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Rearrangements in the *Cf-9* Disease Resistance Gene Cluster of Wild Tomato Have Resulted in Three Genes That Mediate Avr9 Responsiveness

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ABSTRACT

Cf resistance genes in tomato confer resistance to the fungal leaf pathogen *Cladosporium fulvum*. Both the well-characterized resistance gene *Cf-9* and the related *9DC* gene confer resistance to strains of *C. fulvum* that secrete the Avr9 protein and originate from the wild tomato species *Lycopersicon pimpinellifolium*. We show that *9DC* and *Cf-9* are allelic, and we have isolated and sequenced the complete *9DC* cluster of *L. pimpinellifolium* LA1301. This *9DC* cluster harbors five full-length *Cf* homologs, including orthologs of the most distal homologs of the *Cf-9* cluster and three central *9DC* genes. Two *9DC* genes (*9DC1* and *9DC2*) have an identical coding sequence, whereas *9DC3* differs at its 3′ terminus. From a detailed comparison of the *9DC* and *Cf-9* clusters, we conclude that the *Cf-9* and *Hcr9-9D* genes from the *Cf-9* cluster are ancestral to the first *9DC* gene and that the three *9DC* genes were generated by subsequent intra- and intergenic unequal recombination events. Thus, the *9DC* cluster has undergone substantial rearrangements in the central region, but not at the ends. Using transient transformation assays, we show that all three *9DC* genes confer Avr9 responsiveness, but that *9DC2* is likely the main determinant of Avr9 recognition in LA1301.

PLANTS are continuously challenged by a diverse array of pathogens. Resistant plants carry resistance (*R*) genes that enable them to recognize pathogen strains that carry matching avirulence (*Avr*) genes, a phenomenon called the gene-for-gene relationship (Flor 1946). In response to pathogen pressure, sophisticated surveillance systems have evolved to maintain and to generate new *R* genes in plants (Michelmore and Meyers 1998; Hulbert *et al.* 2001). *R* genes often occur in clusters, and extensive sequence exchange between homologs can occur through unequal recombination. This leads to novel sequence combinations and possibly to novel *R* genes. Although many *R* genes and *R*-gene clusters have been isolated, the evolution of *R*-gene clusters in natural populations is still poorly understood.

One of the best-studied pathosystems that follows the gene-for-gene relationship is the tomato-*Cladosporium fulvum* interaction (Joosten and de Wit 1999; Rivas and Thomas 2002). *C. fulvum* is a fungal biotrophic leaf pathogen of tomato (Lycopersicon) species. Resistance of tomato cultivars to *C. fulvum* has been introduced from wild tomato germ plasm. The tomato resistance gene *Cf-9* that originates from *L. pimpinellifolium* (*Lp*) confers resistance to strains of *C. fulvum* that secrete the Avr9 protein (Jones *et al.* 1994). In resistant *Cf-9* plants, a hypersensitive response (HR) is mounted at the infection site upon Avr9 recognition, thereby restricting the growth of the fungus. A high proportion of *Lp* accessions collected from their natural habitat are able to recognize Avr9 (Laugé *et al.* 2000; Van der Hoorn *et al.* 2001a), which suggests that the ability to recognize Avr9 may be beneficial to wild tomato plants.

Cf-9 is a member of the *Hcr9* (**h**omologs of ***C****ladosporium fulvum* **r**esistance gene *Cf-9*) gene family (Parniske *et al.* 1997). *Hcr9*'s encode proteins with a stretch of extracellular leucine-rich repeats, a transmembrane domain, and a short cytoplasmic tail that lacks an obvious signaling signature (Joosten and de Wit 1999; Rivas and Thomas 2002). Tomato plants usually carry several clusters of *Hcr9*'s, and up to five homologs per cluster have been reported (Parniske *et al.* 1997, 1999; Parniske and Jones 1999). The *Cf-9* cluster contains five homologs, *Hcr9-9A–9E*, of which *Hcr9-9C* is the functional *Cf-9* gene (Parniske *et al.* 1997). Comparative analysis of *Hcr9* clusters suggested that point mutation, unequal recombination, gene conversion, gene duplication, and translocation have contributed to the diversification of individual *Hcr9*'s. Orthologous *Hcr9*'s are more similar than *Hcr9* paralogs, suggesting that sequence exchange occurs most frequently between orthologs (Parniske and Jones 1999). We previously identified the natural *Cf-9* variant *9DC* in *Lp* (Van der Hoorn *et al.* 2001a). *9DC* has the same activity and specificity in conferring HR-associated Avr9 responsiveness as *Cf-9* and was suggested to be ancestral to *Cf-9* (Van der Hoorn *et al.* 2001a).

Sequence data from this article have been deposited with the GenBank Data Libraries under accession no. AY569331.

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To date, only *Hcr9* clusters of different species, clusters with different *Cf* genes, or clusters located at different chromosomal positions have been compared to study *Hcr9* evolution (Parniske *et al.* 1997, 1999; Parniske and Jones 1999). Since *9DC* and *Cf-9* are clearly related and both genes confer Avr9 responsiveness (Van der Hoorn *et al.* 2001a), we investigated the relationship between their respective clusters at the individual and at the population level. Isolation of the *9DC* cluster of the *Lp* accession LA1301, from which the *9DC* gene was originally isolated (Van der Hoorn *et al.* 2001a), provided us with a unique opportunity to compare the *Cf-9* and *9DC* clusters in detail. Extensive sequence homology between both clusters was revealed. We identified numerous rearrangements in the central region of the clusters that allowed us to conclude that *Cf-9* is ancestral to *9DC*. Multiple unequal recombination events have resulted in the generation of three *9DC* genes in the *9DC* cluster, which all confer Avr9 responsiveness.

MATERIALS AND METHODS

Plant material: Accessions of *Lp* were donated by the C. M. Rick Tomato Genetics Resource Center of the University of California, Davis (http://tgrc.ucdavis.edu/). The *Cf-9* and *9DC* genes of all Avr9-responsive *L. pimpinellifolium* accessions used in this study have been sequenced (Van der Hoorn *et al.* 2001a). These accessions were collected throughout the natural geographical range of *L. pimpinellifolium* and therefore represent distinct populations (Van der Hoorn *et al.* 2001a). The tomato cultivar MoneyMaker (MM) and the near-isogenic line MM-Cf9 (Tigchelaar 1984), which contains the *Cf-9* cluster (Parniske *et al.* 1997), were used as controls. Plants were grown under standard greenhouse conditions. Avr9-responsive plants were selected by injection of leaflets with Avr9 protein (10 μg/ml) and screened for visible necrosis.

DNA manipulations: DNA manipulations were performed according to standard protocols (Sambrook and Russell 2001). DNA sequence analysis was performed using Lasergene software programs (DNASTAR, Madison, WI). PCRs were performed with AmpliTaq (Perkin-Elmer, Wellesley, MA) or the Expand High Fidelity PCR system (Roche, Basel, Switzerland) for fragments >2 kb. Hybridizations were performed with ^{32}P-labeled probes (Prime-a-gene labeling system, Promega, Madison, WI). Genomic DNA blots were hybridized with either a *9DC* ORF probe or a *Cf-9-Hcr9-9D* (*9D*) intergenic region (IR) probe, which was obtained by PCR with primers IRF and IRR on a pBluescript II SK$^-$ (Stratagene, La Jolla, CA) library clone (see below). Primers were synthesized by Sigma-Genosys (Cambridge, UK). Primer sequences (5′–3′ direction, restriction sites underlined) are: 9AS1, ttttt<u>ccatggg</u>ttgtgtaaaacttata; H5S1, ttttt<u>ccatgg</u>attgtgtagaacttgta; CS1, gccgttcaagttgggtgtt; CS5, tttccaact tacaatcccttc; CS10, aaaccagaagaacctacaatta; CS11, ccccc<u>ctgcag</u> tcactaatatcttttcttgtgc; DS1, gagagctcaacctttacgaa; DS9, ttttt<u>ccat gggttgtgtaaaacttgtg</u>; DS12, ccccc<u>ctgcag</u>taattaatatcttttcttgtgc; DS13, ggaagagaggttcacttcgta; DS14, ccaagtctaacactatcaacatttc; DCS1, gttcttatcctttaacacccaac; IRF, ctaatgtatacaaagcaacaaacc; and IRR, tgaagttgtgaagggaagc.

Library construction, clone selection, and sequencing: Genomic DNA was isolated (Van der Beek *et al.* 1992) from LA1301 plants homozygous for *9DC* and partially digested with *Sau*3*A*I. Fragments were cloned into the Lambda FIX vector and packaged using the Lambda FIX II/*Xho*I Partial Fill-In vector kit (Stratagene) and transfected to *E. coli* KW251 (Promega). Phages carrying an *Hcr9* were identified by hybridization of plaque lift filters with a *9DC* ORF probe. On the first phage isolations, a PCR was performed with the *9DC*-specific primers DS1 and CS1 (Van der Hoorn *et al.* 2001a) to identify phages that contain *9DC*. Pure phages were obtained after two subsequent screens. Phage DNA was isolated using a plate-lysate method (Sambrook and Russell 2001). The tomato genomic DNA inserts were cloned into the *Not*I site of pBluescript II SK$^-$ (Stratagene). End sequences of clones were determined using the universal M13F and M13R primers. Inserts were subcloned in pBluescript II SK$^-$ and sequenced by primer walking (triple-strand coverage, BaseClear, Leiden, The Netherlands).

Agroinfiltration assays: Individual *Hcr9*'s were amplified by PCR with gene-specific primer pairs 9AS1/CS11 ('*9A*'), DS9/DS12 (*9DC3*) and H5S11/CS11 ('*9E*') using library clones as templates. *9D* was amplified from MM-Cf9 genomic DNA with primer pair DS9/DS12. The *Hcr9*'s were cloned in pRH80, sequenced (BaseClear), and subcloned in the binary plasmid pMOG800 (Van der Hoorn *et al.* 2000), yielding overexpression agroinfiltration constructs. The previously described *9DC* overexpression construct (Van der Hoorn *et al.* 2001a) was used for both *9DC1* and *9DC2* genes. Genomic agroinfiltration constructs were made by subcloning of the *Not*I inserts of pBluescript II SK$^-$ clones into pBIVM2 [a pCGN1548 (McBride and Summerfelt 1990) derivative]. Agroinfiltration assays with *Nicotiana tabacum* cv. Petite Havana SR1 were performed as described, with use of the pAvr9 and pCf9 constructs (Van der Hoorn *et al.* 2000), except that *Agrobacterium tumefaciens* strain GV3101 was used. To test the relative activities of *Cf* genes, agroinfiltration dilution series were performed as described previously (Van der Hoorn *et al.* 2001b).

RESULTS

***9DC* genetics and cluster conservation:** Both the *9DC* and *Cf-9* genes confer Avr9 recognition in the *Lp* population. Sequencing of seven alleles of both genes (including the previously isolated *Cf-9* allele by Jones *et al.* 1994) showed only three polymorphism nucleotides in *9DC* and none in *Cf-9* (Van der Hoorn *et al.* 2001a). The high DNA sequence homology (99.8%) among *9DC*, *Cf-9*, and *9D* suggested that these genes are allelic. Selfings of an F_1 of LA1301 and the susceptible cultivar MM-Cf0 showed a 3:1 segregation for HR-associated Avr9 recognition (65:24, $\chi^2 = 0.18$, $P > 0.67$), which indicates that Avr9 recognition is inherited as a monogenic dominant trait in LA1301. LA1301 was crossed to cultivar MM-Cf9 (Tigchelaar 1984) and subsequently backcrossed to MM-Cf0. All 330 BC_1 plants responded with an HR upon injection with Avr9 protein, confirming that *9DC* is allelic, or very closely linked, to *Cf-9* (<1.8 cM, $P = 95\%$) and located at the *Milky Way* locus (Parniske *et al.* 1999).

To assess the possible conservation of the *9DC* and *Cf-9* clusters in the *Lp* population, genomic DNA blots from a sample of *Lp* plants were hybridized with the *9DC* ORF probe (Figure 1). The *Lp* plants that carry either sequenced *9DC* or *Cf-9* alleles represent accessions from geographically distant locations throughout

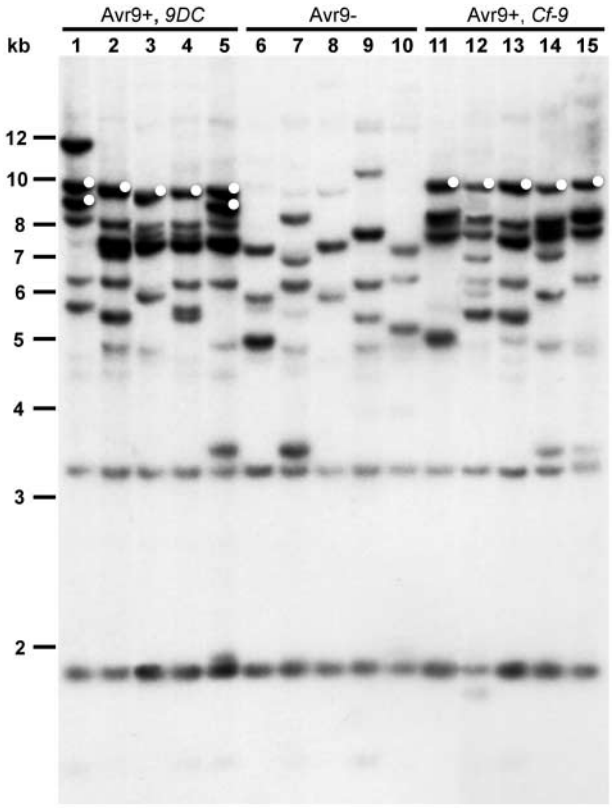

Figure 1.—High-stringency blot of *Eco*RV-digested genomic DNA of a sample of *L. pimpinellifolium* and reference genotypes, hybridized with a *9DC* ORF probe. Lanes 1–5: LA1301, LA0114, LA1629, LA1637, and LA1659 [Avr9-responsive (Avr9+), all contain sequenced *9DC* alleles (Van der Hoorn *et al.* 2001a)]; lanes 6–10: MM-Cf0, LA0400, LA1279, LA1590, and LA2852 [Avr9-nonresponsive (Avr9-), contain neither *9DC* nor *Cf-9*]; lanes 11–15: MM-Cf9, LA1585, LA1614, LA1687, and LA2653 [Avr9-responsive (Avr9+), all contain sequenced *Cf-9* alleles (Van der Hoorn *et al.* 2001a)]. Fragments marked with a white dot also hybridize to the *Cf-9-9D* IR region probe. A DNA size ladder (in kilobases) is shown on the left.

the natural range of *Lp* (Van der Hoorn *et al.* 2001a) and therefore represent different local populations. In addition, *Lp* plants from geographically distinct locations that lack both *Cf-9* and *9DC* were studied (Figure 1). Similar hybridization patterns may be expected if the *Hcr9* clusters are conserved in different *Lp* plants. However, only some hybridizing fragments appear to be conserved, and both *Cf-9*- and *9DC*-containing *Lp* plants display variation in their *Hcr9* hybridization patterns. Avr9-nonresponsive plants show fewer *Hcr9*-hybridizing bands, as does the susceptible cultivar MM-Cf0. This suggests that the nonresponsive *Lp* plants harbor fewer *Hcr9*'s than Avr9-responsive plants, possibly due to a *Milky Way* cluster with only one or a few *Hcr9*'s, as observed in MM-Cf0 (Parniske *et al.* 1997).

Isolation and assembly of the *9DC* cluster: LA1301 was chosen to molecularly characterize the *9DC* cluster, since this was also the genotype from which *9DC* was originally isolated (Van der Hoorn *et al.* 2001a), and it exhibits a strong Avr9 response. A genomic phage library with an approximate fivefold genome coverage was made from LA1301. Thirteen phages were selected on the basis of hybridization with a *9DC* ORF probe and PCR selection with *9DC*-specific primers. The inserts were subcloned in plasmids. Restriction fingerprinting, restriction hybridization, end sequencing of the inserts and AFLP-based fingerprinting of the clones (M. J. D. de Kock, R. Van der Hulst, P. J. G. M. de Wit and P. Lindhout, personal communication) of these plasmids did not result in an unambiguous contig. This suggested that either *9DC* sequences are present at two closely linked loci or sequence duplications are present within the *9DC* cluster. Hybridization of a DNA blot of *Bgl*II-digested clones with a *9DC* probe enabled us to form a contig. However, this contig obscured possible duplications within the *9DC* cluster. Therefore, *Bgl*II subclones (0.7–6.4 kb) were made of selected inserts, which were sequenced by primer walking to prevent sequence assembly artifacts due to repetitive sequences. By sequencing multiple subclones, an 8.7-kb near-perfect direct repeat was detected, which interfered with contig building by conventional methods. A clone containing an insert of 16.5 kb, which almost encompassed the two complete 8.7-kb repeat regions, frequently exhibited recombination in an *Escherichia coli* rec$^-$ strain, which resulted in loss of part of the insert. The size of the remaining insert suggested that one repeat region was lost due to this recombination (data not shown). A similar case of recombination in an *E. coli* clone leading to loss of part of a *Cf* gene cluster was previously described for the *Cf-2* cluster, which also contains repeated sequences (Dixon *et al.* 1996). With the inclusion of the 8.7-kb repeat, an unambiguous contig of the *9DC* cluster was assembled that consists of 44,539 bp (Figure 2A). The overall organization of the cluster, including the 8.7-kb repeat, has been verified by restriction fingerprinting of individual library clones and a LA1301 DNA blot hybridization using 14 different restriction enzymes and a *9DC* ORF probe (data not shown). The near-perfect 8.7-kb repeat includes two *9DC* genes with identical coding sequences (*9DC1* and *9DC2*, sequences described as *9DC* in Van der Hoorn *et al.* 2001a) and a third gene (*9DC3*), which is similar to *9DC1* and *9DC2*. We initially isolated clones containing one of the three *9DC* genes, as PCR with *9DC*-specific primers resulted in the same product for all three *9DC* genes. We conclude that the final contig based on these clones encompasses the complete *9DC* cluster for two reasons. First, the *9DC* cluster harbors several *LipoxygenaseC* (*LoxC*; Heitz *et al.* 1997) exons, which are thought to have coduplicated with *Hcr9*'s (Parniske *et al.* 1997). The identity and orientation of the *LoxC* exons located at the termini of the *9DC* cluster correspond to those found at the termini of the *Cf-4* and *Cf-9* clusters (Parniske *et al.* 1997). Second, the *9DC* cluster harbors orthologs of

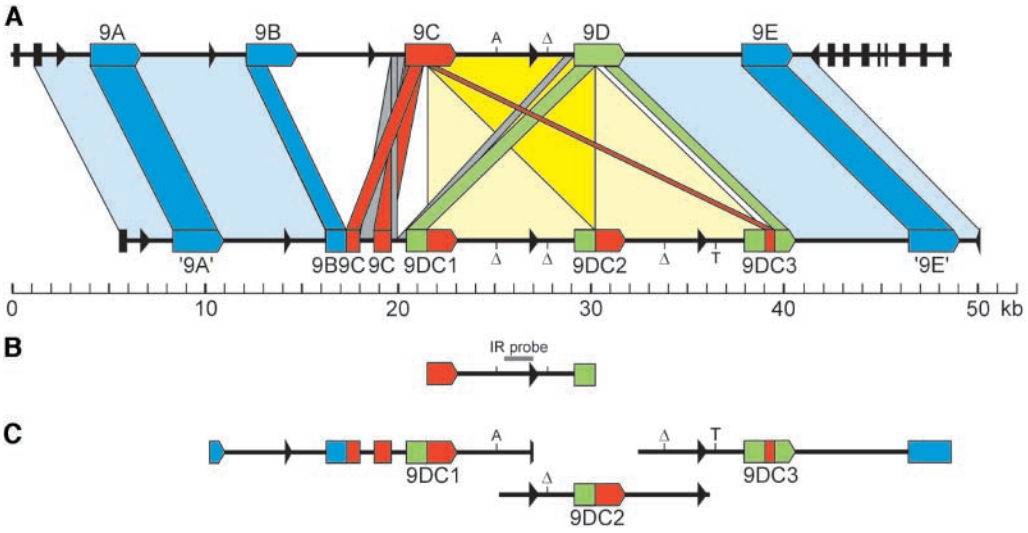

FIGURE 2.—Schematic of the relationships between the *Cf-9* and *9DC* clusters and of several genomic DNA fragments used in this study. (A) Relationships between the *Cf-9* and *9DC* clusters. The *Cf-9* cluster was previously described (PARNISKE *et al.* 1997). Colored arrowed boxes represent complete *Hcr9*'s; colored rectangular boxes represent *Hcr9* pseudogenes. *Cf-9* (*9C*)-like sequences are depicted in red; *9D*-like sequences in green; other *Hcr9* sequences in blue. All *Hcr9*'s and *Hcr9* fragments in the *Cf-9* and *9DC* clusters are in the 5′-3′ orientation. Black arrows and bars represent *LipoxygenaseC* exons, and the arrows indicate the polarity of transcription of the 3′-exon. Boxes connecting the *Cf-9* and *9DC* clusters indicate orthologous regions. Note that in the central part of the *9DC* cluster an 8.7-kb repeat is present that is almost identical to a region in the *Cf-9* cluster (see also B). These regions are connected by light yellow boxes, which overlap in the dark yellow triangle. A and T indicate single polymorphic nucleotides in these regions; "Δ" represents a single nucleotide deletion. (B) The 8.7-kb DNA sequence fragment that is present once in the *Cf-9* cluster and as a direct repeat in the *9DC* cluster. This fragment includes the 3′-half of *Cf-9*, the *Cf-9/9D* intergenic region, and the 5′-half of *9D*. The position of the *Cf-9-9D* IR probe is indicated by a gray horizontal bar above the fragment. (C) Genomic fragments containing one of the three *9DC* genes that were cloned in a binary expression vector for agroinfiltration studies to determine their ability to confer Avr9 responsiveness.

the most distal *Hcr9*'s from the *Cf-9* cluster ('*9A*' and '*9E*'; see Figure 2A).

Features of the *9DC* cluster: The *9DC* cluster contains five full-length *Hcr9*'s (Figure 2A). Most striking is the presence of three *9DC* genes. The *9DC1* and *9DC2* coding sequences are completely identical and are likely the result of a duplication within the *9DC* cluster. *9DC3* is identical to *9DC1* and *9DC2* for the first 1635 nucleotides, encompassing the proposed recombination site within *9DC* (VAN DER HOORN *et al.* 2001a). The remaining 3′-part has five nucleotide differences when compared with the corresponding region in *9D* (PARNISKE *et al.* 1997), and the sequence downstream of *9DC3* is nearly identical to that downstream of *9D*, indicating that the 3′-part of *9DC3* indeed has a *9D*-like origin. Both '*9A*' and '*9E*' differ by only 11 bp from their respective orthologs *Hcr9-9A* and *Hcr9-9E* from the *Cf-9* cluster (PARNISKE *et al.* 1997), including a single nucleotide deletion that leads to a premature stopcodon in '*9E*'.

In addition to the complete *Hcr9*'s found in the *9DC* cluster, several *Hcr9* fragments are present. The 1026-bp '*9B*' part of the '*9B9C*' fragment (Figure 2A) is 91% identical and orthologous to *Hcr9-9B* of the *Cf-9* cluster. This '*9B*' fragment is directly followed by a *Cf-9* (*9C*) fragment (position 206–821 in *Cf-9*), 831 bp of the *Cf-9* promoter directly preceding the *Cf-9* gene, and a second *Cf-9* fragment that comprises the first 821 nucleotides of the *Cf-9* ORF. The two *Cf-9* fragments in the *9DC* cluster (Figure 2A) carry the same nucleotide difference when compared with *Cf-9* itself. The second *Cf-9* fragment is followed by a *Cf-9* promoter fragment that starts at the same point as the first *Cf-9* promoter fragment and merges into the *9D* promoter. This chimeric promoter precedes *9DC1*. The sequences upstream of '*9A*' and downstream of '*9E*' and the '*9A*'-'*9B9C*' and '*9DC3*'-'*9E*' IRs are homologous to the corresponding regions in the *Cf-9* cluster (Figure 2A). The 6-kb IR between *9DC1* and *9DC2* and the IR between *9DC2* and *9DC3* each differ by only a single nucleotide from the IR between *Cf-9* and *9D* (PARNISKE *et al.* 1997) and by a single nucleotide from each other (Figure 2A).

Conservation of *Cf-9*, *9D*, and three *9DC* genes in Avr9-responsive *L. pimpinellifolium* plants: The high sequence conservation in the IRs in the *9DC* and *Cf-9* clusters and coding sequences of the three *9DC* genes and *Cf-9* indicates a clear relationship between the *Cf-9* and *9DC* clusters. Moreover, it suggests that the duplications in the *9DC* cluster are recent events. The near-identical 8.7-kb sequence in the *Cf-9* and *9DC* clusters consists of the 3′-terminal half of *Cf-9*, the IR between *Cf-9* and *9D*, and the 5′-terminal half of *9D* (Figure 2B). This 8.7-kb conserved sequence could provide a means to further unravel the evolutionary relationships between the *9DC* genes and *Cf-9*. Therefore, we decided to study the occurrence of this region in *Lp* by PCR, using the same selection of plants used in the hybridization experiment (Figure 1). We designed a set of specific primer pairs, based on the sequences of the three *9DC* genes, *Cf-9*, and *9D*. With these primer pairs, products can be obtained only if specific combinations of the above-mentioned genes are present (Figure 3). All *9DC*- and *Cf-9*-containing genotypes appear to carry at least one tandem repeat, which comprises the 3′-half of *Cf-9*, the *Cf-9/9D* IR, and the

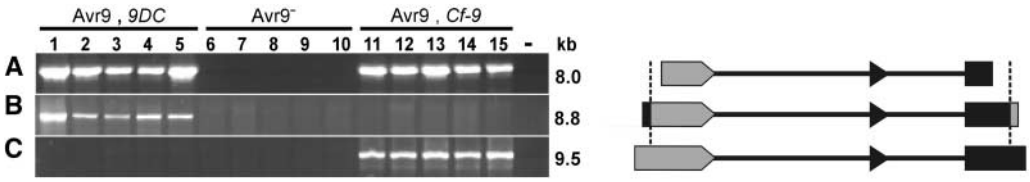

FIGURE 3.—Presence of specific tandem repeats of the three *9DC* genes or *Cf-9* and *9D* in a sample of *L. pimpinellifolium* and reference genotypes, as detected by PCR. Genotypes used for DNA templates are as shown in Figure 1. Lanes 1–5: LA1301, LA0114, LA1629, LA1637, and LA1659 [Avr9-responsive (Avr9+), all contain *9DC*]; lanes 6–10: MM-Cf0, LA0400, LA1279, LA1590, and LA2852 [non-Avr9-responsive (Avr9-), contain neither *9DC* nor *Cf-9*]; lanes 11–15: MM-Cf9, LA1585, LA1614, LA1687, and LA2653 [Avr9-responsive (Avr9+), all contain *Cf-9*]; "—," water control. (Left) PCR products were size separated on 0.7% agarose gels. Fragment sizes were estimated using a DNA size marker (not shown). (Right) Schematics of the structure of the PCR products. *Cf-9* sequences are shaded; *9D* sequences are solid. The vertical dashed lines indicate the boundaries of the 8.7-kb fragment as shown in Figure 2B. (A) PCR products obtained with primers CS10 and DS13. CS10 anneals to the 3′-part of both *9DC* and *Cf-9*, and DS13 to the 5′-part of all *9DC* genes and *9D*. A PCR product (8.0 kb) is obtained if a *9DC1-9DC2*, a *9DC2-9DC3* direct repeat, or a *Cf-9-9D* tandem repeat is present. (B) PCR products obtained with primers DCS1 and CS1, which both anneal to the proposed recombination site (VAN DER HOORN *et al.* 2001a) within all three *9DC* genes. A PCR product (8.8 kb) is obtained only when a *9DC1-9DC2* or a *9DC2-9DC3* direct repeat is present. The 17- and 15-bp fragments adjoining the 8.7-kb fragment are not drawn to scale. (C) PCR products obtained with primers CS5 and DS14. CS5 anneals to the 5′-part of *Cf-9*, and DS14 to the 3′-part of *9D*. A PCR product (9.5 kb) is obtained only when a *Cf-9-9D* tandem repeat is present.

5′-half of *9D*, whereas no PCR fragments were obtained from Avr9-nonresponsive plants (Figure 3A). All *9DC* genotypes carry at least one *9DC1-9DC2* or *9DC2-9DC3* direct repeat, including the *Cf-9/9D* IR. This repeat is not present in either *Cf-9*-containing or Avr9-nonresponsive genotypes (Figure 3B). Only the *Cf-9* genotypes carry a *Cf-9-9D* repeat, including the *Cf-9/9D* IR (Figure 3C). Restriction fingerprinting of the PCR products confirmed that all fragments obtained from a specific primer pair are similar and compose the *Cf-9/9D* IR (data not shown). In addition, control PCR experiments showed the presence of *9D* only in all *Cf-9* genotypes and the presence of *9DC3* only in all *9DC* genotypes. Conversely, *Cf-9* is present in none of the *9DC* genotypes (VAN DER HOORN *et al.* 2001a; data not shown).

To confirm the PCR results and to study the structure of the *9DC* and *Cf-9* clusters in the *Lp* plants, we designed a specific *Cf-9/9D* IR probe (Figure 2B). This probe was hybridized to the DNA blots used for surveying *Hcr9* diversity in *Lp* (Figure 1). Hybridizing fragments were found only in genotypes that carry *9DC* or *Cf-9* (Figure 1). The fragments that hybridized to the *Cf-9/9D* IR probe are a subset of those hybridizing with the *9DC* ORF probe (Figure 1). Of the four *9DC* genotypes that we sampled in addition to LA1301, only LA1659 showed an IR hybridization pattern identical to LA1301. The upper band in LA1301 represents the *9DC2-9DC3* IR, and the lower band the *9DC1-9DC2* IR. This indicates that both a *9DC1-9DC2* and a *9DC2-9DC3* repeat are present, as in LA1301. The remaining three *9DC* genotypes carry only a *9DC2-9DC3* repeat. All *Cf-9* genotypes have a hybridization pattern identical to that of MM-Cf9, in which only hybridization of the *Cf-9/9D* IR is observed. Combined with the PCR data, these results indicate that all sampled *Cf-9* genotypes contain the same *Cf-9-9D* tandem repeat. These data also show that, although the overall structure of the *Hcr9* clusters is not conserved (Figure 1), at least the genes directly downstream of *9DC* and *Cf-9* and the IRs between these genes are conserved in the *Lp* population.

Activity of individual *9DC* genes: Single amino acid changes in Cf proteins have no, or only minor, effects on their activity, whereas multiple changes can lead to a drastic reduction in activity (VAN DER HOORN *et al.* 2001b; WULFF *et al.* 2001). 9DC and Cf-9 have the same activity in conferring Avr9 responsiveness, although they differ in 61 amino acids (VAN DER HOORN *et al.* 2001a). Compared with *9DC1* and *9DC2*, *9DC3* has 33 nucleotide differences, resulting in only 23 amino acid substitutions. Therefore, *9DC3* may confer Avr9 responsiveness as well. This was tested by co-agroinfiltration of *9DC3* and *Avr9*. First, the activity of *9DC3* was tested in an agroinfiltration assay in tobacco with *Hcr9*'s under control of the 35S promoter (VAN DER HOORN *et al.* 2000). In this assay, in addition to *9DC1* and *9DC2*, *9DC3* is active as well (Figure 4A), as cells in the infiltrated leaf section undergo a typical HR (VAN DER HOORN *et al.* 2000). However, in dilution assays the activity of *9DC3* is reduced four- to eightfold as compared to *9DC* and *Cf-9* (data not shown). As expected, *9D* from the *Cf-9* cluster (Figure 4A) and the '*9A*' and '*9E*' homologs from the *9DC* cluster did not confer Avr9 responsiveness (data not shown). An agroinfiltration assay in which *Hcr9*'s are overexpressed cannot distinguish the intrinsic activities of *9DC1* and *9DC2*, which have identical coding sequences. Genomic constructs of the three *9DC* genes (Figure 2C), which likely reflect the intrinsic activity of *Hcr9*'s in tomato plants, were therefore generated. Surprisingly, only the genomic *9DC2* construct conferred Avr9 responsiveness in an agroinfiltration assay with Avr9 (Figure 4B). Therefore, *9DC2* is likely the main determinant of Avr9 recognition in *Lp* LA1301.

DISCUSSION

Numerous *R* genes have been cloned in the past decade and it appears that they frequently occur in clusters

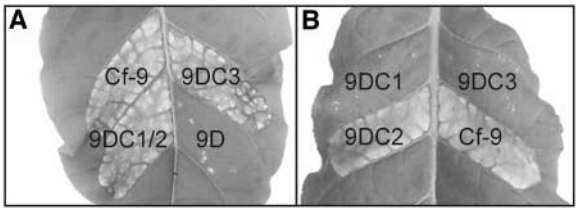

FIGURE 4.—Combined agroinfiltration of *Avr9* under control of the 35S promoter and *Hcr9*'s under control of the 35S or their native promoter. (A) Agroinfiltration of 35S-driven constructs containing the *9DC* genes, *Cf-9* or *9D*. *9DC1/2* represents the identical *9DC1* and *9DC2* coding regions. (B) Agroinfiltration assay of constructs containing *9DC1*, *9DC2*, or *9DC3* under control of their native promoter; 35S-driven *Cf-9* was used as a control. Expression of an *Hcr9* that confers Avr9 responsiveness results in visible necrosis. Both photographs were taken 7 days after infiltration.

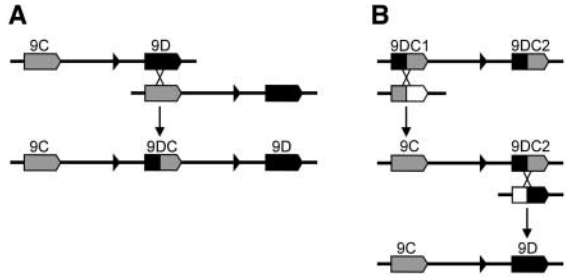

FIGURE 5.—Schematic of the two possible ancestral relationships among *Cf-9* (*9C*), *9D*, and *9DC*. Arrowed boxes represent complete *Hcr9*'s; *Cf-9* sequences are shaded; *9D* sequences are solid; open boxes, unknown *Hcr9* sequences; solid triangles, *LipoxygenaseC* exons. Diagonal crossed lines, (unequal) recombination points; arrows point toward one of the possible recombination products. All recombinations are shown in a homozygous genotype. (A) A single intragenic recombination between *9D* and *Cf-9* could result in the *9DC* gene. (B) The *Cf-9* and *9D* genes can be generated only from two *9DC* genes via two independent unequal recombinations at identical positions within each of these *9DC* genes. Recombination of *9DC1* with an unknown *Hcr9* that carries 5′ *Cf-9*-like sequences would result in *Cf-9*. Recombination between *9DC2* and an unknown *Hcr9* that carries 3′ *9D*-like sequences would generate *9D*.

(TAKKEN and JOOSTEN 2000; HULBERT *et al.* 2001; MARTIN *et al.* 2003). However, evolution of *R*-gene clusters at the population level is still poorly understood. Several studies report on the structural rearrangements in *R*-gene loci (reviewed in MICHELMORE and MEYERS 1998; HULBERT *et al.* 2001). Unequal recombination, gene conversion, point mutation, duplication, and translocation all contribute to the generation of novel *R* genes. The discovery of the *9DC* gene is an example of a recent event leading to a novel *R* gene (VAN DER HOORN *et al.* 2001a). *Cf-9* and *9DC* are related by intragenic recombination, differ in 61 amino acids, but have a similar specificity and activity in conferring Avr9 responsiveness. Here we describe the isolation of the complete *9DC* cluster from *Lp* LA1301 and a detailed comparison with the previously isolated *Cf-9* cluster (PARNISKE *et al.* 1997). We conclude that several unequal recombination events in the *Cf-9* cluster, including two intragenic recombinations, have resulted in three *9DC* genes. Surprisingly, all three genes confer Avr9 responsiveness when overexpressed, but only *9DC2* is active under control of its native promoter. Furthermore, we discuss and reconstruct the evolution of the *9DC* cluster and show that, in contrast to what had been suggested previously (VAN DER HOORN *et al.* 2001a), *Cf-9* is ancestral to *9DC*.

Reconstruction of the evolution of the *9DC* cluster: On the basis of previous data, it was initially assumed that *9DC* is ancestral to *Cf-9* (VAN DER HOORN *et al.* 2001a). Without knowledge of the flanking regions of *9DC*, this could be explained by a single intragenic unequal recombination between *9DC* and an unidentified homolog, which gave rise to both *Cf-9* and *9D*. However, we now show that an 8.7-kb region of the *Cf-9* cluster that comprises the 3′-half of *Cf-9*, the *Cf-9/9D* IR, and the 5′-half of *9D* is duplicated in the *9DC* cluster (Figure 2, A and B). If the sequence in the *Cf-9* cluster is ancestral, a single intragenic unequal recombination event between *9D* and *Cf-9* explains the generation of the first *9DC* gene and its flanking IR sequences (Figure 5A). Conversely, two independent unequal recombinations should have occurred at the same site in the middle of two *9DC* genes to create both *Cf-9* and *9D* from *9DC1* and *9DC2* and two other, unknown *Hcr9*'s (Figure 5B). As this is very unlikely, the sequence in the *Cf-9* cluster should represent the ancestral state. Furthermore, identification of *Cf-9* alleles in the distantly related tomato species *L. hirsutum* confirms that *Cf-9* indeed is an ancient gene (M. KRUIJT, D. J. KIP, M. H. A. J. JOOSTEN, B. F. BRANDWAGT and P. J. G. M. DE WIT, unpublished results).

The recent generation of *9DC* from *Cf-9* and *9D* and the isolation of both the *Cf-9* (PARNISKE *et al.* 1997) and the *9DC* clusters allow a detailed reconstruction of the evolution of the *9DC* cluster. A model that most likely represents the evolutionary events that have created the *9DC* cluster is presented, although alternative explanations cannot be excluded. The termini of the *Cf-9* and *9DC* clusters are similar, and all major rearrangements have occurred in the central region of the clusters (Figure 2A). A single intragenic unequal recombination event in the central region of the *Cf-9* cluster (Figure 6A, cluster 1) would give rise to a cluster with *Cf-9*, a single *9DC* gene, and *9D* (Figure 6A, cluster 2). This cluster harbors two identical *Cf-9/9D* IRs and would therefore be prone to further intragenic unequal recombination due to mispairing of individual homologs. Indeed, a second *9DC* copy was generated (Figure 6A, cluster 3). Two scenarios may explain the presence of a third *9DC* gene in some of the *9DC* clusters (Figure 6, A and B). In scenario A (Figure 6A), *9DC3* was generated before *9DC1*. Intragenic recombination between the second *9DC* gene and the *9D* ortholog of cluster 3 (Figure 6A)

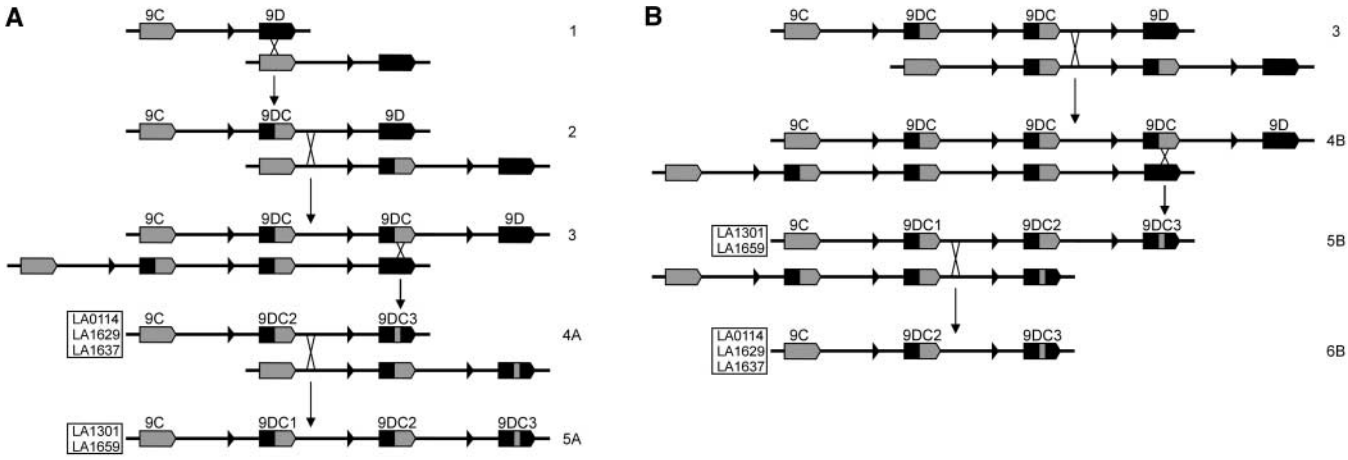

FIGURE 6.—Schematic of two scenarios for the formation of the central region of the *9DC* cluster, which involve unequal recombinations due to mispairing of individual *Hcr9*'s. Arrowed boxes represent complete *Hcr9*'s; *Cf-9* (*9C*) sequences are shaded and *9D* sequences are solid. Solid triangles, *LipoxygenaseC* exons. Diagonal crossed lines indicate (unequal) recombination points; arrows point toward one of the possible recombination products. All recombinations are shown in a homozygous genotype. Only the positions of the intragenic recombination sites that generated the first of the two identical *9DC* genes and *9DC3* are known exactly. The other recombination sites may be located anywhere within the colinear sequence stretches and are drawn at arbitrary positions within these stretches. Boxed LA numbers to the left of clusters indicate the *L. pimpinellifolium* accessions that contain these clusters. To the right, numbers indicate the different clusters. The first three clusters are identical in both scenarios. (A) Scenario A, in which *9DC3* was generated prior to *9DC1*. (B) Scenario B, in which *9DC3* was generated after *9DC1*. (Recombinations that may lead to deletion of *Cf-9* or to the formation of the *Cf-9* fragments present in the *9DC* cluster of LA1301 are not indicated.)

generated a cluster that contains the *9DC2* gene and *9DC3* (Figure 6A, cluster 4A), which is present in three of the *L. pimpinellifolium* genotypes studied here. A final unequal recombination event generated a cluster that contains the two identical *9DC1* and *9DC2* genes and *9DC3* (Figure 6A, cluster 5A), which is present in two of these *L. pimpinellifolium* genotypes, including LA1301. Alternatively, in scenario B (Figure 6B), all genotypes first accumulated the three *9DC* genes, and some have subsequently lost *9DC1* or *9DC2*. A recombination point in cluster 3 (Figure 6B) different from that shown in scenario A resulted in a cluster that contains three identical *9DC* genes (Figure 6B, cluster 4B). Intragenic recombination between the third *9DC* gene and the *9D* ortholog of cluster 4B generated a cluster that contains the two identical *9DC1* and *9DC2* genes and *9DC3* (Figure 6B, cluster 5B), which is present in two of the *L. pimpinellifolium* genotypes studied here, including LA1301. A final unequal recombination event within cluster 5B resulted in loss of *9DC1* and in generation of cluster 6B, which is present in three of the *L. pimpinellifolium* genotypes studied here.

In all five tested *9DC* genotypes, *Cf-9* and *9D* have been lost. *9D* was lost through unequal recombination with *9DC2*, yielding *9DC3* (Figure 6, A and B). The sequences upstream of *9DC1* that compose the '*9B9C*' fragment, the two *Cf-9* fragments, and the two *Cf-9* promoter sequences can be explained only by numerous recombinations. These at least constitute truncation of *Cf-9* due to unequal recombination with the *Cf-9* promoter; duplication of this *Cf-9* fragment-*Cf-9* promoter sequence; fusion of the first *Cf-9* fragment with an *Hcr9-9B* ortholog, which would yield the '*9B9C*' fragment; and fusion of the second *Cf-9* promoter fragment with the *9DC1* promoter, which would yield the short chimeric *Cf-9/9D* promoter.

Mispairing of *R*-gene homologs is known from several *R*-gene families (HULBERT *et al.* 2001). Inter- and intragenic recombinations give rise to novel *R*-gene homologs and novel combinations of *R*-gene homologs and thus contribute to diversity at *R*-gene loci. However, if the unequal recombination rate within an *R*-gene cluster is too high, this may lead to homogenization of the *R*-gene sequences. Consistent with this idea, it was previously suggested that sequence exchange between orthologous *Hcr9*'s occurs more frequently than that between paralogs (PARNISKE and JONES 1999). However, the generation of three *9DC* genes from a cluster that contained only a single *9DC* gene by mispairing of and unequal recombination among homologs is unprecedented and suggests that the initial *9DC* cluster was unstable due to the presence of the 8.7-kb repeat. In contrast, *Cf-9* was found to be very stable in a homozygous background, whereas the meiotic stability of *Cf-9* was dramatically reduced in a *Cf-4/9* heterozygous background (PARNISKE *et al.* 1997). This indicated that unequal mispairing in *Hcr9* clusters in a homozygous background is rare, which can be explained by diverged IRs that prevent mispairing of homologs and subsequent homogenization of the homologs. However, the repetitive IR structure of the initial *9DC* cluster (Figure 6A) appears to be prone to mispairing, which resulted in

unequal recombination and the generation of three *9DC* genes. *L. pimpinellifolium* is a facultative outcrosser (Rick *et al.* 1977), and therefore the *9DC* cluster could be present in heterozygous plants, which would have increased the frequency of mispairing. Therefore, sequence exchange by inter- and intragenic recombination and gene conversion among the *9DC* genes and homologs that occupy orthologous positions may lead to further *Hcr9* sequence homogenization and a decrease of *Hcr9* variation at the *MW* locus.

The termini of the *9DC* cluster (5′ of the '*9B*' fragment and 3′ of *9DC3*) are similar to those of the *Cf-9* cluster (Figure 2A). The ORFs of the '*9A*' and '*9E*' genes each differ from their orthologs *Hcr9-9A* and *Hcr9-9E* of the *Cf-9* cluster by only 11 bp, whereas the intergenic regions show a higher proportion of nucleotide differences, as well as some insertions and deletions. In contrast, the 8.7-kb repeat regions in the *9DC* cluster, including IRs of almost 6 kb, differ by only one or two nucleotides from the corresponding region in the *Cf-9* cluster, which suggests that the formation of the three *9DC* genes in the *9DC* cluster is a relatively recent event. It further suggests that gene conversion and/or intergenic recombination have occurred at the termini of the *Cf-9* and *9DC* clusters. However, the termini of the *Cf-9* cluster may not be ancestral to those of the *9DC* cluster, but more likely represent variation in the *Hcr9-9A*-like and *Hcr9-9E*-like homologs in the *L. pimpinellifolium* population.

Activity of *Hcr9*'s mediating Avr9 recognition in *L. pimpinellifolium*: In an agroinfiltration assay, all three *9DC* genes confer Avr9 responsiveness under control of the 35-S promoter, although the activity of *9DC3* is four- to eightfold reduced when compared to *Cf-9*, *9DC1*, and *9DC2*. Previously, only the two *Cf-2* genes from the *Cf-2* cluster were found to have the same function in conferring resistance to *C. fulvum* strains that express the Avr2 protein (Dixon *et al.* 1996; Luderer *et al.* 2002), and therefore the three *9DC* genes that share the same function represent a unique situation among all known *C. fulvum* resistance gene clusters. In an agroinfiltration assay with genomic constructs, however, only *9DC2* conferred Avr9 responsiveness. Thus, *9DC2* is likely the main determinant of Avr9 recognition in *Lp* LA1301. Since *9DC1* and *9DC2* have identical downstream sequences, but *9DC1* has a promoter region of only 797 bp, the observed difference in activity may be attributed to a lower expression level of *9DC1*. Unfortunately, this cannot be verified in LA1301 plants, as both genes and their 5′- and 3′-untranslated regions do not contain any polymorphic nucleotides that would enable a discriminatory RT-PCR analysis. *9DC3* has the same promoter as *9DC2*, but the terminators of these two genes differ considerably. Therefore, its inactivity when expressed by agroinfiltration under its native promoter may be explained by a combination of the lower activity of the 9DC3 protein and a lower *9DC3* expression level. The agroinfiltration assay with genomic constructs, however, is less sensitive compared to that with overexpression constructs and therefore does not exclude that, in addition to *9DC2*, *9DC1* and/or *9DC3* may also be active in Avr9 recognition upon *C. fulvum* infection of LA1301. Unfortunately, the intolerance of LA1301 to the high humidity used in the standard infection assay prevented successful *C. fulvum* infections.

Avr9 recognition in tomato: All isolates of *C. fulvum* collected to date originate from commercially grown tomatoes. At least one of these strains can overcome the resistance provided by the *Cf-9* cluster without an apparent loss of pathogenic fitness (Laugé *et al.* 1998). Moreover, *Avr9* gene replacement did not affect the pathogenic fitness of *C. fulvum* in greenhouse infection assays (Marmeisse *et al.* 1993). This suggests that, at least in greenhouse assays, *Avr9* may be dispensable. However, Avr9 recognition is present in a high proportion of *Lp* plants and based on the highly conserved *Cf-9* and *9DC* genes (Laugé *et al.* 2000; Van der Hoorn *et al.* 2001a). Avr9 recognition is also present in several other tomato species and functional *Cf-9* alleles have also been identified in the distantly related tomato species *Lycopersicon hirsutum* (M. Kruijt, D. J. Kip, M. H. A. J. Joosten, B. F. Brandwagt and P. J. G. M. de Wit, unpublished results). This suggests that in wild tomato plants *Hcr9*'s that confer Avr9 recognition may have been maintained by selection. In addition to *Cf-9* itself, the *Cf-9* cluster also harbors the partial resistance gene *Hcr9-9B* (Parniske *et al.* 1997; Laugé *et al.* 1998; Panter *et al.* 2002). Although the *9DC* cluster does not harbor a complete *Hcr9-9B* ortholog, it is possible that the other *9DC* cluster homologs encode novel resistance specificities. The selective advantage of such a *9DC* cluster may explain the prevalence of *9DC* over *Cf-9* in the *Lp* population (Van der Hoorn *et al.* 2001a).

To further study the evolutionary forces that drive the evolution of *Cf* gene clusters, it would be highly interesting to collect natural *C. fulvum* strains from wild tomato plants and characterize both the *Avr* and the *R* genes. This would add greatly to our knowledge of natural selection and co-evolution in plant-pathogen populations (Bergelson *et al.* 2001; DeMeaux and Mitchell-Olds 2003; Thrall and Burdon 2003). It may also shed light on the relative importance of the different *C. fulvum* Avr factors within the *C. fulvum* population in a natural situation, including Avr9.

Our present study enabled a unique detailed reconstruction of the evolution of a single *R*-gene cluster at the species level and has shown that unequal recombination can have a major impact on the evolution of *R*-gene clusters. A great challenge in the near future will be to study *R*-gene clusters on an even larger scale by using novel *R*-gene cluster fingerprinting methods.

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