1997). A common feature of most R genes is that the encoded proteins recognise target molecules delivered by invading pathogens. The specific interaction of both molecules results in changes in certain components of the plant's signal transduction pathway, which ultimately leads to localised cell death at the site of infection and thus prevents further growth of the pathogen (Hammond-Kosack and Jones 1997; Schulze-Lefert and Vogel 2000).

The isolated R genes can be classified on the basis of common sequence characteristics of the encoded protein. A majority of R-gene products group to the class containing a nucleotide-binding site (NBS) and leucine-rich repeats (LRR). These proteins are further characterised by an N-terminal domain with homology to the Toll/interleukin receptor (TIR) found, for example, in the *N* gene product (Whitham et al. 1994), or a coiled-coil motif (CC) such as the leucine zipper present in *RPS2* (Bent et al. 1994). Genes of this class confer resistance to pathogens of diverse origin such as viruses (*N*,

Whitham et al. 1994), bacteria (*RPS2*, Bent et al. 1994) and fungi (*I2C-2*, Ori et al. 1997). Moreover, the *Mi-1* gene confers resistance to both a nematode and an insect, indicating that one and the same NBS-LRR gene may encode resistance even to different pathogens (Rossi et al. 1998).

NBS-LRR genes are characterised by highly conserved regions as well as domains of high sequence divergence (Ellis et al. 2000). Conservation of amino acid sequences in proteins encoded by different genes is restricted to short and presumed functional domains such as the three kinase domains (Bent 1996) and a functionally uncharacterised GLPL motif (Meyers et al. 1999). Degenerate primers targeted at the underlying short conserved DNA sequences allowed the isolation of resistance gene analogues (RGAs) by the polymerase chain reaction (PCR) (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996). Several of these domains have been successfully exploited for candidate R-gene isolation in *Brassica* (Vicente and King 2001), bean (Rivkin et al. 1999) and soybean (Kanazin et al. 1996; Yu et al. 1996).

The potential functions of candidate R genes have been evaluated by genetic analyses. Several RGAs were linked to characterised resistance loci (Kanazin et al. 1996; Leister et al. 1996; Yu et al. 1996; Shen et al. 1998), and some were perfectly co-segregating, so that the isolated gene fragments obviously represented parts of the functional R genes (Leister et al. 1996; Shen et al. 1998). These results strongly recommend exploitation of conserved domains for the isolation of candidate gene fragments. This approach, together with genetic analyses of segregating material, allows direct access to R genes, given that RGAs are transformed into applicable molecular markers (Michelmore 1996).

Chickpea (*Cicer arietinum* L., $2n = 2x = 16$ chromosomes) is a self-pollinated, diploid annual species and ranks third worldwide as a food legume crop after pea and common bean (FAO 1988). Its major production areas are the Indian sub-continent, West Asia and North Africa (WANA). Chickpea breeding aims at high yield combined with resistance to biotic stresses and tolerance to drought and cold. Despite its agronomical importance and the international efforts in conventional breeding, productivity of the crop has not yet been significantly improved. Major constraints for increasing seed production are the fungal diseases Ascochyta blight, caused by the pathogen *Ascochyta rabiei* (Pass) Labr., and fusarium wilt, caused by *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *ciceris* (Padwick) Matuo K. Sato. Ascochyta blight, a widespread foliar disease, is predominant in WANA countries and restricted only by low annual rainfall and elevated temperatures (Singh et al. 1992). Resistance genetics of Ascochyta blight in chickpea seems to be complex and does not fit a simple "gene-for-gene" interaction. Moreover, Santra et al. (2000) provided evidence that at least three quantitative trait loci (QTLs) control Ascochyta blight resistance in chickpea.

Seven different races (0–6) of *Fusarium* have been reported, with race 0 being the least virulent and race 5 the most virulent (Kraft et al. 1994). Only a few DNA markers are linked to *Fusarium* R genes, with randomly amplified polymorphic DNA (RAPD) marker CS-27 successfully being converted into an ASAP marker, which in turn was also linked to race 4 and 5 fusarium wilt R genes together with an inter-simple sequence repeat (ISSR) marker in the inter-specific cross *C. arietinum* × *C. reticulatum* used in our study. Inter-specific crosses are superior segregating material for genetic mapping, since variation within cultivated chickpea is minimal (Udupa et al. 1993). Therefore, this inter-specific population of recombinant inbred lines (RILs) was previously selected for the construction of a first dense genetic map of the chickpea genome. We mapped a total of 303 different co-dominant (STMS, isozymes, cDNAs) and dominant markers (DAF, AFLP, ISSR, RAPD, SCAR), which were organised in eight large linkage groups, most probably resembling the number of chickpea chromosomes (Winter et al. 2000). However, the majority of the loci are anonymous, uncharacterised DNA markers, and neither sequence-characterised cDNAs nor candidate R genes have been mapped so far in chickpea. Characterised RGAs, mapped in defined populations, will be useful tools in marker-assisted selection (MAS) and may support the cloning of chickpea disease R genes.

Here we present the isolation of RGAs using degenerate primers targeting conserved domains in the NBS region. Eight different *C. arietinum* RGAs were selected for the isolation of orthologous sequences in *C. reticulatum*. Sequence analysis yielded a total of 13 different RGAs, which grouped into nine different classes. For the first time, RGAs were mapped together with cleaved amplified polymorphic sequences (CAPS) and restriction fragment length polymorphisms (RFLPs) in an inter-specific cross segregating for race-specific *Fusarium* R genes. Our results revealed clustering of RGAs, their association with a *Fusarium* R-gene cluster as well as their distribution on four of eight already established linkage groups.

Materials and methods

Plant material and DNA extraction

For candidate gene isolation and genetic mapping, we used chickpea cv. ICC 4958 and a cross between this line and the closely related wild species *C. reticulatum* PI489777. The segregating material consisted of 131 RILs derived from this inter-species cross. For RIL development, F_2 plants were propagated to the F_7 or F_8 using single-seed descent. Plants were propagated in the greenhouse, and DNA was isolated from young leaflets using a modified cetyl-trimethyl-ammoniumbromide (CTAB) protocol (Weising et al. 1995). Contaminating polysaccharides were selectively precipitated according to Michaels et al. (1994).

Isolation of RGAs by PCR with degenerate domain-specific primers

Two combinations of degenerate primers were used to amplify RGA sequences by PCR. The first combination was deduced from conserved amino acids of putative P-loop and kinase-3 sequences

of R genes *RPS2* and *N* (NBS-F1 5′-GGAATGGGNGGNGTN-GGNAARAC-3′ and NBS-R1 5′-YCTAGTTGTRAYDATDAY-YYTRC-3′; Yu et al. 1996), whereas the second primer combination targeted at the P-loop and a conserved GLPL(T/A) amino acid motif (primers LM638 5′-GGIGGIGTIGGIAAIACIAC-3′ and LM637 5′-ARIGCTARIGGIARICC-3′, Kanazin et al. 1996). The temperature regime during PCR was identical to conditions described by these authors. Amplifications were performed on a Perkin Elmer Geneamp 9700 thermal cycler in a 50-µl reaction volume. Each reaction contained 50 m*M* Tris-HCl (pH 8.3), 2.5 m*M* $MgCl_2$, 150 µ*M* dNTPs, 1 µ*M* forward and reverse degenerate primers, 25 ng of genomic DNA, and 1 U *Taq* DNA polymerase (Gibco-BRL, Karlsruhe, Germany). PCR products were separated on agarose electrophoresis gels, and target bands were excised and purified before cloning.

The vectors pGem-T and pGem-T Easy (Promega, Mannheim, Germany) were used for DNA cloning. For discrimination between the different RGA sequences usually 96 or 184 clones were characterised by restriction analyses with *Hinf*I and *Rsa*I and classified according to their restriction pattern. After visual inspection, at least one clone of each class was selected for sequencing. Double-stranded plasmid DNA was sequenced using fluorescent-labelled dideoxy terminator mix (Perkin Elmer) with M13 forward and reverse sequencing primers and separated on an ABI 377 sequencing apparatus. Open reading frame analysis and editing of sequences was executed with OMIGA1.1 software (Oxford Molecular Group, Oxford). Database searches for homologies were performed using standard gapped BLAST algorithms (Altschul et al. 1997; see URL http://www.ncbi.nlm.nih.gov/blast/). Phylogenetic analysis was done with CLUSTALX software using default parameters (Thompson et al. 1997).

Analysis of RGA alleles of *C. reticulatum*

For the development of PCR-based genetic markers, RGA-specific primer pairs were deduced from isolated *C. arietinum* sequences, and for the design of primers, RGA sequences were aligned using CLUSTALX software. Sequence regions specific for each identified *C. arietinum* RGA were used as start points for the deduction of specific primers using primer 3 software (S. Rozen, available at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi). The criteria for primer design were annealing temperatures around 55 °C and a primer length of 19–21 bases. Other parameters were identical to the default criteria of the program.

Alleles of *C. reticulatum* were amplified in 30-µl aliquot PCR reactions consisting of 20 ng genomic DNA, 0.2 µ*M* of each gene-specific primer, 150 µ*M* of each dNTP, 1.5 m*M* $MgCl_2$, 1× PCR buffer and 1 U *Taq* DNA polymerase (Gibco-BRL, Karlsruhe, Germany). PCR was performed in a Perkin Elmer 9700 Geneamp thermal cycler with initial denaturation (96 °C for 4 min), followed by 35 cycles at 94 °C for 15 sec, 55 °C for 23 sec and 30 sec at 72 °C, and a final elongation step for 1 min at 72 °C. For confirmation of the expected allele size for *C. reticulatum* the amplified products were separated on agarose gels, and target fragments were recovered and purified. Products were cloned with the pGEM-T vector system (Promega), and clone status was checked with colony PCR using M13 forward and reverse primers. Amplification conditions were identical to the previous protocol except that the reaction volume was increased to 50 µl. Amplified products of at least eight independent clones were restricted with *Hinf*I and *Rsa*I to assure the presence of a unique allele, i.e. the expected *C. reticulatum* orthologue and at least two clones were subjected to sequencing.

RGA sequence comparison and CAPS analysis

Sequences from *C. arietinum* and *C. reticulatum* were aligned and screened for polymorphic sites, which were then monitored for the presence of restriction enzyme recognition sequences potentially differentiating alleles of the two *Cicer* species. A total of seven allelic RGA sequences were compared with RGA-A alleles differing by an *Nla*III restriction site. This polymorphism was then exploited for genetic mapping by analysis of alleles in segregating material. The employed protocol was identical to the amplification procedure of *C. reticulatum* alleles (see previous section). For CAPS analysis (Konieczny and Ausubel 1993), PCR products were first purified through ethanol precipitation, then dissolved in 1× reaction buffer in a final volume of 15 µl and incubated with 2 U of *Nla*III overnight at 37 °C according to the supplier's instructions (New England Biolabs, Wiesbaden, Germany). DNA fragments were separated on 1.5% agarose gels, and segregation was scored visually.

RFLP analysis

Southern blotting was essentially performed as described (Sambrook et al. 1989). Briefly, 10 µg genomic DNA was restricted according to the supplier's instructions, electrophoresed on 1% agarose gels and documented and the fragments blotted overnight onto Hybond N+ membrane by capillary transfer (Amersham Pharmacia Biotech, Freiburg, Germany). Five restriction endonucleases were selected for the identification of polymorphisms (*Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I). Cloned RGA fragments were labelled by incorporation of α-[^{32}P]dCTP following the RadPrime DNA labelling System protocol (Gibco-BRL). Prehybridisation and hybridisation were each performed for 16 h at 65 °C in standard buffer [5× SSPE, 5× Denhardt's solution, 0.5% (w/v) SDS, 100 µg/ml salmon sperm DNA]. Blots were treated with 2× SSC at RT for 15 min followed by a stringent wash in 1× SSC for 30 min at 65 °C. Filters were briefly dried, exposed to X-ray film (Kodak X-AR), and autoradiographed.

Linkage analysis

For linkage analysis, MAPMAKER V3.0 (Lander et al. 1987) was used. Loci were first divided into linkage groups at a LOD-score of 4 by two-point analysis using the "group" command. Marker order within the linkage groups was determined using the "try" command of the programme, with the previously published order used as starting order (Winter et al. 2000). The obtained marker orders were scrutinised by multi-point analysis applying the "ripple" function using default values. Final map distances were calculated with the Kosambi mapping function (Kosambi 1944) provided by the programme.

Results

PCR amplification and molecular cloning of chickpea RGA sequences

Chickpea RGA sequences were amplified from genomic DNA using two primer combinations targeting three different conserved domains in the NBS region. Of all the combinations tested, the degenerate primer pair targeting the putative P-loop and kinase-3a sequences yielded major amplification products of 340, 420, 550, 620 and 900 bp in size, while a second primer combination targeting at the P-loop and a conserved GLPL(T/A) amino acid motif resulted in a single amplified band of about 540 bp. The expected sizes of PCR products amplified from these domains in the nucleotide-binding site region are approximately 340 bp and 500 bp, considering the R-gene sequences of *RPS2*, *N* and *L6* as references. We selected all of the major fragments for cloning, especially since the presence of intron-containing

Table 1 RGA-specific primer sequences, designed from isolated *Cicer arietinum* candidate R genes, for the amplification and isolation of orthologous sequences of *C. reticulatum*

RGA sequence	Forward primer (5′ – 3′)	Reverse primer (5′ – 3′)
A	GGGGGTGGGTAAGACAACTA	CTTCCTGGACCAAACCAATC
B	GGGTGGGTAAGACAACCCTT	TCTTGCTTCCAATTTTTCCG
C	GGGTGGGGAAAACCACTCTA	CTGCTTGCTCTAAGGTTGCC
D	GGGTGTAGGGAAGACAACCA	GCTCCCTGAACCAAACCAT
E	GGGGTCGGGAAGACAACTAT	TCTGCTTCCTTTACCAAACCA
F	AATTTGATTTGAAAGGGTGGG	GGGGAAGCCATCACATTTTT
G	CCAATTTCCTGTGTACTGCC	GGGAGGCCATTAGCATACCT
H	GAAGACAGCCATAGCCGAAG	TTTGCTACCTGCACCAAACC

amplification products might result in larger PCR products. The restriction analysis, performed to differentiate between putative chickpea RGAs, revealed that the cloned loci were heterogeneous. Therefore, clones of all different restriction patterns were sequenced.

Sequence analysis of chickpea RGAs

A total of 60 putative *C. arietinum* RGA clones were sequenced. BLAST algorithms served to identify resistance gene analogues as well as other homologous sequences in the GenBank. For clones amplified with a primer combination targeting the P-loop and kinase-3a motifs and expected to encode the majority of chickpea RGAs, we found that the 340-bp sequence class had either no hits in the database or a homology to sequences binding ATP, for example, ATP-dependent Clp proteinases or the chloroplast photosystem-I apoprotein of pea (AF223227). However, a total of six sequences showed homologies to R genes or R-gene analogues isolated by similar experimental approaches (CaRGA-A to -E and CaRGA-H for *C*icer *a*rietinum *R*esistance *G*ene *A*nalogue; Fig. 1). Analyses of the remaining amplicons differing from 340 bp identified no additional RGA sequences but did identify several gene sequences as well as a retroelement with homology to *Tto*1 from tobacco. Two additional RGAs were detected from the sequences amplified with primers derived from the P-loop and GLPL(T/A) motifs (CaRGA-F and -G; Fig. 1). For eight additional clones with unique restriction patterns and sizes expected from consensus domain spacing, we could find no significant hits to GenBank entries.

The resulting eight distinct RGA sequences were aligned using CLUSTAL X software, and primer pairs discriminating between these eight RGA sequences were designed (Table 1). *C. reticulatum* alleles were cloned and sequenced to provide sequence information of the presumptive RGA orthologues. Open reading frame analysis of cloned *C. reticulatum* amplicons revealed four previously unknown RGAs, even though sequence-specific primer pairs were used for amplification (CrRGA-D0, -D2, F3, -F4; Fig. 1). Also, PCR with an RGA-D-specific primer pair resulted in an additional *C. reticulatum* amplicon (CrRGA-Ds; Fig. 1). This fragment was also cloned and sequenced and is coding for an RGA. Together with the first isolation series, we therefore identified a total of 13 different *Cicer* RGAs (Table 2, Fig. 1). Multiple alignments of the peptide sequences between the kinase1 and kinase3 domains of *Cicer* RGA-encoded peptides and six characterised plant R gene-encoded peptide sequences identified the highest homology in the characteristic kinase2 motif of the NBS region. P-loop and kinase3 sequences were excluded, since they were introduced by the selection of degenerate primers. However, when amino acids in the highly conserved kinase2 domain were compared to the multi-level consensus proposed by Meyers et al. (1999), we found almost perfect identity to the kinase2 consensus sequence K(K/R)(F/V)L(L/I)VLDDV. The highest homology to any functionally characterised plant disease R gene was 51% amino acid identity (CaRGA-B and *I2C-2 Fusarium* R gene of tomato) followed by 38% identity between CaRGA-A and the NBS region of the *N* gene of tobacco.

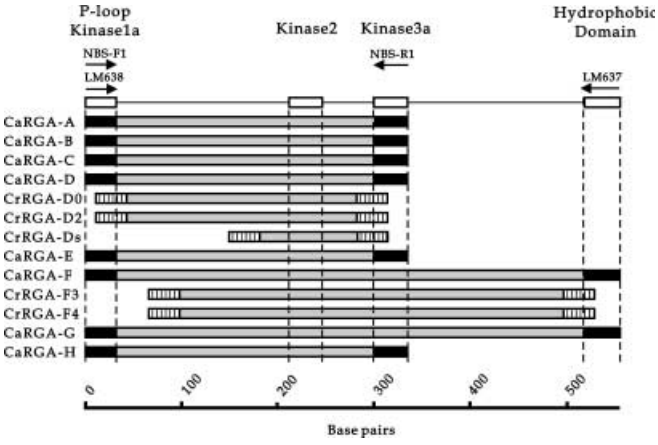

Fig. 1 Features of isolated resistance gene analogues (RGAs) of *Cicer*. Two degenerate primer combinations were used for initial RGA isolation, resulting in six RGAs derived from degenerate primers targeting at P-loop and kinase 3a (NBS-F1 and NBS-R1) and two for the combination P-loop and the hydrophobic GLPL(T/A) (LM637 and LM638) domain. The positions of the selected degenerate primers are given as *solid black boxes* at the end of isolated RGA sequences. These *C. arietinum* sequences – denoted as *CaRGA* – served to design specific primers for amplification of *C. reticulatum* alleles, yielding five additional RGAs (CrRGA) symbolised by *hatched boxes* at the respective flanks. Canonical arrangement of domains and idealised spacing between domains can be quantified by the *scale* at the *bottom* of the figure

Table 2 Characteristics of different RGA classes of *Cicer*. Classification of RGA sequences is based on a minimum threshold value of 68% amino acid identity of the encoded proteins. Copies indicate numbers of hybridising genomic fragments estimated by Southern analysis with five different restriction enzymes. Sequences of degenerate primers targeting P-loop and kinase-3a domains (NBS-F1–NBS-R1), and P-loop and the GLPL(T/A) motif (LM637–LM638) selected for isolation of *C. arietinum* RGAs were as described by Yu et al. (1996) and Kanazin et al. (1996), respectively. Sequence analysis of PCR products amplified with RGA sequence-specific primers from chickpea (see Table 1) revealed five additional RGA sequences from *C. reticulatum* (*n.d* not determined)

RGA class	RGA sequence	Primer combination targeting at	*Cicer* species	Copies
I	A	P-loop and kinase-3a	*C. arietinum*	1
II	B	P-loop and kinase-3a	*C. arietinum*	6
III	C	P-loop and kinase-3a	*C. arietinum*	2
IV	D	P-loop and kinase-3a	*C. arietinum*	10
IV	D0	RGA-D specific sequence	*C. reticulatum*	n.d.
IV	D2	RGA-D specific sequence	*C. reticulatum*	n.d.
IV	Ds	RGA-D specific sequence	*C. reticulatum*	n.d.
V	E	P-loop and kinase-3a	*C. arietinum*	1
VI	F	P-loop and GLPL(T/A) motif	*C. arietinum*	6
VII	F3	RGA-F specific sequence	*C. reticulatum*	n.d.
VII	F4	RGA-F specific sequence	*C. reticulatum*	n.d.
VIII	G	P-loop and GLPL(T/A) motif	*C. arietinum*	1
IX	H	P-loop and kinase-3a	*C. arietinum*	1

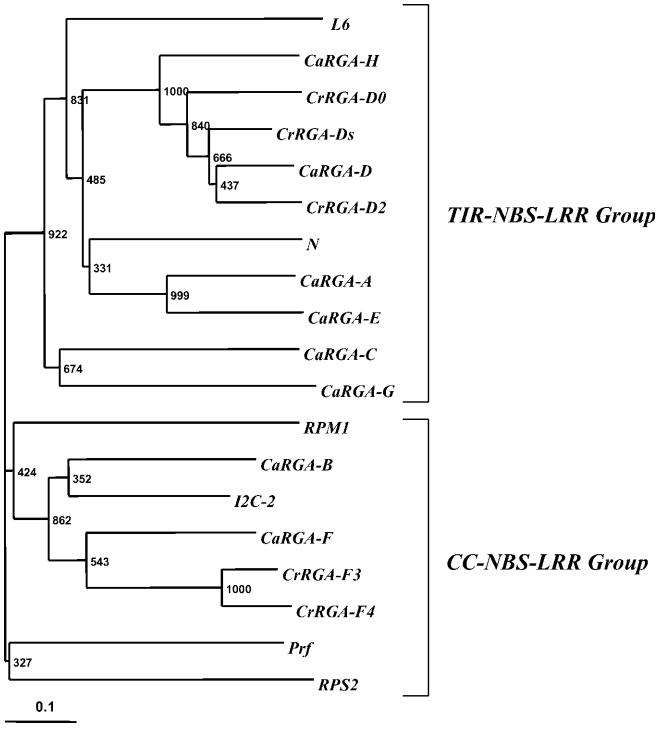

Fig. 2 Similarities in amino acid sequences of RGA-encoded proteins of *Cicer* and some plant resistance proteins visualised as a phylogenetic tree. Abbreviations for *C. arietinum* (*CaRGA*) and *C. reticulatum* (*CrRGA*) R-gene analogues are followed by a *symbol* for a specific sequence character, for example, *-A* or *-H*. *Cicer* sequences are compared to additional NBS-LRR type sequences with known disease resistance functions such as *L6* (Lawrence et al. 1995), *N* (Whitham et al. 1994), *RPM1* (Grant et al. 1995), *I2C-2* (Ori et al. 1997), *Prf* (Salmeron et al. 1996) and *RPS2* (Bent et al. 1994). *Numbers* at nodes are bootstrap values, supporting the final grouping after 1,000 bootstraps. The *scale* at the *lower part* of the figure indicates genetic distance

The genetic relationships between RGA sequences were investigated using CLUSTAL and the neighbour-joining method, which clearly separated two groups (Fig. 2). These were identical to different NBS subgroups containing either N-terminal conserved sequences with homology to TIR or the coiled-coil (CC) peptide motif, respectively (Pan et al. 2000). The division into two groups is also reflected by conserved amino acids within the NBS regions specific for each group. For the TIR-NBS-LRR group, the characteristic consensus motif FXXXXF and a highly conserved glycine is present between kinase1 and kinase2 domains, whereas the CC-NBS-LRR group contains the consensus sequence FXXXXW (Pan et al. 2000). Of 13 *Cicer* RGAs, nine grouped to the TIR class (Fig. 2). The corresponding proteins are characterised by the presence of a conserved glycine, which is not detectable at this position in CC-NBS-LRR sequences.

For sequence similarity identification and classification of *Cicer* RGAs, we performed a pair-wise comparison of all translatable sequences. In general, sequence identity was low, ranging from 14% to a mean value of 30%. Based on a sequence identity threshold of 68% for peptide sequence and 80% for nucleotide sequence, nine different classes were defined, of which seven consisted of a single RGA sequence. Two classes contained several members (Table 2). Of the latter, one group consists of two RGA sequences detected by sequence analysis of PCR products amplified by CaRGA-F specific primers. Identified ORFs of CrRGA-F3 and -F4 are only 51% and 44% identical to CaRGA-F, whereas 75% identity was detected between sequences on the amino-acid level (Table 3). The members of a second group are homologous to the CaRGA-D sequence. Amino acid sequence identity within this group ranged from 68% to 85% for CaRGA-D and CrRGA-Ds, respectively (Table 3). High homology within this group was also found at the nucleic acid level (>80%). Therefore, these sequences were grouped into a single class

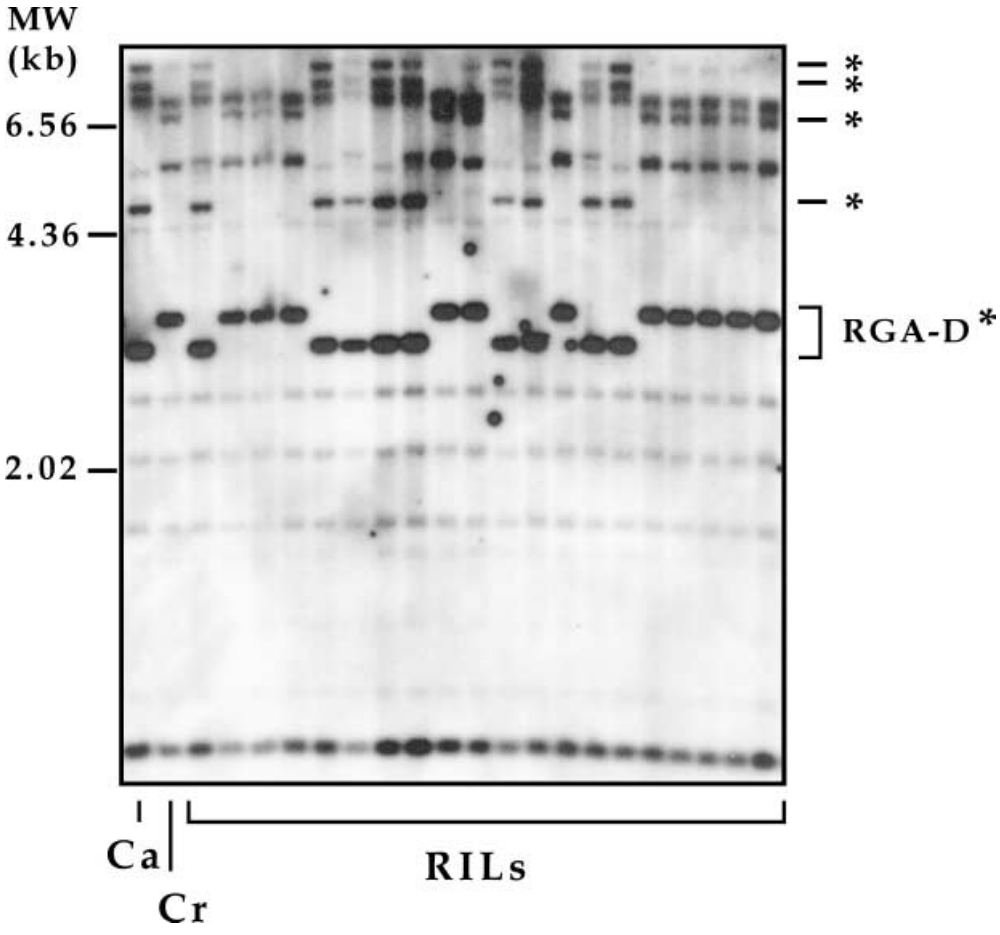

Fig. 3 RGA profile of a segregating progeny from *C. arietinum* ICC4958 × *C. reticulatum* PI489777. DNA of the parents and segregants was restricted with *Xba*I, the fragments electrophoresed and blotted, and the blot hybridised to a [^{32}P]-labelled CaRGA-D probe. The multi-copy RFLP profile was then detected by autoradiography. *Asterisks* indicate RGA-D loci linked to *Fusarium* R genes in LG2 as well as positions of additional linked fragments. Only genomes of *C. arietinum* ICC4958 (*Ca*) and *C. reticulatum* PI489777 (*Cr*) as well as a subset of RI lines are presented

Table 3 Percentage amino acid identities of RGA-encoded proteins from *Cicer* in a pair-wise comparison. Identical positions were identified by alignments using CLUSTAL X software. Comparison was restricted to amino acids between P-loop and kinase 3a domains since these are represented in a majority of the isolated 13 RGAs. Sequence similarity between RGAs equal or above the selected classification threshold value of 68% is presented in bold

	A	B	C	D	D0	D2	Ds	E	F	F3	F4	G
B	26											
C	35	20										
D	24	24	33									
D0	38	16	29	**71**								
D2	38	22	27	**76**	**68**							
Ds	43	28	30	**85**	**72**	**77**						
E	65	25	36	42	39	37	40					
F	29	54	23	23	16	18	26	24				
F3	18	34	15	14	18	16	32	25	51			
F4	15	21	13	15	19	15	26	22	44	**75**		
G	33	20	33	27	25	23	32	29	21	16	14	
H	40	23	30	61	59	59	59	43	21	20	19	26

consisting of one *C. arietinum* sequence (CaRGA-D) and three *C. reticulatum* sequences (CrRGA-D0, -D2, and -Ds; class IV; see Table 2). Moreover, these three *C. reticulatum* sequences are perfectly conserved in *C. arietinum* (i.e. no sequence variation detectable in *C. arietinum* orthologues). The sequence counterparts of the *C. reticulatum* RGAs were identified through pair-wise alignments and we found CrRGA-D0 to be identical to cp8, CrRGA-Ds to cp2 and CrRGA-D2 to cp3 (GenBank accessions: cp8 AAF36337, cp2 AAF36332, and cp3 AAF36333; Meyers et al. 1999).

Marker development and genetic mapping of RGAs

For the genetic mapping and identification of RGA sequences associated with disease resistance in chickpea, we followed two strategies: (1) CAPS analysis, including

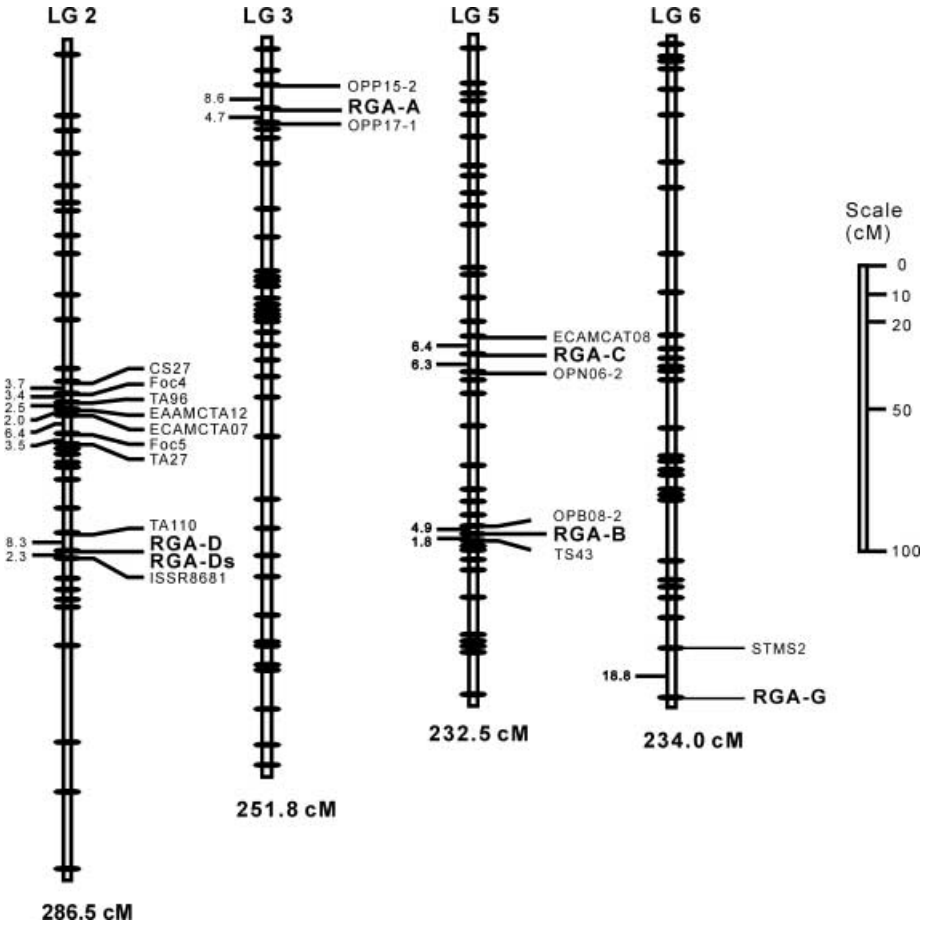

Fig. 4 Localization of RGA loci on the genetic map of the chickpea genome. Markers flanking the RGA loci and the *Fusarium* R-gene region are presented in detail, additional markers as *bar* symbols (for details, see Winter et al. 2000). Microsatellite markers are described as *STMS* (Hüttel et al. 1999), or *TA* and *TS* (Winter et al. 1999), AFLP markers are characterised by the abbreviation for the rare cutting enzyme *Eco*RI (*E*) and selective bases (as, for example *C* and *A*), followed by the abbreviation for the f requently cutting enzyme *M*seI (*M*) and selective bases (as, for example, *T* and *C*). DAF and RAPD are designated *OP* or *CS*, respectively, followed by a *number*. ISSR markers are identified by *ISSR* and a *number*. Loci for resistance to *Fusarium* wilt races 4 and 5 are abbreviated as *Foc4* and *Foc5*, respectively (Winter et al. 2000). *Numbers* to the *left* of each linkage group (*LG*) represent distances between adjacent marker loci (in centiMorgans) and were calculated using the Kosambi mapping function

Table 4 Variation of RGA alleles in *C. arietinum* and *C. reticulatum*. The alleles of seven RGA sequences were compared, and similar numbers of synonymous and nonsynonymous polymorphisms were identified

RGA sequence	Base pairs compared	Polymorphic sites	Synonymous exchange	Non-synomymous exchange *C. arietinum*. *C. reticulatum*	CAPS analysis
A	285	1	0	GTT to CTT: V → L	+
B	291	3	2	GCT to ACT: A → T	–
C	187	0	0	0	–
D	291	0	0	0	–
F	486	1	1	0	–
G	372	1	0	AAT to AGT: N → S	–
H	297	0	0	0	–

the identification of polymorphic sites in NBS regions in alleles of two different *Cicer* species, and (2) classical RFLP analysis with a set of restriction endonucleases.

PCR amplification of RGA alleles from genomic DNA of *C. reticulatum* with primers designed for specific *C. arietinum* RGAs resulted in single products, except for RGA-D primers (see above). A total of seven different *C. reticulatum* alleles were cloned, and unique fragments were verified through restriction analysis. Polymorphic sites were identified through multiple sequence alignment. Four out of seven different sequences harboured variable sites, of which three represented single nucleotide polymorphisms (SNPs). The alleles of CaRGA-B and CrRGA-B differed at three sites. The majority of nucleotide variations between RGAs resulted in non-synonymous amino acid substitutions (Table 4). Of the variable sites in the four different alleles, a unique *Nla*III restriction site in the *C. arietinum* allele of RGA-A was identified and exploited for genetic mapping. CAPS analysis of PCR products amplified in the *Cicer* population resulted in two clearly distinguishable fragments of 147 bp and 186 bp, respectively.

RGA sequences were also exploited for genetic mapping in a classical RFLP approach. The eight *C. arietinum* RGAs were selected as suitable probes, since their overall similarity at the nucleotide level was low, with a maximum value of 77% for CaRGA-A and -E. For RFLP mapping, we first searched for different probe/enzyme combinations and their potential to detect polymorphisms between lines of *C. arietinum* and *C. reticulatum*. Screening of parental material included the random selection of five different restriction enzymes in

combination with eight *C. arietinum* CaRGAs, where each sequence represented a unique class. Southern analysis resulted in single bands, when CaRGA-A, -E, -G, and -H were used as probes, while multiple bands were produced with the other RGAs (up to six bands for CaRGA-B, two for CaRGA-C, up to ten for CaRGA-D and six for CaRGA-F). The total number of homologous copies in the *Cicer* genome was independent of the restriction endonuclease. Probes CaRGA-A, -E, -F and -H revealed only monomorphic hybridisation patterns on both intra- and inter-specific levels for all of the restriction endonucleases used. Polymorphism on an inter-specific level was detected for CaRGA-B, -C, -D and -G with B, resulting in polymorphic patterns for all of the tested enzymes (C for *Eco*RI and *Hin*dIII, D for *Eco*RI, *Hin*dIII and *Xba*I and G for *Eco*RI and *Eco*RV). RFLP analysis therefore allowed the mapping of four RGAs in the inter-specific cross *C. arietinum* ICC4958 × *C. reticulatum* PI489777, which segregates for three *Fusarium* resistances (Fig. 3). We determined the RGA map positions in the context of segregation data of 303 different markers on the established integrated genetic map (Winter et al. 2000). All six RGAs mapped in this population and grouped to major linkage groups, but no tight clustering of the different RGAs could be observed. With the exception of RFLP marker RGA-G, which maps to the distal part of LG6 and thus extends the genetic map by 18.8 cM, the remaining RGAs are closely linked to other DNA markers. A characteristic feature of this inter-specific genetic map is a high degree of distorted segregation in general and, more specifically, for three of six RGA markers with RGA-C (4.35; χ^2-test with $P<0.05$) and RGA-B (8.67) mapping to linkage group 5. The various loci in this genomic region display similar levels of segregation distortion. Also, RGA-A maps in a genomic region of LG3 with similar attributes (RGA-A, 9.56). However, no segregation distortion was detected for RGA-G and two co-segregating markers belonging to RGA class IV (RFLP marker CaRGA-D and PCR-based marker CrRGA-Ds).

Previous studies of the inter-specific *C. arietinum* × *C. reticulatum* population located two race-specific *Fusarium* R genes on linkage group 2 (Fig. 4; Winter et al. 2000). Both RGA-D markers are mapping to the same linkage group, but no tight linkage or even co-segregation with resistance was found.

Discussion

Isolation and sequence analysis of RGAs

Different PCR strategies were successfully used to isolate 13 different RGA sequences from two *Cicer* species. PCR with degenerate primers derived from three different conserved motifs of the NBS region of known disease R genes amplified several fragments from *C. arietinum* that contained different sequences. Most of these showed similarity to known R genes. Based on such sequence similarities, eight different *Cicer* RGAs were identified. Sequence analysis of the products which deviate in size from the consensus spacing of NBS domains used for priming, yielded no RGAs with intron sequences, as has been reported for *A. thaliana* (Pan et al. 2000). Except for RGA-encoding sequences, a few additional coding sequences – for example, for presumed ATP-binding proteins – as well as retrotransposable elements were detected. The presence of such retroelements as by-products of PCR amplification with highly degenerate primers is frequent, especially for more complex genomes such as barley (Leister et al. 1999).

Initially, two different primer combinations were chosen, with the first combination targeting the P-loop and presumed kinase3a domains, and the second one directed towards the P-loop and a conserved hydrophobic domain. Identical combinations were used for the isolation of NBS-LRR candidate gene fragments from soybean and resulted in 9 and 11 different gene classes, respectively (Kanazin et al. 1996; Yu et al. 1996). Since a sequence comparison of their data revealed that only 3 of the 20 sequences belonged to the same class, thus resulting in a total of 17 different classes for soybean, we selected two primer combinations for the isolation of RGAs from chickpea. While the P-loop/kinase3a combination produced six RGAs, P-loop/GLPL(T/A) yielded two different RGAs from *Cicer*. This unexpected result might be caused by different degrees in degeneracy of the selected primers, since this is the only difference between both pairs with P-loop/kinase3a (512-/576-fold degeneracy) and P-loop/GLPL(T/A) (4,096-/1,024-fold degeneracy). The degree of primer degeneracy is a critical parameter for candidate gene isolation, as has also been shown by Meyers et al. (1999), who isolated chickpea RGAs with the only moderately degenerate primers published by Shen et al. (1998).

The 13 different RGA sequences were grouped into nine classes, whose identity was generally less than 65% at the amino acid level. Classification thresholds for *Cicer* RGA sequences were defined by cross-hybridisation, and thus practical aspects were taken into consideration to identify homologues in, for example, BAC libraries. Similar criteria were chosen for soybean RGAs (Kanazin et al. 1996; Yu et al. 1996). These threshold values are significantly higher than those selected for lettuce (50% amino acid identity). However, Shen et al. (1998) additionally considered the clustering of RGA sequences in the same genomic region as a criterion for a classification.

Sequence similarity between 13 isolated NBS sequences from *C. arietinum* and *C. reticulatum* and several known R genes was investigated by multiple alignments. The resulting phylogenetic tree clearly supports two groups with either an N-terminal region with homology to TIR or a coiled-coil sequence motif such as leucine zippers (Pan et al. 2000). Each N-terminal sequence motif is correlated with a high probability of specific amino acids at conserved positions within the NBS region (Meyers et al. 1999). Pan et al. (2000) identified a total of 11 conserved

domains, including functional kinase domains, as well as additional amino acids indicative of either the TIR or CC group. *Cicer* NBS sequences own the consensus for a conserved domain located between P-loop and kinase2 with the sequence FXXXXF typical for TIR, whereas FXXXXW is indicative for the CC group.

RGA sequences have been isolated from a variety of species, including legumes – soybean (Kanazin et al. 1996; Yu et al. 1996), common bean (Geffroy et al. 1999, 2000; Rivkin et al. 1999), common pea (Timmerman-Vaughn et al. 2000) and cowpea (Meyers et al. 1999). However, when comparing our *Cicer* RGAs with sequences in public domain databases (Genbank), the overall similarity at the nucleic acid level is low because of the evolutionary dynamics of NBS-LRR type genes (Meyers et al. 1999). The homology of *Cicer* NBS regions was highest to RGAs of other leguminous species with, for example, a sequence identity of up to 61% between CaRGA-G and a pigeonpea NBS sequence (pp3, Genbank accession no. AF186636), whereas maximum identity of this RGA to other *Cicer* RGAs was only 33%. Similar observations have been previously reported for bean RGAs (Rivkin et al. 1999). The divergence of these regions in related species nevertheless reduces similarities such that they are not useful for candidate gene identification. The exploitation of functionally defined heterologous R genes for RGA isolation has been studied in detail for lettuce but without the detection of expected candidate gene sequences. Obviously, high-level similarity between different NBS-LRR-type R genes is restricted to only a few, short, presumed functional domains (Shen et al. 1998). A high degree of sequence divergence is even more prominent in functional R genes of the LRR class, such as the *Cf* genes in tomato (Parniske et al. 1999). Since the exploitation of gene fragments such as the numerous isolated NBS domains of related species or functionally characterised disease R genes does not effectively allow the identification of an R gene harbouring genomic loci, *de novo* isolation of RGAs from any crop species of interest via PCR with domain-specific primers is obviously one of the successful strategies to select for and tag potential R genes.

Inter-specific sequence variation in RGA alleles of *Cicer*

Sequence variation within the NBS regions of different alleles is mandatory for mapping RGAs in segregating material by CAPS analysis, which is a rapid technique compared to time-consuming standard techniques like RFLP analysis. The potential application of CAPS markers has recently been shown for a highly indicative virus R gene in potato (Sorri et al. 1999). Once identified, these markers can easily be transferred to other inter- and intra-specific segregants if a polymorphism exists between parental lines. In most of the investigations reported, the identification of informative sites relied on the random selection of a set of restriction endonucleases and thus is more or less left to chance (Joyeux et al. 1999; Deng et al. 2000). However, a more systematic search for sequence variation between alleles will include the design of allele-specific primers, PCR amplification and cloning of the respective allele. For CAPS identification, we exploited eight *C. arietinum* RGA sequences to analyse the respective *C. reticulatum* alleles. The variation in RGA sequences at the inter-specific level was high, with four alleles showing at least SNPs and a similar rate of synonymous and non-synonymous substitutions (3:3). A high degree of variation was also found in different alleles of the R gene *RPS2* of different *Arabidopsis* ecotypes (Caicedo et al. 1999). Compared to nuclear genes, the sequence variation was one order of magnitude higher, with 70% of the scored polymorphic sites leading to amino acid changes. This result again supports the high evolutionary dynamics of plant NBS-LRR genes (Caicedo et al. 1999; Ellis et al. 2000). An even higher degree of variation in NBS sites as compared to *Cicer* was identified in intergeneric *Citrus/Poncirus* hybrids. Out of 11 different NBS regions amplified with RGA allele-specific primer pairs, polymorphism was identified for nine RGAs using restriction endonucleases with 4-bp recognition sites. However, sequence identity of these digested, presumed NBS sequences was not validated (Deng et al. 2000).

Our data prove that non-degenerate allele-specific primers amplify products related to targeted RGA loci, thus increasing the total number of RGA sequences from 8 to 13 (Fig. 1). Similar observations were made in *Brassica*, using *A. thaliana RPS2* -derived specific primers, which resulted in PCR products with no sequence homology to expected NBS regions (Joyeux et al. 1999).

Genomic organization of RGAs and genetic association with a *Fusarium* resistance gene cluster

RFLP analysis revealed two types of genomic organisation of RGAs in the *Cicer* genome. They were either present as single copies, and therefore resemble classes with unique members (e.g. CaRGA-A and -G), or as multiple copies with copy numbers varying between two (CaRGA-C) and ten (CaRGA-D). A similar organisation has been reported for soybean, pea and potato, where RGAs also occur frequently in multi-copy families, with some loci tightly linked to the trait of interest such that they cannot be resolved in typical mapping populations (Kanazin et al. 1996; Leister et al. 1996). Such multi-copy, closely linked RGA families were also detected in *Cicer* (CaRGA-B and CaRGA-D), since hybridisation with these probes produced multiple bands and several copies are tightly linked to each other. In fact, for probes CaRGA-B and CaRGA-D at least four additional homologues were not resolved in RI lines of the population, and therefore their map positions on linkage groups represent a cluster of tightly linked NBS-LRR genes. In addition to CaRGA-D homologues identified through RFLP analysis, another PCR-based RGA marker – a PCR product obtained through allele-specific amplifi-

cation, referred to as CrRGA-Ds – also mapped to this cluster without recombination. Therefore, this marker already serves to tag this RGA-rich region in the *Cicer* genome. However, genetic clustering of these RGAs can either result from physical clustering with multiple homologous sequences in close vicinity or be the result of suppressed recombination in this genomic region. Such clusters with reduced recombination rates were in fact identified in other crops such as soybean and lettuce (Kanazin et al. 1996; Meyers et al. 1998). Genetic mapping of *Cicer* RGAs in other inter-specific crosses will be useful to clarify their genomic organisation.

A major goal of breeding and marker-assisted selection in chickpea is the tagging of genes controlling resistance to the most severe fungal pathogens *Ascochyta* and *Fusarium*. Until now only a very few functional R genes have been mapped in *Cicer* species (including the population used in this study, which segregates for *Fusarium* resistances). In a previous study, we presented a detailed genetic map with tags for race 4 and 5 R genes (Winter et al. 2000). There is also evidence that LG2 with both R genes harbours additional race-specific *Fusarium* R genes. Recently, Tekeoglu et al. (2000) integrated data of Tullu et al. (1998) with a *Fusarium* race-1 R gene linked to the race-4 R gene with 5% recombination in an intraspecific *C. arietinum* cross. The *Fusarium* R genes in both intra- as well as inter-specific populations were characterised by linkage to the same RAPD marker CS27 (Tekeoglu et al. 2000). Of the several RGAs mapped in our inter-specific population, linkage to Foc4 and Foc5 R genes was identified with the RFLP probe CaRGA-D and the PCR marker CrRGA-Ds. However, tighter linkage or even co-segregation of this identified NBS-LRR cluster with additional race-specific *Fusarium* R genes relies on phenotypic scoring of segregating material for resistance reaction.

Up to now, most race-specific R genes belong to the NBS-LRR class. For example, race-specific resistance of tomato to *Fusarium* is controlled by a unique NBS-LRR gene localised in a cluster of several homologues (Ori et al. 1997). In two segregating populations of pea, an RGA mapped to the same genomic region, which is characterized by molecular markers linked to *Fusarium* resistance (Timmerman-Vaughan et al. 2000). We have not been able to identify polymorphic *Cicer* RGAs as candidate R genes for *Fusarium*. However, the total number of NBS-LRR genes in *Cicer* certainly exceeds the number of already isolated and characterised candidate genes. If we simply consider the number of NBS-LRR genes in the *A. thaliana* genome – the only plant genome completely sequenced to date – as a minimum, then we can expect around 120 TIR-NBS-LRR and CC-NBS-LRR genes in *Cicer* (The *Arabidopsis* Genome Initiative 2000). Thus, the isolation of target RGAs and their identification through, for example, RFLP analysis of the target gene co-segregating with the resistance phenotype, to some extent relies on chance. On the other hand, NBS-LRR genes do not necessarily control *Fusarium* resistance in *Cicer*. Other functional plant R genes code for serine/threonine kinases (*Pto*; Martin et al. 1993), for LRRs (*Cf9*; Jones et al. 1994), for cell-death controlling factors (*mlo*; Büschges et al. 1997) or for detoxifying enzymes (*Hm1*; Johal and Briggs 1992). Exploitation of such candidate R-gene classes for the identification of R genes in chickpea, as well as not yet isolated RGAs with NBS regions, relies on the identification of conserved domains which can serve as target sites for inter-domain amplification. The products are cloned, sequenced and then exploited for R-gene identification by for example, RFLP-based mapping. Alternatively, PCR products might be separated on high-resolution gels without DNA cloning (Chen et al. 1998). For chickpea, experiments exploiting conserved domains, especially in combination with resistant and susceptible segregating material, are underway and expected to produce tightly linked or even co-segregating candidate R genes.

In summary, we have isolated and mapped a set of *Cicer* RGAs for the first time. The results presented here provide a candidate R-gene pool that serves as a basis for the identification of R genes in this genus. Chickpea yield is reduced by numerous pests, for which only few resistances have yet been described. Therefore, our study is a starting point for the identification and isolation of R genes in segregating chickpea populations, ultimately aiming at the widespread use of R genes in adapted breeding material.

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