The *Cf-4* and *Cf-9* Resistance Genes Against *Cladosporium fulvum* are Conserved in Wild Tomato Species

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Submitted 1 February 2005. Accepted 11 May 2005.

The *Cf-4* and *Cf-9* genes originate from the wild tomato species *Lycopersicon hirsutum* and *L. pimpinellifolium* and confer resistance to strains of the leaf mold fungus *Cladosporium fulvum* that secrete the Avr4 and Avr9 elicitor proteins, respectively. Homologs of *Cf-4* and *Cf-9* (*Hcr9s*) are located in several clusters and evolve mainly through sequence exchange between homologs. To study the evolution of *Cf* genes, we set out to identify functional *Hcr9s* that mediate recognition of Avr4 and Avr9 (designated *Hcr9-Avr4s* and *Hcr9-Avr9s*) in all wild tomato species. Plants responsive to the Avr4 and Avr9 elicitor proteins were identified throughout the genus *Lycopersicon*. Open reading frames of *Hcr9s* from Avr4- and Avr9-responsive tomato plants were polymerase chain reaction-amplified. Several *Hcr9s* that mediate Avr4 or Avr9 recognition were identified in diverged tomato species by agroinfiltration assays. These *Hcr9-Avr4s* and *Hcr9-Avr9s* are highly identical to *Cf-4* and *Cf-9*, respectively. Therefore, we conclude that both *Cf-4* and *Cf-9* predate *Lycopersicon* speciation. These results further suggest that *C. fulvum* is an ancient pathogen of the genus *Lycopersicon*, in which *Cf-4* and *Cf-9* have been maintained by selection pressure imposed by *C. fulvum*.

Plants are continuously challenged by a wide variety of pathogens in their natural habitats. Nevertheless, most plants are resistant to these pathogens. One way plants can achieve resistance is described by the gene-for-gene model (Flor 1946). In this model, the pathogen contains an avirulence (*Avr*) gene and the plant carries a matching resistance (*R*) gene that mediates specific recognition of the *Avr* gene product. Upon recognition, the plant mounts various defense responses that often include the hypersensitive response (HR) that restricts pathogen growth (Hammond-Kosack and Jones 1996). The presence of *R* genes in host plants imposes selection pressure on their pathogens to inactivate the corresponding *Avr* genes by mutation or deletion in order to gain virulence. Plants, in turn, are under pressure to maintain *R* genes or generate novel *R* genes that enable them to recognize new strains of the pathogen and to remain resistant.

Cladosporium fulvum is a biotrophic leaf pathogen of the tomato (*Lycopersicon*) genus. The *C. fulvum*–tomato interaction is a versatile gene-for-gene pathosystem to study plant–pathogen interactions (Joosten and De Wit 1999; Rivas and Thomas 2002) and *R* gene evolution (Kruijt et al. 2004, 2005; Parniske and Jones 1999; Parniske et al. 1997, 1999; Van der Hoorn et al. 2001a). Upon infection, *C. fulvum* secretes various small proteins into the apoplast of the tomato leaf that act as elicitors of defense in tomato genotypes that carry the corresponding *C. fulvum* resistance genes (*Cf* genes) (Joosten and De Wit 1999). The Avr4 and Avr9 elicitor proteins have been extensively studied. Avr4 was shown to bind chitin oligomers and may protect the cell wall of *C. fulvum* against plant chitinases during infection of tomato leaves, or against chitinases of hyperparasitic fungi (Van den Burg et al. 2003). Avr9 is related structurally to other small cysteine-rich peptides, such as serine protease inhibitors, ion channel blockers, and growth factors, and is most related to carboxy peptidase inhibitor (Vervoort et al. 1997). Although no other function besides causing avirulence has been reported for Avr9 yet, its homology to other proteins suggests that it may interfere with physiological processes in the tomato leaf. Whereas no Avr4-responsive *Lycopersicon pimpinellifolium* accessions were identified (Laugé et al. 2000), Avr9 recognition was found in 72 of 231 *L. pimpinellifolium* accessions tested (Laugé et al. 2000; Van der Hoorn et al. 2001a). All other known *C. fulvum* elicitors are also specifically recognized in certain *L. pimpinellifolium* accessions (Laugé et al. 2000). These data suggest that, within the population of this wild tomato species, many different *Cf* genes exist.

Cf genes encode membrane-anchored proteins that carry extracellular leucine-rich repeats (LRRs) and lack an obvious cytoplasmic signaling domain. The *Cf-2*, *Cf-4*, *Cf-4E*, *Cf-5*, and *Cf-9* genes all have been isolated from near-isogenic lines of the tomato cv. MoneyMaker (MM) (Boukema 1981; Dixon et al. 1996, 1998; Jones et al. 1994; Takken et al. 1999; Thomas et al. 1997; Tigchelaar 1984). The *Cf-4* gene from *L. hirsutum* and the *Cf-9* gene from *L. pimpinellifolium* have been studied extensively, because *Cf-9* was the first isolated *Cf* gene (Jones et al. 1994) and *Cf-4* is highly homologous to *Cf-9* (Thomas et al. 1997). *Cf-4*, *Cf-4E*, and *Cf-9* are all members of the *Hcr9* (homolog of *C. fulvum* resistance gene *Cf-9*) gene family, whereas *Cf-2* and *Cf-5* are members of the *Hcr2* gene family. Clusters containing up to five *Hcr9s* are found at several loci on the short arm of chromosome 1 of tomato, and many different *Hcr9s* have been published to date (De Kock et al. 2005; Jones et al. 1994; Kruijt et al. 2004; Parniske and Jones 1999; Parniske et al. 1997; Thomas et al. 1997; Van der Hoorn et al. 2001a; Wulff et al. 2004). Most of these *Hcr9s* do not confer

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GenBank accession numbers: *Hcr9-Avr4-chl1*: AY634610, *Hcr9-Avr4-chl1*: AY634611, *Hcr9-Avr4-par1*: AY634612, *Hcr9-Avr4-par1*: AY634613, *Hcr9-Avr9-hir1*: AY634614, *Hcr9-Avr9-hir2*: AY634615, *Hcr9-Avr9-hir3*: AY634616, and *Hcr9-Avr9-hir4*: AY634617.

resistance to known isolates of *C. fulvum*. However, sequence exchange between homologs was shown to contribute greatly to *Hcr9* diversity (Kruijt et al. 2004, 2005; Parniske and Jones 1999; Parniske et al. 1997), and may result in the generation of novel *Cf* genes. This was exemplified by the discovery of the *9DC* gene from *L. pimpinellifolium*, which is composed of the 5' half of *Hcr9-9D* from the *Cf-9* cluster and the 3' half of *Cf-9*, and has a similar activity and specificity in mediating Avr9 responsiveness as *Cf-9* (Van der Hoorn et al. 2001a). The isolation of the complete *9DC* cluster from *L. pimpinellifolium* LA1301, which contains three *9DC* genes, showed that the *9DC* gene is the result of intragenic recombination between *Cf-9* and *Hcr9-9D*.

The precise mechanism of Avr recognition by Cf proteins is not known yet, but several studies have reported specific features in *Cf-4* and *Cf-9*, including several solvent-exposed amino acids in the LRR region, that are required for their function (Van der Hoorn et al. 2001b; Wulff et al. 2001). Furthermore, these experiments, as well as studies on the naturally occurring *9DC* gene, showed that considerable variation in *Cf* genes is allowed without loss of function. Recently, it was shown that, by *Hcr9* gene shuffling, so-called auto-activators of HR could be generated (Wulff et al. 2004). These auto-activators induce an HR in *Nicotiana* spp. in the absence of an elicitor. Many different auto-activators were recovered; however, despite limited sequence variation in the auto-activators, extensive sequence analysis failed to reveal which residues were responsible for the necrosis-inducing phenotype (Wulff et al. 2004).

Although much is known about the genetic mechanisms involved in the evolution of *Hcr9*s, it is not known how *Cf* genes are maintained in wild tomato populations. The birth-and-death model (Michelmore and Meyers 1998) describes the traditional view of *R* gene evolution, and complies with the arms race model (Dawkins and Krebs 1979). The birth-and-death model implies that an individual *R* gene emerges in a plant population, will disappear from it when the resistance is broken, and subsequently is replaced by new *R* genes. The *Rps4* gene from *Arabidopsis thaliana* may have evolved according to this arms race model (Bergelson et al. 2001). Several recent studies, however, suggest that resistance genes are maintained in plant populations over long periods of time (Mauricio et al. 2003; Riely and Martin 2001; Rose et al. 2004; Stahl et al. 1999; Tian et al. 2002; Van der Hoorn et al. 2001a). Selection for *Hcr9*s that confer resistance to *C. fulvum* could maintain these genes in the *Lycopersicon* population, resulting in ancient *Cf* genes. Another intriguing possibility is convergent

evolution of *R* genes toward specificity of recognition for the same pathogen-derived elicitor molecule. This was shown for two *R* genes in soybean and *A. thaliana*, which have evolved independently to recognize the AvrB elicitor from *Pseudomonas syringae* (Ashfield et al. 2004). The versatile nature of *Hcr9* clusters and the fact that large variation in *Cf* genes is allowed without loss of function implies that convergent evolution could have resulted in independently evolved *Hcr9*s with the same specificity for a particular *C. fulvum* elicitor.

To study which of these scenarios applies to the *Hcr9*s, we set out to study how *Hcr9*s that mediate recognition of the *C. fulvum* elicitors Avr4 and Avr9 have evolved. We describe the isolation of functional homologs of *Cf-4* and *Cf-9* of diverged tomato species. These homologs show few polymorphisms compared with *Cf-4* and *Cf-9*, respectively. Therefore, we conclude that the *Hcr9*s that confer Avr4 or Avr9 recognition pre-date *Lycopersicon* speciation. The presence of ancient *C. fulvum* resistance genes in wild tomato species further suggests that *C. fulvum* is an ancient pathogen that has co-evolved with the genus *Lycopersicon*.

RESULTS

Avr4 and Avr9 recognition is found throughout the tomato genus.

A genus-wide screen has been performed to identify wild tomato plants that recognize the two best-characterized *C. fulvum* elicitors, Avr4 and Avr9. Representative accessions collected throughout the natural range of each of the eight wild tomato species were screened. *L. esculentum* var. *cerasiforme* was not included, because its natural geographic distribution is not clear and it is not clearly separated from cultivated tomato (*L. esculentum*) and *L. pimpinellifolium* (Warnock 1988, 1991). Plants were tested for Avr4 and Avr9 responsiveness by protein injections, which allowed a large number of plants to be tested, and plants could be injected multiple times with several proteins simultaneously in a nondestructive way. The presence of a *Cf* gene will result in visible necrosis (HR) throughout the injected leaf section (Fig. 1). Four to six plants of each accession were injected. Most accessions that contained Avr4- or Avr9-responsive plants also contained nonresponsive plants, indicating heterozygosity for recognition of Avr proteins within most accessions. Only some Avr9-responsive *L. pimpinellifolium* accessions did not exhibit this heterozygosity. Protein injections enabled us to identify Avr9-responsive plants in more *L. pimpinellifolium* accessions when compared with a potato virus X (PVX)-based screen (Lauzé et al. 2000; Van der Hoorn et al. 2001a).

A schematic overview of the presumed phylogenetic relationship between the eight wild tomato species is shown in Figure 2, as well as the distribution of specific Avr4- and Avr9-responsiveness within 188 accessions of these species. Avr4-responsiveness was observed throughout the genus *Lycopersicon*, with the exception of *L. pimpinellifolium* (consistent with Lauzé et al. 2000). In addition to the Avr9-responsiveness reported in *L. pimpinellifolium*, we also observed Avr9-responsive *L. hirsutum*, *L. peruvianum* (both in a northern and a southern accession), and *L. pennellii* plants (Fig. 2). No *Lycopersicon* plants or accessions were found that recognized both Avr4 and Avr9.

Isolation of novel *Hcr9*s that mediate Avr4 or Avr9 recognition.

Cf-4 and *Cf-9* both are members of the well-studied *Hcr9* family. Therefore, a polymerase chain reaction (PCR)-based method for the isolation of novel *Hcr9*s that mediate Avr4 or Avr9 recognition was developed, based on sequence informa-

Fig. 1. Examples of a specific hypersensitive response in wild *Lycopersicon* plants upon injection of the *Cladosporium fulvum* Avr4 and Avr9 elicitor proteins. **A**, Primary leaf of an Avr4-responsive *Lycopersicon parviflorum* LA2190 plant. **B**, Primary leaf of an Avr9-responsive *L. hirsutum* LA2155 plant. Sections of both leaves were injected with Avr4 (left side) and Avr9 (right side). Arrows indicate the visible necrotic leaf sectors. Photographs were taken 4 days after injection.

tion of previously published *Hcr9s* at the *Milky Way*, *Northern Lights*, and *Southern Cross* loci (Jones et al. 1994; Kruijt et al. 2004; Parniske and Jones 1999; Parniske et al. 1997; Thomas et al. 1997; Van der Hoorn et al. 2001a), and three *Hcr9s* located at the *Cf-Ecp2 Orion* locus (De Kock et al. 2005). A balanced mix of degenerate *Hcr9* primers was developed (discussed below) to maximize the number of different open reading frames amplified from each tomato genotype and to allow cloning of these *Hcr9s*. Patterns of nucleotides were incorporated in separate primers, to prevent high degeneracy in a single primer. Furthermore, the PCR conditions were optimized to yield high quantities of PCR products, using control genomic DNA preparations of MM-Cf4, MM-Cf9, and several randomly selected wild tomato plants as a template (data not shown). From each *Lycopersicon* sp. in which Avr4 recognition was found, a single Avr4-responding plant was selected for further analysis and, likewise, from each species in which Avr9 recognition was found, a single Avr9-responding plant was selected. With this mix of degenerate *Hcr9* primers, PCR was performed on genomic DNA of these Avr4- and Avr9-responsive plants. PCR generally resulted in products of approximately 2.6 kb, which is the length of *Hcr9s* that encode proteins with 27 LRRs, such as *Cf-9*. A slightly smaller fragment of approximately 2.4 kb also was amplified in some cases, which might represent *Hcr9s* that

Fig. 2. Tentative phylogenetic relationship between the *Lycopersicon* spp. and Avr4 and Avr9 responsiveness within these species. The figure does not represent a true phylogenetic tree, but is a synthesis of inter-species crossability, morphological data, and phylogeny of nuclear genes (Alvarez et al. 2001; Marshall et al. 2001; Miller and Tanksley 1990; Nesbitt and Tanksley 2002; Peralta and Spooner 2001; Rick 1979). Four to six plants of each accession were injected with the Avr4 and Avr9 elicitor proteins and scored for specific recognition, resulting in a hypersensitive response 2 to 4 days after injection. The total number of accessions injected with Avr4 and Avr9 protein per species (# acc. tested) and the number of accessions per species responding to injections (# acc. responding) of Avr4, Avr9, or both elicitors (Avr4/9) are indicated. *: Avr4 and Avr9 recognition in cultivated tomato (*Lycopersicon esculentum*) has been introgressed from the wild tomato species *L. hirsutum* (*Cf-4*) and *L. pimpinellifolium* (*Cf-9* and *9DC*), but *L. esculentum* was not tested (–) in this study; †: *L. pimpinellifolium* has previously been screened for Avr9 recognition (Van der Hoorn et al. 2001a).

encode proteins with 25 LRRs, such as *Cf-4* (data not shown). These fragments were cloned into pRH80 (Van der Hoorn et al. 2000), and the resulting 35S-*Hcr9*-terminator cassettes were subcloned into the binary vector pMOG800 (Honée et al. 1998), which has been used extensively in agroinfiltration studies for *Cf* gene activities (Kruijt et al. 2004; Van der Hoorn et al. 2000, 2001a,b,c; Wulff et al. 2004).

An initial number of approximately 10 *Hcr9s* per selected plant was tested for activity. For most species–Avr combinations, screening of 10 to 20 *Hcr9s* was sufficient to identify an active *Hcr9*. From a selected *L. cheesmanii* plant responding to Avr4, a total of 45 clones was tested for activity by agroinfiltration without identifying an *Hcr9*–Avr4. However, due to internal *KpnI* or *XbaI* restriction sites, a number of *Hcr9s* from this plant could not be tested for functionality. For some species–Avr combinations (*L. pennellii*–Avr4, *L. pennellii*–Avr9, and *L. peruvianum*–Avr9), approximately 20 *Hcr9s* were tested per selected plant, and no active *Hcr9s* were identified. Again, many of the *Hcr9s* from these plants could not be tested due to internal restriction sites. To overcome these problems, several other binary vectors containing alternative restriction sites were used for cloning of *Hcr9s* and expression via agroinfiltration, but all were less efficient than pMOG800 (data not shown). Therefore, single pMOG800 clones carrying an *Hcr9* insert were tested in an agroinfiltration assay for their ability to confer Avr4 or Avr9 responsiveness in tobacco, which is a well-established tool to study *Cf-4* and *Cf-9* activity (Kruijt et al. 2004; Thomas et al. 2000; Van der Hoorn et al. 2000, 2001a,b; Wulff et al. 2001, 2004). Co-infiltration of a matching *Cf*–Avr gene pair (e.g., *Cf-4*–Avr4) resulted in an HR (visible necrosis) throughout the infiltrated leaf sector, whereas agroinfiltration of only *Cf-4*, *Cf-9*, Avr4, or Avr9 did not result in an HR (Van der Hoorn et al. 2000; data not shown). Following this approach, *Hcr9s* that mediate Avr4 responsiveness (designated *Hcr9*–Avr4s) were isolated, and subsequently sequenced, from *L. chilense* LA2759, *L. chmielewskii* LA1316, *L. hirsutum* LA0361, *L. parviflorum* LA2190, and (northern) *L. peruvianum* LA0441 (Table 1). No *Hcr9*–Avr4s could be isolated from *L. cheesmanii* and *L. pennellii*, possibly due to the presence of restriction sites used for cloning in these *Hcr9*–Avr4s. Likewise, *Hcr9s* that mediate Avr9

Table 1. Overview of all isolated *Hcr9*–Avr4s and *Hcr9*–Avr9s and their relative hypersensitive response (HR)-inducing activities upon agroinfiltration

<i>Lycopersicon</i> accession	<i>Hcr9</i>	Relative activity ^a
<i>L. hirsutum</i> LA0361	<i>Hcr9</i> –Avr4– <i>hir1</i> (<i>Cf-4</i>) ^b	+++++
<i>L. chilense</i> LA2759	<i>Hcr9</i> –Avr4– <i>chl1</i>	+++++
<i>L. chmielewskii</i> LA1316	<i>Hcr9</i> –Avr4– <i>chl1</i>	++++
<i>L. parviflorum</i> LA2190	<i>Hcr9</i> –Avr4– <i>par1</i>	+++++
<i>L. peruvianum</i> LA0441 ^c	<i>Hcr9</i> –Avr4– <i>per1</i>	+
<i>L. pimpinellifolium</i> PI126915	<i>Cf-9</i> ^d	+++++
<i>L. pimpinellifolium</i> LA1301	<i>9DC</i> ^e	+++++
<i>L. pimpinellifolium</i> LA1301	<i>9DC3</i> ^f	+++
<i>L. hirsutum</i> LA2155	<i>Hcr9</i> –Avr9– <i>hir1</i>	+++++
<i>L. hirsutum</i> LA2155	<i>Hcr9</i> –Avr9– <i>hir2</i>	+++++
<i>L. hirsutum</i> LA2155	<i>Hcr9</i> –Avr9– <i>hir3</i>	+++++
<i>L. hirsutum</i> LA1347	<i>Hcr9</i> –Avr9– <i>hir4</i>	+++++

^a The HR-inducing activity of *Hcr9*–Avr4s is compared with *Cf-4* in a transient agroinfiltration assay; the HR-inducing activity of *Hcr9*–Avr9s with *Cf-9*. An extra “+” relative to the controls indicates a double relative HR-inducing activity, one less “+” indicates half of that activity. Relative HR-inducing activities are based on at least three independent agroinfiltration experiments.

^b Sequence of *Hcr9*–Avr4–*hir1* is identical to *Cf-4* (Thomas et al. 1997).

^c Northern accession of *L. peruvianum* (Peralta et al. 2001).

^d Jones et al. 1994.

^e Van der Hoorn et al. 2001a.

^f Kruijt et al. 2004.

known *Hcr9s*, suggesting that most SNPs found within the five *Hcr9-Avr4s* represent novel point mutations. Moreover, the *Hcr9-Avr4s* carry conserved nucleotides at five positions that are not found in any other reported *Hcr9* (not shown), which reinforces the idea of a common ancestry of the *Hcr9-Avr4s*.

At the protein level, only 66 of 808 amino acids were polymorphic within the five *Hcr9-Avr4s* (Fig. 3B), and 19 of these are located at putatively solvent-exposed positions, mainly in LRRs 1 to 9, which are part of the variable domain of *Hcr9* proteins. The remaining polymorphic amino acids are scattered throughout other protein domains. The isolated *Hcr9-Avr4s* are polymorphic compared with the previously isolated *Cf-4* gene. Therefore, their activity relative to *Cf-4* was determined by agroinfiltration. Dilution series of *Agrobacterium tumefaciens* cultures carrying the *Hcr9-Avr4* constructs were combined with a culture that carries an *Avr4* construct and infiltrated in tobacco leaves. Their ability to confer *Avr4* responsiveness was compared with that of the *Cf-4* control (Van der Hoorn et al. 2001b). The results of these experiments are summarized in Table 1, and examples of agroinfiltration dilution series with *Hcr9-Avr4-chm1* and *Hcr9-Avr4-chl1* are shown in Figure 4. *Hcr9-Avr4-per1* was considerably less active in conferring *Avr4* responsiveness than *Cf-4*. Interestingly, *Hcr9-Avr4-chm1* was slightly less active than *Cf-4* (Fig. 4A), whereas *Hcr9-Avr4-chl1* was slightly more active than *Cf-4* (Fig. 4B). The activity of *Hcr9-Avr4-par1* was similar to that of *Cf-4*.

Features and activities of the *Hcr9-Avr9s*.

Seven identical *L. pimpinellifolium* *Cf-9* alleles as well as seven *9DC* alleles have been isolated previously (Jones et al. 1994; Van der Hoorn et al. 2001a). In this study, four additional *Hcr9-Avr9s* from the distantly related tomato species *L. hirsutum* have been isolated. A DNA sequence alignment of all SNPs within the newly isolated *Hcr9-Avr9s* is shown in Figure 5A, in which *Cf-9* was chosen as the reference sequence. Overall, the DNA sequences of all *Hcr9-Avr9s* isolated from *L.*

hirsutum are over 98% identical to *Cf-9*, and these genes cluster in a phylogenetic analysis of all known *Hcr9s* (data not shown), strongly suggesting that the *Hcr9-Avr9-hir* genes are orthologous to *Cf-9*. Most striking is that *Hcr9-Avr9-hir1* of *L. hirsutum* LA2155 differs only by one silent mutation from *Cf-9* (Fig. 5A; SNP 177), which indicates a common ancestry between these two genes. From the same LA2155 plant, two additional *Hcr9-Avr9s* (*-hir2* and *-hir3*) were isolated, which also carry SNP 177 from *Hcr9-Avr9-hir1*. SNP 177 is not found in any of the previously described *Hcr9s*, or the other *Hcr9s* described in this study, which suggests that it is the result of a recent point mutation that has occurred in the *Hcr9-Avr9-hir* genes. In addition to SNP 177, *Hcr9-Avr9-hir2* and *-hir3* differ from *Cf-9* by 13 and 15 additional SNPs, respectively, which also are present in other *Hcr9s*. *Hcr9-Avr9-hir2* and *-hir3* are likely derived from the same ancestral *Hcr9*. *Hcr9-Avr9-hir4* was isolated from *L. hirsutum* LA1347, and differs from *Cf-9* by 38 SNPs, including SNP 1,721, which is shared by *Hcr9-Avr9-hir2* and *-hir3*. *Hcr9-Avr9-hir4* further shares the two SNPs with *Hcr9-Avr9-hir3* that distinguishes it from *Hcr9-Avr9-hir2*. Only 7 of these 38 SNPs between *Cf-9* and *Hcr9-Avr9-hir4* are unique for *Hcr9-Avr9-hir4*, indicating that most of these SNPs are the result of sequence exchange with other homologs. Indeed, *Hcr9-Avr9-hir4* has an exclusive perfect sequence affiliation with *Hcr9-9D* from nucleotide 1,827 (surpassing SNP 1,857 in Figure 5A) up to nucleotide 2,000 (preceding SNP 2,001 in Figure 5A), suggesting a possible recombination between the two genes.

At the protein level (Fig. 5B), *Hcr9-Avr9-hir1* is identical to *Cf-9*. The identical *Hcr9-Avr9-hir2* and *-hir3* proteins differ from *Cf-9* by only nine amino acids, which are located in LRRs 18 to 22. Only two of these are located at solvent-exposed positions (Fig. 5B). The polymorphic residues of *Hcr9-Avr9-hir4* relative to *Cf-9* are more scattered throughout the protein (Fig. 5B). Overall, the *Hcr9-Avr9s* carry six conserved amino acids that are not present in any other known *Hcr9* protein, of

Fig. 4. Hypersensitive response (HR) induced by agroinfiltration of dilution series of *Hcr9-Avr4s* and *Hcr9-Avr9s* in combination with the matching *Avr4* and *Avr9* elicitor genes. All genes are under control of the 35S promoter. The activity of the *Hcr9-Avr4s* was compared with that of *Cf-4*, and the activity of the *Hcr9-Avr9s* was compared with that of *Cf-9*. Dilution series were as follows: *Cf* culture:*Avr* culture ratios; 1:1; 1:3; 1:7; 1:15; 1:31; and 1:63 (dilutions 1 to 6). Dilutions were infiltrated in separate *Nicotiana tabacum* leaf sections from top to bottom. **A**, The HR-inducing activity of *Hcr9-Avr4-chm1* (*chm1*) is approximately half of that of *Cf-4*. Dilutions 3 to 6 were infiltrated. **B**, The HR-inducing activity of *Hcr9-Avr4-chl1* (*chl1*) is approximately twice that of *Cf-4*. Dilutions 1 to 6 were infiltrated. **C**, The HR-inducing activity of *Hcr9-Avr9-hir4* (*hir4*) is equal to that of *Cf-9*. Dilutions 3 to 6 were infiltrated.

which four are located at solvent-exposed positions in LRRs 7 to 13. The activities of the *Hcr9-Avr9*s relative to *Cf-9* were determined by agroinfiltration of dilution series with *Avr9* (Van der Hoorn et al. 2001b). All four *Hcr9-Avr9*s from *L. hirsutum* are as active in conferring *Avr9* responsiveness as *Cf-9* (Table 1), including *Hcr9-Avr9-hir4* (Fig. 4C), which is most diverged from *Cf-9* (Fig. 5). This suggests that none of the amino acid substitutions relative to *Cf-9* alter the protein activity.

DISCUSSION

Isolation of *Hcr9-Avr4s* and *Hcr9-Avr9s* from wild tomato species.

With Avr4 protein injections, Avr4 responsiveness was found in all *Lycopersicon* spp., except for *L. pimpinellifolium* (Fig. 2). This is consistent with a PVX-based screen of *L. pimpinellifolium*, in which no Avr4-responsive plants were identified (Laugé et al. 2000). Therefore, it is likely that Avr4 recognition is completely absent in this species. Avr9 recognition was found in only a few, evolutionarily diverged species (Fig. 2). The method for isolation of *Hcr9*s that mediate recognition of the *C. fulvum* elicitors Avr4 and Avr9 from different tomato species was shown to be successful. It allowed relatively fast isolation of these *Hcr9-Avr4*s or *Hcr9-Avr9*s and, therefore, enabled population studies of these *Hcr9*s, which would not have been feasible by map-based cloning of these genes or by screening of genomic libraries. However, this method depends heavily on known *Hcr9* sequences, which might prohibit the isolation of some more diverged *Hcr9-Avr4*s or *Hcr9-Avr9*s.

Fig. 5. Alignment of *Hcr9-Avr9* DNA and protein sequences. All sequences are compared with the reference *Cf-9* **A**, DNA or **B**, protein sequence of *Lycopersicon pimpinellifolium* (Jones et al. 1994). Only residues that deviate from these reference sequences are shown in the alignment; residues identical to those in *Cf-9* are indicated by a dot. The *9DC* genes from *L. pimpinellifolium* were not included in this alignment, because single nucleotide polymorphisms in these genes relative to *Cf-9* are derived from *Hcr9-9D* by intragenic recombination between *Cf-9* and *Hcr9-9D* (Kruijt et al. 2004; Van der Hoorn et al. 2001a). *Hcr9-Avr9-hir1* to *-hir4* all were isolated from *L. hirsutum*. **A**, Nucleotide alignment of the *Hcr9-Avr9*s. The position of each polymorphic nucleotide is relative to the first nucleotide of the start codon of the *Hcr9-Avr9* consensus sequence, and is indicated by vertical numbers above each nucleotide. **B**, Amino acid alignment of the *Hcr9-Avr9*s. The positions of the polymorphic sites in the different structural domains (A-G) (Jones and Jones 1997) are indicated above each amino acid residue. Numbers indicate individual leucine-rich repeats (LRRs) (within domain C); L refers to the loop-out region within the LRR-domain. Putative solvent-exposed residues are shown in boldface black.

sources of *Cf* genes, which explains why *Cf-4* and *Cf-9* are present in many tomato breeding lines (Haanstra et al. 2000; Van der Hoorn et al. 2001a), rather than other *Hcr9-Avr4s* and *Hcr9-Avr9s*. Identical *Cf-4* and *Cf-9* alleles have been isolated from different accessions of *L. hirsutum* and *L. pimpinellifolium*, respectively, as well as highly conserved *9DC* alleles (this study; Van der Hoorn et al. 2001a), suggesting low allelic diversity among *Hcr9-Avr4s* and *Hcr9-Avr9s* within *Lycopersicon* spp. It recently was shown that the *9DC* gene has evolved from the *Cf-9* gene (Kruijt et al. 2004). Moreover, *Cf-9* appears to be conserved between *L. pimpinellifolium* and *L. hirsutum*. Whereas, in seven *Cf-9* alleles, no variation was found (Jones et al. 1994; Van der Hoorn et al. 2001a), the four *Cf-9* alleles from *L. hirsutum* reported here are all different. *L. pimpinellifolium* is mostly self-compatible, and *L. hirsutum* is mostly self-incompatible (Rick 1979; Rick et al. 1977) which may explain why the *L. hirsutum* *Cf-9* alleles are more polymorphic. Therefore, it is likely that *Cf-9* was present in a *Lycopersicon* ancestor and was maintained in distinct species.

The high sequence conservation of *Cf-9* between *L. pimpinellifolium* and *L. hirsutum* and the fact that *9DC* exhibits more polymorphism within *L. pimpinellifolium* than *Cf-9* (Van der Hoorn et al. 2001a) seem contradictory to the finding that *Cf-9* is ancestral to *9DC* (Kruijt et al. 2004). However, in the present study, a considerable variation within *Cf-9* alleles of only two different *L. hirsutum* accessions was encountered. The variation in *L. hirsutum* *Cf-9* alleles likely is maintained and possibly increased by the highly self-incompatible nature of the species, which favors polymorphic loci and recombination events. However, in *L. pimpinellifolium*, *Cf-9* is found primarily in self-compatible accessions (Van der Hoorn et al. 2001a), limiting sequence exchange between alleles. Moreover, genetic drift may have resulted in prevalence of the only known *L. pimpinellifolium* *Cf-9* allele over other *Cf-9* alleles. *9DC*, however, is present mostly in facultative outcrossing accessions of *L. pimpinellifolium*, increasing chances for sequence exchange between alleles. Furthermore, from a study on the *9DC* gene cluster, it was clear that this cluster carries three *9DC* genes, all of which potentially confer recognition of *Avr9* (Kruijt et al. 2004). The repetitive structure of this cluster is likely to increase sequence exchange between the *9DC* genes, and also between the *9DC* genes and other *Hcr9s*, thus further contributing to intraspecific *9DC* variation.

The *9DC* cluster is not the only known *C. fulvum* resistance gene cluster that carries multiple genes with the same resistance specificity. Two highly homologous *Cf-2* genes from the *Cf-2* cluster both confer resistance to strains of *C. fulvum* that express *Avr2* (Dixon et al. 1996; Luderer et al. 2002). In this study, three distinct *Hcr9-Avr9s* (*Hcr9-Avr9-hir1*, *-hir2*, and *-hir3*) were isolated from a single self-incompatible and, therefore, heterozygous *L. hirsutum* LA2155 plant. *Hcr9-Avr9-hir2* and *-hir3* may be the result of a duplication event similar to those found in the *9DC* and *Cf-2* clusters, and *Hcr9-Avr9-hir1* may be located at the orthologous *MW* locus. The few SNPs in the *Hcr9-Avr9s* suggest that limited sequence exchange has occurred, both between these genes and with other *Hcr9s*. However, a sequence stretch within *Hcr9-Avr9-hir4* shows a perfect sequence affiliation with *Hcr9-9D*. The 10 SNPs found in *Hcr9-Avr9-hir4* relative to the other *Hcr9-Avr9s* between nucleotides 1,858 and 2,000 (Fig. 5A) might indicate a recombination event with *Hcr9-9D*. The *Hcr9-Avr9-hir4* sequence 3' of nucleotide 2,000, however, cannot be found within the known *Hcr9s*, and could well be derived from yet-unidentified homologs. Indeed, five of the seven SNPs exclusive to *Hcr9-Avr4-hir4* are located here. We previously showed that *Cf-9* and *Hcr9-9D* are ancestral to *9DC* (Kruijt et al. 2004), and we now found *Hcr9-Avr9-hir1* in *L. hirsutum*, which differs from

the *L. pimpinellifolium* *Cf-9* by only one nucleotide. This indicates that *Hcr9-Avr9-hir4* may be derived from *Hcr9-Avr9-hir1* or related alleles, and has obtained a sequence stretch from *Hcr9-9D* and possibly from one or more other, unknown *Hcr9s*. However, for *Hcr9-Avr9-hir4*, no clear ancestral relationship with known *Hcr9s* can be deduced, as was the case for *Cf-9*, *Hcr9-9D*, *9DC*, and *9DC3* (Kruijt et al. 2004). Divergence of the individual *Hcr9-Avr4s* from an ancient *Hcr9-Avr4* likely has been a combination of point mutations and sequence exchange by gene-conversion or recombination with other *Hcr9s*, because many of the discriminatory SNPs between the *Hcr9-Avr4s* are shared with other *Hcr9s*. Likewise, the overall high sequence conservation of the *Hcr9-Avr9s* from *L. hirsutum* and *Cf-9* from *L. pimpinellifolium* suggests that *Cf-9* was present in an ancestral *Lycopersicon* sp. and has not diverged to a great extent since. The sequence polymorphisms in the *Hcr9-Avr4s* coincide with the evolutionary relationships among the *Lycopersicon* spp. from which these genes were isolated (Figs. 2 and 3). For example, *Hcr9-Avr4-chl1* is most homologous to *Cf-4*, and *L. chilense* and *L. hirsutum* are closely related, whereas *Hcr9-Avr4-chm1*, *-par1*, and *-perl* differ only little, which fits with the close relationship among the three species.

The genera *Solanum* and *Lycopersicon* have a common ancestry and are highly related (Taylor 1986). Recently, tomato species have been included in the genus *Solanum* (Peralta and Spooner 2000), integrating the two genera and emphasizing the close relationship between tomato species and traditional *Solanum* spp. *Avr9* recognition also has been observed in four tuber-forming *Solanum* spp. upon PVX::*Avr9* infection (R. Laugé, personal communication). This was confirmed for *Solanum hannemanii* and *S. leptophyes* by *Avr9* protein injections (data not shown), indicating that *Avr9* recognition may be conserved between the genera *Lycopersicon* and *Solanum*. Previously, three linked loci with *Cf* gene homologs have been reported on the short arm of chromosome 1 of cultivated potato (*S. tuberosum*) (Leister et al. 1996). Moreover, in several solanaceous species, including *S. tuberosum*, fragments that hybridize to *Cf-9* have been identified. Furthermore, *S. tuberosum* contains a high-affinity binding site for *Avr9* (Kooman-Gersmann et al. 1996), and *Cf-9* is functional in conferring *Avr9* responsiveness in several solanaceous species, including *S. tuberosum* (Hammond-Kosack et al. 1998; Van der Hoorn et al. 2000). Therefore, *Avr9* responsiveness in some *Solanum* spp. may well be based on conserved *Hcr9-Avr9s*.

We did not find evidence for convergent evolution of the *Hcr9-Avr4s* and the *Hcr9-Avr9s*; however, this cannot be excluded, because our screen was limited to a subset of *Hcr9s* that could be amplified with degenerate *Hcr9* primers. The isolation of additional *Hcr9-Avr4s* and *Hcr9-Avr9s*, and possibly the corresponding complete *Hcr9* clusters, would yield insight into the population structure of the *Hcr9-Avr4s* and *Hcr9-Avr9s* in individual species, as was shown for *Cf-9* and *9DC* in *L. pimpinellifolium* (Kruijt et al. 2004; Van der Hoorn et al. 2001a). PCR amplification of *Cf-2* homologs from 16 *L. pimpinellifolium* accessions yielded at least 26 unique homologs (Caicedo and Schaal 2004). Sequence analysis of these homologs revealed that the *Cf-2* gene family is evolving actively and is affected by heterogeneous evolutionary processes. However, no conclusion could be drawn specifically on the evolutionary forces acting upon functional *Cf-2* genes, because the homologs were not tested for their ability to confer *Avr2* responsiveness. For such analysis, it is crucial to distinguish functional and nonfunctional homologs. Agroinfiltration assays were shown to provide an effective tool for identification of *Hcr9-Avr4s* and *Hcr9-Avr9s* and comparison of functional homologs. Sequencing of nonfunctional *Hcr9-Avr4s* and *Hcr9-*

Avr9s orthologs and comparison of these with the functional homologs will contribute to the understanding of specificity and evolution of *Avr4* and *Avr9* responsiveness mediated by *Hcr9s*. This analysis may be broadened further by including *Hcr9s* derived from other members of the family *Solanaceae*, such as *Solanum* spp.

Activity of the *Hcr9-Avr4s* and the *Hcr9-Avr9s*.

Expression in tobacco of *Cf-4* and *Cf-9* by agroinfiltration in the absence of *Avr4* or *Avr9* does not result in an HR. However, using the same techniques as described here, two natural *Hcr9s* from *L. peruvianum* were isolated that induce an elicitor-independent HR upon agroinfiltration in tobacco (Wulff et al. 2004). None of the *Hcr9-Avr4s* and *Hcr9-Avr9s* identified in the present study induces such an elicitor-independent HR, and the activity observed in the agroinfiltration experiments could be attributed wholly to an *Avr*-dependent induction of HR.

By domain swapping and gene shuffling between *Cf-4* and *Cf-9*, several features crucial for conferring recognition of *Avr4* were identified in *Cf-4* (Van der Hoorn et al. 2001b; Wulff et al. 2001). All four novel *Hcr9-Avr4s* share these essential features, including i) 25 LRRs, which is unique to *Cf-4*; ii) a deletion of 10 amino acids in the B-domain relative to other *Hcr9s*; and iii) three specific amino acids at solvent-exposed positions in the LRR domain of *Cf-4* (Van der Hoorn et al. 2001b; Wulff et al. 2001). Solvent-exposed residues within the LRRs of *Hcr9* proteins are thought to interact with other proteins (Jones and Jones 1997; Kobe and Deisenhofer 1994). However, in this study, no additional specific solvent-exposed residues could be identified in the *Hcr9-Avr4s* that affect their activity. However, in the agroinfiltration overexpression assay, the activity of *Hcr9-Avr4-per1* was strongly reduced compared with *Cf-4*. *Hcr9-Avr4-per1* contains two unique proline residues that are located in the loop-out region and at a nonsolvent-exposed position in LRR 23 (Fig. 3B), which may well disrupt the tertiary structure of the protein and, thereby, reduce its activity. Only *Hcr9-Avr4-par1* is as active as *Cf-4* in conferring *Avr4* responsiveness, whereas the activities of *Hcr9-Avr4-chm1* and *Hcr9-Avr9-chl1* differ only slightly from *Cf-4*. Therefore, these three *Hcr9-Avr4s* may well function in resistance against *C. fulvum* strains that express *Avr4*.

All *Hcr9-Avr9s* from *L. hirsutum* are as active as *Cf-9* in agroinfiltration overexpression assays with *Avr9*. Therefore, none of the amino acid substitutions alters the activity of the *Hcr9-Avr9s* relative to *Cf-9*. Moreover, it indicates that all these genes can be functional in resistance against *Avr9*-expressing strains of *C. fulvum*. The number of amino acid substitutions in the *Hcr9-Avr9-hir* proteins compared with *Cf-9* is relatively low when compared with those found in the *Hcr9-Avr4s*. This suggests that sequence exchange that occurs between *Cf-9* and other *Hcr9s* in wild tomato may disrupt *Cf-9* function, consistent with the observation that *Cf-9* specificity is scattered throughout the protein (Van der Hoorn et al. 2001b; Wulff et al. 2001). However, the 9DC protein is as active as *Cf-9*, although it differs by 61 amino acids from *Cf-9* (Van der Hoorn et al. 2001a). The differences in 9DC are located in the N-terminal half of the protein up to LRR 11 and apparently are not located at positions essential for conferring *Avr9* responsiveness, whereas the SNPs in the *Hcr9-Avr9s* are located mostly in the C-terminal domains.

C. fulvum selection pressure may maintain *Cf-4* and *Cf-9* in wild tomato plants.

A high proportion of all sampled tomato species and accessions specifically recognize the *Avr4* or *Avr9* elicitor proteins of *C. fulvum* (Fig. 2), and *Avr9* recognition is even found outside the genus *Lycopersicon*. Moreover, the *Hcr9-Avr4s* and

Hcr9-Avr9s described in this study are highly conserved, suggesting that these genes precede *Lycopersicon* speciation. Conservation of an ancient *R* gene specificity in tomato also has been demonstrated for the *Pto* gene (Riely and Martin 2001). This suggests that tomato *R* genes are not rapidly replaced by novel ones, likely reflecting a selection pressure on the plant to maintain functional *R* genes. Not only does this suggest that *Avr4* and *Avr9* responsiveness is beneficial to wild tomato plants, it also suggests that *C. fulvum* must have been, and still is, an important pathogen of these species, and that *Avr4* and *Avr9* are beneficial to the pathogen. Maintenance of *Avr* genes in a pathogen population indicates that they contribute to virulence or saprophytic fitness (Brown 2003; Van der Hoorn et al. 2002). Indeed, for *Avr4*, a role in protection of *C. fulvum* hyphae against plant chitinases has been proposed (Van den Burg et al. 2003). The differential fixation of *Cf-4* and *Cf-9* alleles in the *Lycopersicon* spp. might reflect the necessity of *C. fulvum* to maintain *Avr4*, *Avr9*, or both for survival on these different *Lycopersicon* hosts. In greenhouse infection assays, *L. hirsutum*, *L. pimpinellifolium*, *L. pennellii*, and *L. peruvianum* plants have been reported to be susceptible to *C. fulvum* (Boukema 1981; Kerr et al. 1971; Leski 1969). It remains to be determined whether *C. fulvum* is pathogenic on all wild *Lycopersicon* spp.; however, in all wild tomato species, recognition of either of the *C. fulvum* elicitors *Avr4* or *Avr9* was found and, in some species, recognition of both elicitors was found. Therefore, all *Lycopersicon* spp. may be a host for *C. fulvum* or for highly related pathogens that share a common ancestry with *C. fulvum* and secrete *Avr4*- and *Avr9*-like proteins. We identified *Avr9*-responsive *Solanum* spp., and these may be host to such *C. fulvum*-related pathogens. Alternatively, the *Hcr9-Avr4s* and *Hcr9-Avr9s* might mediate resistance to a variety of different pathogens which produce *Avr4*- and *Avr9*-like proteins. Possibly, the *Hcr9-Avr4s* and *Hcr9-Avr9s* guard a virulence target (Van der Biezen and Jones 1998) that is attacked by virulence proteins from different pathogens.

In conclusion, this study has broadened our understanding of the evolution of *R* genes in natural plant populations. A high proportion of wild tomato plants specifically recognize the *C. fulvum* *Avr4* and *Avr9* elicitors, and functional homologs of the *C. fulvum* resistance genes *Cf-4* and *Cf-9* are conserved in diverged tomato species. This suggests that these genes were present before tomato speciation occurred and that they are maintained in the tomato population, possibly due to selection imposed by *C. fulvum*. A population survey of *C. fulvum* and related pathogens on different wild *Lycopersicon* and *Solanum* spp. would provide insight into the variation of *Avr* genes in natural populations of *C. fulvum*. The variation in *Avr* genes in wild *C. fulvum* populations then could be compared with the variation in *Cf* genes in wild tomato species, to determine whether the tomato *Cf* genes and *C. fulvum* *Avr* genes are maintained by balancing selection.

MATERIALS AND METHODS

Plant screening.

Seed of wild *Lycopersicon* accessions, consisting of the core collections of eight wild species, were kindly provided by the C. M. Rick Tomato Genetics Resource Center of the University of California, Davis. Plants were grown in trays and pots under standard Dutch greenhouse conditions. Using a micro-syringe (Ito Corporation, Fuji, Japan), four to six plants of each accession were injected in the first expanded leaf with approximately 10 μ M *Avr4* and *Avr9*, which is sufficient to induce an HR in the control plants MM-*Cf4* and MM-*Cf9*, respectively. Wild-type *Avr4* and FLAG-*Avr4*-

S107A (a glycosylation-minus Avr4 mutant, with an N-terminal FLAG tag) were produced in *Pichia pastoris* as described by Van den Burg and associates (2001). *P. pastoris* culture filtrates containing Avr4 or FLAG-Avr4-S107 were dialyzed against water, and subsequently purified on a G-25 column (26 mm in diameter, 40 cm in length) (Amersham-Pharmacia, Buckinghamshire, U.K.). The column was eluted with water at a rate of 1 ml/min and 5-ml fractions were collected. Fractions were tested for necrosis-inducing activity in MM-Cf4 plants and active fractions were pooled. Wild-type Avr4 and FLAG-Avr4-S107A were similarly active in inducing an HR. Avr9 was obtained from culture filtrates of a *C. fulvum* Avr9-overproducing transformant (Van den Ackerveken et al. 1993), or chemically synthesized and folded in vitro (Van den Hooven et al. 1999). Plants that responded with an HR within 4 days upon injection of Avr4 or Avr9 were selected (one or two per accession). Injections were repeated at least three times to confirm the observed HR. For the initial screens, the FLAG-Avr4-S107 and *C. fulvum*-derived Avr9 were used. For subsequent injections, the wild-type Avr4 and chemically synthesized Avr9 were used.

Hcr9 amplification and cloning.

All DNA manipulations were performed using standard protocols (Sambrook and Russell 2001). Restriction enzymes and T4 DNA ligase were obtained from Promega Corp. (Madison, WI, U.S.A.). Primers were synthesized by Sigma-Genosys (Cambridge, U.K.). To amplify as many *Hcr9s* per genotype as possible, primers were designed that anneal to the start and stop codon of published *Hcr9s* at the *Milky Way*, *Northern Lights*, *Southern Cross*, and *Orion* loci (De Kock et al. 2005; Kruijt et al. 2004; Parniske and Jones 1999; Parniske et al. 1997; Van der Hoorn et al. 2001a). Several mixes of different primers in different concentrations were tested for amplification of *Hcr9s* from several *Lycopersicon* genotypes to give an optimal yield of PCR products. The final primer mix contained degenerate forward and reverse primers with 5' terminal *NcoI* and *PstI* cloning sites, respectively, for subsequent cloning of the generated PCR product. Both forward and reverse primers end at three nucleotides that encode a conserved methionine residue, ensuring that the three terminal nucleotides are invariant. The following primers were used (in 5'-to-3' direction; restriction sites underlined; start and stop codons in bold):

ATG-N1: tttttc**catg**grrtgtgtaaaacttrtrttttrtg,
 ATG-N2: tttttc**catg**grrtgtktaraacttrttccctatg,
 ATG-N3: tttttc**catg**grrtgttttagaacttrtrttttrtg,
 ATG-N4: tttttc**catg**gttactgramactgttdttttttag,
 TAG-P1: tttttc**gcagc**taatactctttctgtgcttttcat, and
 TAG-P2: tttttc**gcagc**taayatcttctygtgyttttcat.

Primers were used in the following ratios: ATG-N1:N2:N3:N4, 4:1:1:1; TAG-P1:P2, 2:1; and at a total concentration in the PCR mix of 28 ng/μl for the ATG-mix and 24 ng/μl for the TAG-mix. PCR experiments were performed on genomic tomato DNA (isolated as described by Van der Beek and associates 1992) using the Expand high fidelity PCR system (Roche, Basel, Switzerland) according to the manufacturer's instructions, with an annealing temperature of 58°C. DNA fragments of the expected sizes (2.4 to 2.6 kb) were excised from gel and purified using the GFX PCR DNA and gel band purification kit (Amersham-Pharmacia). Purified PCR products were digested with *NcoI* and *PstI* and ligated into the *NcoI* and *PstI* sites of pRH80 (Van der Hoorn et al. 2000). Individual clones containing a 35S promoter-*Hcr9*-PI-II terminator cassette subsequently were digested with *XbaI* and *KpnI* and the fragments were cloned into the binary expression vector pMOG800 (Honée et al. 1998).

Agroinfiltration for selection of *Hcr9-Avr4s* and *Hcr9-Avr9s*.

Individual pMOG800 clones containing a single *Hcr9* amplified from an Avr4- or Avr9-responsive *Lycopersicon* plant were transformed into *A. tumefaciens* GV3101. These constructs subsequently were tested for their ability to confer Avr4 or Avr9 responsiveness using agroinfiltration in *Nicotiana tabacum*, including the 35S-overexpression constructs pAvr4, pAvr9, pCf-4, and pCf-9, as described before (Van der Hoorn et al. 2000). In a first screen for *Hcr9s* active in Avr4 or Avr9 recognition, *A. tumefaciens* colonies containing a single *Hcr9* were grown overnight in 3-ml cultures containing 2 mM acetosyringone. The bacteria were pelleted and resuspended in MMA and used for agroinfiltration in leaves of *N. tabacum* cv. Petite Havana SR1 (Van der Hoorn et al. 2000). *Hcr9s* derived from Avr4-responsive plants were tested for Avr4 recognition by co-infiltration with pAvr4, and *Hcr9s* derived from Avr9-responsive plants for Avr9 recognition by co-infiltration with pAvr9. For confirmation of the activity of single *Hcr9s* and for a reproducible comparison of their relative activity, clones were grown in 100-ml cultures as described by Van der Hoorn and associates (2000). Relative activities of *Hcr9s* mediating Avr4 or Avr9 recognition were determined in agroinfiltration dilution series (Van der Hoorn et al. 2001b).

Sequence analysis.

pMOG800 clones containing *Hcr9s* active in Avr4 or Avr9 recognition were selected for sequencing. pMOG800 is a low-copy plasmid, which resulted in low-quality sequence data. Therefore, the corresponding high-copy pRH80 clone was sequenced double stranded. Sequencing was carried out by Base-Clear (Leiden, The Netherlands) with the following primers (presented in 5'-to-3' direction, length of the sequence run in parentheses):

pWLSF4 (800): cctctatataaggaagttcatttc,
 SF1 (800): ctaagaacttgacccaattaa,
 SF2 (800): tataccttctggatattc,
 SF3 (500): gatcaataagtgcattgctc,
 SF4 (500) cttggaaggtcatatacc,
 pWLSR (800): aacacaactttgatgcccac,
 SR1 (800): ctcttaattgggtcaagttct, SR2 (800):
 gaatatccaggaaggtata,
 SR3 (800): ccatgcaacttattttgatctc, and
 SR4 (500): ggtatatgacctccaag.

Sequences were analyzed using Lasergene software programs (DNASTAR, Madison, WI, U.S.A.). To verify that the obtained sequences did not contain PCR-induced mutations, at least one or two additional independently isolated, active clones were sequenced. In the case of *Hcr9-Avr4-chl1*, all five sequenced clones were different from the wild-type (consensus) sequence (all contained one or two independent mutations); in the case of *Hcr9-Avr4-per1*, four isolated clones differed from the wild-type (consensus) sequence. For both *Hcr9-Avr4-chl1* and *Hcr9-Avr4-per1*, a wild-type sequence could be deduced and, to test the activity of these *Hcr9-Avr4s*, clones that contained only one silent mutation were used.

ACKNOWLEDGMENTS

We thank R. Chetelat (Tomato Genetic Resource Center, Davis, CA) for providing the *Lycopersicon* seed; the Center for Genetic Resources (Wageningen University and Research Center, Wageningen, The Netherlands) for providing the *Solanum* seed; R. Laugé for sharing unpublished data; M. Berns, B. Essenstam, R. Janssen, and H. Smid for excellent plant care; P. van Baarlen and M. Staats for help with phylogenetic analysis; and R. van der Hoorn for helpful suggestions. M. Kruijt and B. F. Brandwagt were financially supported by the Dutch Foundation for Applied Sciences

(STW), and M. H. A. J. Joosten was financially supported by a VIDI grant from the Netherlands Organization for Scientific Research (NWO).

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