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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Manuscript received March 20, 2004
Accepted for publication July 23, 2004

ABSTRACT

Avr9 protein (C. fulvum Cf-9 gene) of tomato cultivars to C. fulvum interaction (Cladosporium gene-for-gene relationship is the tomato- Hcr9 clusters in natural populations is still poorly understood. clusters of phenomenon called the gene-for-gene relationship (Avr- recognition in LA1301. This 9DC cluster harbors five full-length Cf homologs, including orthologs of the most distal homologs of the 9DC 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. pinnellifolium (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 accesses collected from their natural habitat are able to recognize Avr9 (LAUGE 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 (homologs of Cladosporium fulvum resistance 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 Her9 clusters of different species, clusters with different Cf genes, or clusters located at different chromosomal positions have been compared to study Her9 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-9and 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 SunMaker (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 32P-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 ORF probe (Stratagene), 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 Synthegen (Cambridge, UK). Primer sequences (5′–3′ direction, restriction sites underlined) are: 9AS1, ttttctcagtagggtaaactattta; HS1, tttt ccattgtgtctaaactattta; CS1, gcgcgttcaggtcctgtgttt; CS5, ttctcact cacaatctccct; CS10, aaaaacagaaacattcactattta; CS11, ccccccggcg agcctataactctctctgtt; DS1, gagagctcaacctttacgaa; DS9, ttttctcat ggtttgtctaaactattta; DS12, cccccctcaggaataatctttctctgtt; DS13, gggaagaggtttacgttt; DS14, ccaagctcaaccctataactattta; DCS1, gtctctttcattcacaacaccac; IRF, caatgtattataaagaacaaac; and IRR, ttgatttggagaggaag.

Library construction, clone selection, and sequencing: Genomic DNA was isolated (Van der Beek et al. 1992) from LA1301 plants homozygous for 9DCand partially digested with Sau3AI. Fragments were cloned into the Lambda FIX vector and packaged using the Lambda FIX II/XhoI Partial Fill-In vector kit (Stratagene) and transfected to E. coli KW251 (Promega). Phages carrying an Her9 were identified by hybridization of plaque lift filters with a 9DC ORF probe. On the first plaques, 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 screenings. Phage DNA was isolated using a plate lysate method (Sambrook and Russell 2001). The tomato genomic DNA inserts were cloned into the Ncol 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 Her9’s were amplified by PCR with gene-specific primer pairs 9A51/CS11 (‘9A’), DS9/ DS12 (9DC3) and H551/CS11 (‘9E’) using library clones as templates. 9D was amplified from MM-Cf9 genomic DNA with primer pair DS9/DS12. The Her9’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 Ncol inserts of pBluescript II SK+ clones into pBI201 (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 9DC (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 F1 of LA1301 and the susceptible cultivar MM-Cf0 showed a 3:1 segregation for HR-associated Avr9 recognition (65.24, χ² = 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 BC1 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 accesses from geographically distant locations throughout
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 *Her9* 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 *Her9* hybridization patterns. *Avr9*-nonresponsive plants show fewer *Her9*-hybridizing bands, as does the susceptible cultivar MM-Cf0. This suggests that the nonresponsive *Lp* plants harbor fewer *Her9*’s than *Avr9*-responsive plants, possibly due to a Milky Way cluster with only one or a few *Her9*’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 *BglII*-digested clones with a *9DC* probe enabled us to form a contig. However, this contig obscured possible duplications within the *9DC* cluster. Therefore, *BglII* 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 *Lipoxygenase C* (*LoxC*; Heitz et al. 1997) exons, which are thought to have coduplicated with *Her9*’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...
and bars represent *LipoxigenaseC* exons, and the arrows indicate the polarity of transcription of the 3′-exon. Boxes connecting the *Cf-9* and *9D* clusters indicate orthologous regions. Note that in the central part of the *9D* 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* and *9D* clusters. 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 *9D* genes that were cloned in a binary expression vector for agroinfiltration studies to determine their ability to confer *Avr9* responsiveness.

**Features of the 9D cluster:** The *9D* cluster contains five full-length *Her9*′s (Figure 2A). Most striking is the presence of three *9D* genes. The *9DC1* and *9DC2* coding sequences are completely identical and are likely the result of a duplication within the *9D* cluster. *9DC3* is identical to *9DC1* and *9DC2* for the first 1635 nucleotides, encompassing the proposed recombination site within *9D* (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 *Her9*9A and *Her9*9E from the *Cf-9* cluster (Parniske et al. 1997), including a single nucleotide deletion that leads to a premature stop codon in ‘9E’.

In addition to the complete *Her9*′s found in the *9D* cluster, several *Her9* fragments are present. The 1026-bp ‘9B’ part of the ‘9B9C’ fragment (Figure 2A) is 91% identical and orthologous to *Her9*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 *9D* 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 9D genes in Avr9-responsive *L. pinninellifolium* plants:** The high sequence conservation in the IRs in the *9D* and *Cf-9* clusters and coding sequences of the three *9D* genes and *Cf-9* indicates a clear relationship between the *Cf-9* and *9D* clusters. Moreover, it suggests that the duplications in the *9D* cluster are recent events. The near-identical 8.7-kb sequence in the *Cf-9* and *9D* 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 *9D* 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 *9D* 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 *9D* 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...
plates 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-)]. 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 Her9 clusters is not conserved (Figure 1), at least the genes directly downstream of 9D 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. 2001a; 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 Her9’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 Her9’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 Her9’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 Her9 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 Her9 that confers Avr9 responsiveness results in visible necrosis. Both photographs were taken 7 days after infiltration.

(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 LpLA1301 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 2A 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 Her9’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. Krujt, 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)
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 (Riick 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 Her9 sequence homogenization and a decrease of Her9 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 Her9-9A and Her9-9E 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 intragenic 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 Her9-9A-like and Her9-9E-like homologs in the *L. pimpinellifolium* population.

**Activity of Her9’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, 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 (Marmesse 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 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, B. F. Brandwagt and P. J. G. M. de Wit, unpublished results). This suggests that in wild tomato plants Her9’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 Her9-9B (Parniske et al. 1997; Laugé et al. 1998; Panter et al. 2002). Although the 9DC cluster does not harbor a complete Her9-B 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 9DCover 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-Olens 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.

We thank Matthieu Joosten and Maarten Koornneef for critically reading the manuscript; Roger Chetelat (Tomato Genetic Resource Center, Davis, CA) for providing the *Lp* seeds; Kees Spelt for providing...
the pBVM2 vector; Maarten de Kock for help with the AFLP-based clone fingerprinting method; Tim Lindhout for helpful discussions; Mart Berns, Bert Essenstam, Ronald Janssen, and Henk Smid for excellent plant care; and Diana Kip for technical assistance. This study was financially supported by the Dutch Foundation for Applied Sciences.

LITERATURE CITED


