Cladosporium fulvum Effectors: Weapons in the Arms Race with Tomato

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Abstract

In this review, I recount my personal history. My drive to study host-pathogen interactions was to find alternatives for agrochemicals, which was triggered after reading the book "Silent Spring" by Rachel Carson. I reflect on my research at the Laboratory of Phytopathology at Wageningen University, where I have worked for my entire career on the interaction between *Cladosporium fulvum* and tomato, and related gene-for-gene pathosystems. I describe different methods used to identify and sequence avirulence (*Avr*) genes from the pathogen and resistance (*R*) genes from the host. The major genes involved in classical gene-for-gene interactions have now been identified, and breeders can produce plants with multiple *R* genes providing durable and environmentally safe protection against pathogens. In some cases, this might require the use of genetically modified plants when *R* genes cannot be introduced by classical breeding.

MY MOTIVATION TO STUDY PLANT PATHOLOGY

I grew up after World War II on a farm in Limburg, the most southern province of The Netherlands. In those days farming was very labor-intensive and still very much dependent on horsepower. I loved to work with horses, but soon they were replaced by tractors. In Limburg, farms were relatively small and not specialized. Apart from growing crops like cereals, sugar beets, and potatoes, most farmers also kept dairy cattle, pigs, and chickens. My eldest brother was going to succeed my father, and I knew that my future would not be in farming. After primary school, I went to high school, where although I spent my time mainly studying languages (Dutch, English, French, German, Greek, and Latin), I was more interested in chemistry and biology. I was fascinated by crop plants that could grow out of tiny seeds into adult plants within one season, and by majestic trees like the chestnut tree that overlooked our farm for more than hundred years. If that chestnut tree could talk, it would have told me how it survived shell bursts during World War II while the farm was completely destroyed. I had a small garden of my own where I experimented with seeds of pea and bean, digging them up from the soil a few days after I had sowed them, curious to find out how far they had developed. My father and my biology teacher at high school stimulated my interest in plant biology. After high school, I went to Wageningen University, where I followed a broad BS program in (bio)chemistry and biology. I did my MS in crop protection, which included three research projects of six months at the Laboratories of Biochemistry, Plant Physiology, and Phytopathology. Apart from these research projects, the MS program included practical training abroad, for which I spent three months at Imperial Chemical Industries (ICI) in Bracknell, UK, working on a bioassay to test the effectiveness of fungicides against powdery mildew of wheat. In those days, protection of crops against fungal pathogens was mainly achieved by applying fungicides. After having read the book "Silent Spring" by Rachel Carson (8), I was convinced that alternatives must be sought to protect crop plants against diseases and pests. I conducted my last MS research project at the Laboratory of Phytopathology under the daily supervision of Maarten de Waard and followed advanced courses on chemical and biological control, pathophysiology, and genetics of host-pathogen interactions. The latter course I liked best. The book "Genetics of Host-Parasite Interaction" by Peter Day (14) gave a good overview of the state of the art of the research in 1974, the year I finished my MS. The gene-for-gene hypothesis especially intrigued me. It was formulated by Harold Flor working on flax and the flax rust fungus *Melampsora lini* (38) and Arend Joan Petrus Oort, emeritus professor of Phytopathology at Wageningen University, working on wheat and the wheat smut fungus *Ustilago tritici* (72). I am fortunate to have personally known Professor Oort, who still kept a laboratory after his retirement. I vividly recall discussions with him during coffee breaks. In biochemical terms, the gene-for-gene hypothesis proposes that the product of a dominant avirulence (*Avr*) gene of the fungus interacts with the product of a dominant resistance (*R*) gene of the plant, triggering defense responses leading to resistance. The products of a recessive *avr* gene and a recessive *r* gene would not interact and would not trigger defense responses, thus leading to disease (**Figure 1**). However, apart from the genetics, not much was known about the molecular basis of the gene-for-gene hypothesis, and my dream was to study this further.

CHOOSING A RESEARCH TOPIC FOR MY PHD

After my MS, it was not clear whether I could do a PhD in phytopathology at Wageningen University, as few PhD positions were available. I had just applied for a job as a teacher in biology and chemistry at a junior college in Den Bosch, The Netherlands, when Professor Johan Dekker, the successor of Professor Oort, offered me a PhD position at the Laboratory of Phytopathology, which I gladly accepted. I was very privileged that he gave me the freedom to choose my own research topic. I spent many days in the library and discussed with colleagues the advantages and

Genetic model

Fungus: Avirulence gene / Plant: Resistance gene	*R*	*r*
Avr	Resistance	Disease
avr	Disease	Disease

Biochemical model

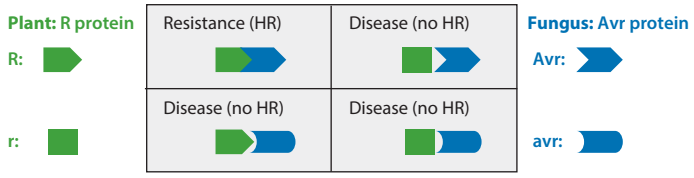

Figure 1

Genetic and biochemical models for Flor's gene-for-gene hypothesis. Abbreviation: HR, hypersensitive response.

disadvantages of particular pathosystems. I narrowed my choices to following the pathosystems: *Cladosporium fulvum*–tomato, *Venturia inaequalis*–apple, and *Ascochyta pisi*–pea, which were all amenable to study. I chose *C. fulvum*–tomato for several reasons.

Tomato leaf mold caused by *C. fulvum* was an important disease (7), and at Wageningen several researchers worked on this disease. Ietje Boukema at the Institute of Vegetable Genetics (IVT) had developed near-isogenic lines of tomato in a Moneymaker background carrying the *Cf-2*, *Cf-4*, *Cf-5*, or *Cf-9* gene for resistance against *C. fulvum* (3). Nico Hubbeling at the Institute of Phytopathological Research (IPO) had accumulated a collection of physiological races of *C. fulvum* (42). He regularly performed virulence assays with *C. fulvum* for tomato breeders. Finally, at the University of Utrecht, Anton van Dijkman had completed his PhD thesis on *C. fulvum* in 1972 (102) and had found evidence for high molecular weight compounds (HMWCs) produced by this fungus in vitro that could cause leakage of electrolytes from tomato leaves of cultivars carrying different *Cf* genes in a race-specific way.

DID I MAKE THE RIGHT CHOICES?

My PhD research on *C. fulvum* started in September 1974. The Laboratory of Phytopathology hosted only a few PhD students in those days, which is very different from the 20–25 students working on their PhD theses nowadays. I enjoyed the discussions with colleagues Adriaan Fuchs, Leen Davidse, Tijmen Hijwegen, and Maarten de Waard about my research; however, a PhD study was very much a one-person enterprise in those days. The research of my colleagues was mainly focused on the mechanisms of action of fungicides and the development of resistance against these compounds in different fungi. Some physiological research on host-pathogen interactions was performed by Adriaan Fuchs, who had a keen interest in phytoalexins, and Tijmen Hijwegen, who was interested in co-evolution between pathogens and their host plants. I also interacted with Dr. Ant Kaars Sijpesteijn at the University of Utrecht, the daily supervisor of van Dijkman (102). There were bimonthly joint meetings between Utrecht University and Wageningen University of the Working Group Internal Therapy of Plants, which was founded by Professor Oort in 1950. Van Dijkman had developed a bioassay to measure leakage of radioactivity from tomato disks

labeled with ^{32}P upon treatment with HMWCs from culture filtrates (CFs) of different races of *C. fulvum* grown in vitro. HMWCs from CFs of avirulent races of *C. fulvum* caused more ^{32}P leakage from leaf disks of resistant tomato plants than HMWCs from CFs of virulent races (102). These results were in agreement with Flor's gene-for-gene hypothesis. Similar findings were reported in the same research group by former PhD student Jan Raa for the *V. inaequalis*–apple pathosystem (75). However, during the first half year of my PhD I could not repeat the experiments and confirm their results, and I was reluctant to report my negative results during the joint meetings of the Working Group. I decided to visit van Dijkman, who was no longer working as a scientist, to discuss my results, but the discussions did not shed new light on my data and did not help me further. I then decided to visit Jan Raa, who, after his PhD, had moved to the University of Tromsö, Norway, the northern-most university in the world. At the beginning of June 1975, I traveled in a Renault 4 with my wife Els to Tromsö, a journey of 3,000 kilometers. It was an unforgettable journey driving along the Gulf of Bothnia via Lapland to Tromsö. In Tromsö, I was disappointed to hear from Jan Raa that he had stopped working on the *V. inaequalis*–apple pathosystem and had started to work on fish, for the simple reason that fish was more important for Tromsö than apple. The stay in Tromsö was pleasant and during free hours and on weekends Els and I played tennis in the midnight sun, or fished for cod in the fjords. Along with discussions I had with Jan Raa, I also conferred with Björn Solheim, who worked on *Rhizobium* sp. and allowed me to work on his scanning electron microscope when I had decided to start a microscopic study of infection of tomato by *C. fulvum*. After three months we returned to Wageningen, where I continued the microscopic work on compatible and incompatible interactions between *C. fulvum* and tomato. I could confirm that *C. fulvum* is an extracellular pathogen that, after penetration of stomata, colonizes the apoplast of tomato leaves without forming haustoria (19). In incompatible interactions, fungal growth is inhibited very soon after stomatal penetration, and the invading hyphae in contact with epidermal and mesophyll cells quickly induce strong accumulation of callose and a hypersensitive response (HR). Not much later, I found out from a conference abstract that at the University of Toronto, in the research group of Verna Higgins, George Lazarovits was also working on *C. fulvum* for his PhD. Soon thereafter he published two very nice papers on light and transmission electron microscopy of compatible and incompatible interactions (58, 59). The ultrastructural studies gave good insight in the timing of the infection process and formed a solid basis for future physiological and molecular work. Lazarovits & Higgins (60) tried to repeat the experiments reported by van Dijkman & Kaars Sijpesteijn (102), as well as Dow & Callow (35, 36) in the United Kingdom, but without success. Indeed, they all identified HMWCs from CFs of *C. fulvum* that induced electrolyte leakage and HR-like defense responses in tomato leaves, but they were not different for leaves of susceptible and resistant tomato plants. The HMWCs from *C. fulvum* isolated by the three research groups were reported to contain mainly (glyco)proteins (25, 27, 36, 57).

Halfway through my PhD, I worried that I may not successfully complete my PhD thesis within four years. I also had to give lectures and practical training to BS students for a few months every year. Then, something very fortunate happened to me. In 1976, I obtained a permanent position as a researcher at the Laboratory of Phytopathology. This appointment allowed me to set up new research lines for continuation of my PhD study. My career would have gone in a completely different direction without this appointment.

The Phytoalexin Era

Müller (67) defined the term phytoalexin, and Cruickshank & Perrin (12) established the phytoalexin concept at the chemical level. Cruickshank & Perrin also isolated the phytoalexin pisatin

from inoculated pea pods and established its structure. Phytoalexins were interconnected with the gene-for-gene hypothesis when in 1975 Noël Keen coined the term elicitor to describe chemical compounds released from the invading pathogen that elicited accumulation of phytoalexins in resistant host plants (51). Elicitors were mainly fungal cell wall–derived or secreted (glyco)proteins that would likely interact with an *R* gene–encoded plant receptor to induce the accumulation of phytoalexins. In the 1970s, every respected plant pathologist working on the physiology of host-pathogen interactions studied the induction of phytoalexins and aimed at their characterization. I also tested whether elicitors present in CFs and cell wall preparations of virulent and avirulent races of *C. fulvum* could differentially induce phytoalexins in leaves and fruits of resistant tomato cultivars (25). I identified two new phytoalexins from tomato leaves that were strongly induced in incompatible interactions but not, or much less so, in compatible interactions (26); however, elicitors isolated from virulent and avirulent races of *C. fulvum* grown in vitro induced similar amounts of phytoalexins and thus were called nonspecific elicitors. In 1978, at the third International Congress of Plant Pathology in Münich, I met for the first time leading scientists in this area of research, including Peter Albersheim, Noël Keen, and Joseph Kuć. It was the first congress I attended as a PhD student. There were many presentations on the induction of phytoalexins by elicitors, but also on the induction of other defense responses, including the accumulation of pathogenesis-related (PR) proteins and the HR. In the mid-1980s together with colleagues Frank van der Meer and Matthieu Joosten, I also reported on the quicker and higher accumulation of PR proteins, including P-14, chitinases, and β-1,3-glucanases, in incompatible interactions than in compatible ones (29, 49). In microscopic studies, we observed that all in planta defense responses mentioned above were always associated with incompatible interactions (26, 29), suggesting that indeed specific compounds are produced by avirulent races and recognized by resistant cultivars carrying the matching *Cf* genes. However, nobody had ever been able to identify race-specific elicitors from fungal plant pathogens when grown in vitro. This led us to hypothesize that, apart from nonspecific elicitors, race-specific suppressors of plant defense encoded by virulence genes are required to explain the lack of defense responses in compatible interactions. In the general discussion of my PhD thesis (30), I presented a model that fit all the observations that were made for *C. fulvum* elicitors until 1980. Together with my colleague Leen Davidse, I published this model (23).

Secretion of Race-Specific Elicitors by *Cladosporium fulvum* In Planta

I wondered why most physiological plant pathologists tried to isolate race-specific elicitors from plant-pathogenic fungi grown in vitro, whereas specific defense responses occur only in planta during infection. Therefore, I decided to study the interaction in planta and take advantage of the fact that *C. fulvum* colonizes only the apoplast of tomato leaves. This enabled us to isolate compounds present in apoplastic fluids (AFs) from *C. fulvum*–infected tomato leaves and study their biological activities. When I infiltrated AFs, obtained from a universally susceptible cultivar lacking any *Cf* gene (cv. Moneymaker; Cf-0) inoculated with races carrying different *Avr* genes, into leaflets of near-isogenic lines of tomato carrying different *Cf* genes, race-specific HR-like responses were observed (28) (**Figure 2**). The responses were induced by proteins secreted by the fungus into the apoplast, as treatment of AFs with commercial proteases destroyed the HR-inducing activity. The near-isogenic line carrying the *Cf-9* gene responded with a very strong HR when infiltrated with AFs from races carrying the *Avr9* gene (28). Verna Higgins, who later spent a sabbatical in Wageningen, obtained similar results with AFs obtained from leaves infected by Canadian isolates of *C. fulvum* (40). I started purification of the Avr9 protein when I was on sabbatical in the laboratory of Professor Joseph Kuć at the University of Kentucky. My decision

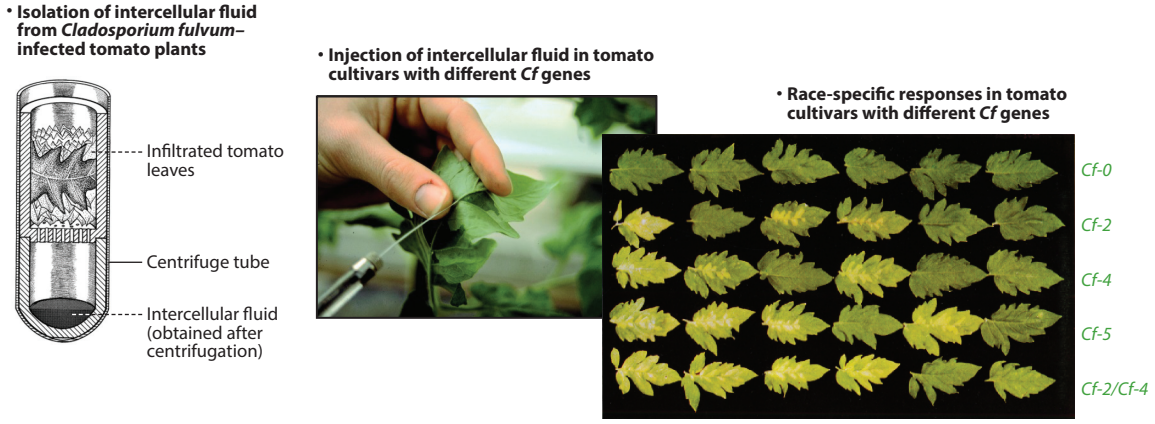

Figure 2

Intercellular fluids of *Cladosporium fulvum*–infected plants contain race-specific elicitors. Adapted from Reference 28.

to go to his laboratory was made before we had found race-specific elicitors in AFs. His coworkers had isolated race-specific suppressors from *Phytophthora infestans* (34), and as we initially expected to find suppressors of defense responses in AFs of infected leaves, a stay in his laboratory was a good choice. It took several years to purify the Avr9 protein (24) and even longer to determine its amino acid (aa) sequence, which required an extremely pure protein to be analyzed by Edman degradation. Eventually, we purified the Avr9 protein using reversed phase chromatography (79) and were able to determine its aa sequence. It contained 28 aa, including 6 cysteine residues. The cloning of the *Avr9* gene was also very difficult, as most of the aa sequence information of the Avr9 protein was not helpful in designing oligonucleotide probes to isolate its encoding cDNA from a library of *C. fulvum*–infected tomato. The *Avr9* gene was cloned by PhD student Guido Van den Ackerveken and postdoc Jan van Kan using several molecular tricks that are beyond discussion here (91, 106). *Avr9* encodes a secreted protein of 63 aa, including a signal sequence of 23 aa, indicating that the Avr9 protein that we purified from AFs was processed, as it lacked 12 aa at the N terminus. Altogether it took nearly 10 years from the discovery of the Avr9 product in AFs to clone the first fungal *Avr* gene. When analyzing the genomic DNA from *C. fulvum* races that could overcome the *Cf-9* resistance gene, we were able to show that they all lacked the *Avr9* gene (91, 106). To prove that the *Avr9* gene was the only gene involved in *Cf-9*-mediated resistance, transformation of *C. fulvum* was crucial. We were lucky to collaborate with Richard Oliver at the University of East Anglia, who had developed a transformation system for *C. fulvum* (71). Indeed, virulent races transformed with the *Avr9* gene became avirulent on *Cf-9* plants, and avirulent races in which the *Avr9* gene was replaced by a selectable marker became virulent (65).

Avr9 Gene-Enabled Cloning of the *Cf-9* Resistance Gene

In 1987, before he took on a group leader position at the Sainsbury Laboratory in 1988, where he initiated the cloning of *Cf* genes by both map-based and transposon-tagging approaches, Jonathan Jones visited our lab. We had long discussions on the *C. fulvum* project. Map-based cloning was labor-intensive, but when the *Avr9* gene became available, the transposon tagging approach accelerated the cloning of the *Cf-9* gene. His research group developed a tomato line that contained a maize dissociator (Ds) element 4 cM away from the *Cf-9* gene. To activate Ds, this line was crossed

to a line that contained a maize activator (Ac) element. This new line was crossed to a transgenic tomato line expressing the *Avr9* gene. The seeds of this cross were viable, but seedlings died when expressing a functional Cf-9 and Avr9 protein, as their interaction killed the seedlings via a systemic HR, unless the *Cf-9* gene was inactivated by Ds. From a progeny of 160,000 seeds, a total of 63 independent mutants (i.e., plants that survived without HR or expressed variegated HR) were obtained (45). From one mutant line, the tagged *Cf-9* gene was identified and was one of the first fungal resistance genes cloned (45). In the summer of 1993, I was on sabbatical in the laboratory of Jonathan Jones when the first mutants were discovered. It was exciting to see the efficiency of the transposon-tagging method. It also showed that sometimes a researcher must have some luck. A similar transposon tagging approach would not have worked for cloning of the *Cf-2* gene with the *Avr2* gene of *C. fulvum* because the near-isogenic Cf-2 line contains two functional copies of the *Cf-2* gene (32). Jonathan once expressed in a commentary: "I am eternally grateful to Pierre de Wit for isolating *Avr9* before *Avr2*!" (46). Having the fungal *Avr9* gene and matching tomato *Cf-9* gene cloned caused much excitement in both laboratories. In those days, both laboratories were very successful in obtaining grants from their national science foundations for continuation of research on *Avr-Cf* gene pairs. We collaborated also with many research groups in Europe supported by EU grants on elicitors, elicitor receptors, and elicitor-triggered defense responses, including the research groups of Jonathan Jones, Richard Oliver, Dierk Scheel, Thorsten Nürnberger, Giulia de Lorenzo and Felice Cervone, Isabelle Roncero, Pierre Ricci, and Regine Kahmann and others.

The *Avr9* Gene Cassette

I was very excited to have cloned the first fungal *Avr* gene in 1991, and together with colleagues I filed a patent on exploiting this gene in molecular disease resistance breeding (20). It became known as the *Avr9* gene cassette (41, 83) (**Figure 3**). We proposed to transform *Cf-9* tomato plants with the *Avr9* gene under the control of a pathogen-inducible promoter. When a biotrophic pathogen enters such an *Avr9*-transgenic *Cf-9* plant an HR is induced, leading to resistance. We found proof of concept (41). Selling the patent to the biotechnology company Mogen International provided the funds for two new postdocs at the Laboratory of Phytopathology. The patent has not been developed further after the merger of Mogen with Zeneca and later Zeneca with Syngenta, as the latter company was concerned that the Avr9 protein produced in transgenic vegetables, such as tomato and potato, could cause allergy to consumers.

In Search of Functions of the Avr9 Protein

Once the *Avr9* gene was cloned, new research questions could be addressed on the structure and function of its encoded protein. Avr9 is a cystine knot protein, as was determined by nuclear magnetic resonance by Jacques Vervoort and colleagues at the Wageningen University, Laboratory of Biochemistry (96, 108). It is surprising that no Avr9 mutants were found in natural populations carrying single or multiple aa polymorphisms. The *Avr9* gene is located in a repeat-rich region of the *C. fulvum* genome, and its loss might occur more frequently than the occurrence of DNA mutations, causing aa substitutions in the Avr9 protein. Artificial Avr9 mutants that showed a strong reduction or increase in Avr9-triggered, Cf-9-dependent HR are easily created, but such mutants were never found in nature (53).

We never obtained evidence for direct interaction between Avr9 and the Cf-9 protein. In collaboration with other laboratories, we used different vectors to produce the Cf-9 protein (insect cells, yeast, or COS cells) but never detected interaction with Avr9 (63). Likely, the Cf-9 protein is not correctly glycosylated and folded when produced in these different heterologous expression

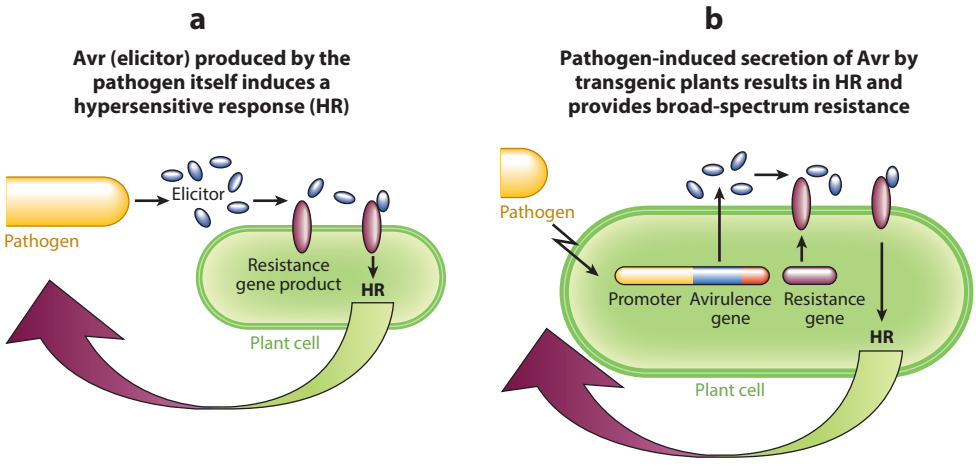

Figure 3

The gene cassette. Generating broad-spectrum disease resistance using an elicitor and resistance gene. (*a*) The hypersensitive response (HR) is triggered by the highly specific recognition of a pathogen-derived elicitor by a plant resistance gene product. The concerted defense that constitutes the HR stops the pathogen. (*b*) The components involved in the basic switch of the HR can be used to create a more nonspecific defense system. A plant-derived, pathogen-inducible promoter drives expression of a pathogen avirulence (*Avr*) gene. The produced elicitor will trigger the HR when the plants also contain the resistance gene. Adapted from References 20 and 83.

systems. It was also hypothesized that Avr9 could be a virulence factor interacting with a host target that was guarded by the Cf-9 protein (97). Interaction of Avr9 with this target would trigger a Cf-9-dependent HR. This model proposed by van der Biezen & Jones (97) became known as the "Guard Hypothesis". By using ^{125}I-Avr9, a high-affinity binding site (HABS) was identified by PhD student Miriam Kooman-Gersmann in microsomal fractions of tomato and tobacco (52), but it has never been characterized further.

We have also never been able to identify the biological function of the Avr9 protein in *C. fulvum*. Deletion of the *Avr9* gene did not significantly reduce virulence of the fungus, suggesting that the Avr9 protein plays only a minor role in the infection process. The *Avr9* gene is highly expressed during infection of tomato and is induced in vitro in media containing minimal nitrogen sources (90). PhD student Sandor Snoeijers and postdoc Alejandro Pérez-García have studied *Avr9* regulation in more detail and found that its expression is dependent on the global nitrogen response factor NRF1 (73). Several international colleagues have tried to identify fungal *Avr* genes by growing pathogenic fungi under nitrogen-limiting conditions, but only a few were found to be induced under these conditions.

More *Avr* and *Ecp* Genes of *Cladosporium fulvum*

Throughout the years, many PhD students have worked on the *C. fulvum*–tomato pathosystem. My first PhD student was Matthieu Joosten, who identified several tomato PR proteins induced by *C. fulvum* during infection (49). After his PhD, he stayed as a postdoc and cloned the *Avr4* gene (48). The *Avr4* gene was cloned by methods similar to those used to clone the *Avr9* gene, but it was achieved more quickly, as molecular techniques became more advanced. Avr4 is a small, secreted, cysteine-rich protein that is processed in planta at both the N and C termini. In races

overcoming Avr4-triggered Cf-4-mediated resistance, the *Avr4* gene was found to carry nucleotide polymorphisms that cause aa substitutions mostly in cysteine residues of the Avr4 protein, causing instability in the apoplast and preventing Avr4 from being recognized by the Cf-4 protein (50). Later, Matthieu became a staff member at the Laboratory of Phytopathology and cosupervised many of the PhD students who have worked on the *C. fulvum*–tomato pathosystem. He was a great daily cosupervisor, especially when I became full professor and head of the Laboratory of Phytopathology in 1990.

The *Avr4E* gene was cloned by PhD student Nienke Westerink, together with postdoc Bas Brandwagt, using methods similar to those used to clone the *Avr9* and *Avr4* genes (110). Again, *Avr4E* encodes a small, secreted cysteine-rich protein. Races overcoming the *Cf-4E* gene either carry an *Avr4E* gene that encodes an effector with two aa substitutions or completely lack the gene. The *Avr2* and *Avr5* genes were cloned much later and required other strategies, as we could not purify sufficient amounts of their encoded proteins from AFs. PhD student Rianne Luderer and postdoc Frank Takken cloned the *Avr2* gene by functional cloning of its cDNA, based on its property to induce an HR in Cf-2 tomato plants (64). A cDNA library of the fungus, grown in vitro under growth-limiting conditions, was made in recombinant *Potato virus X* (PVX) and was inoculated onto Cf-2 plants to detect and isolate clones that caused an HR (64). Also Avr2 is a small, secreted cysteine-rich protein. Races overcoming the *Cf-2* gene all contained frame-shift mutations in *Avr2*, leading to truncated proteins, whereas in a few cases *Avr2* contained a transposon. Later, postdoc Carl Mesarich cloned the *Avr5* gene by a combined bioinformatics and transcriptome sequencing approach (66). This was possible after sequencing the genome of *C. fulvum*. RNA-Seq was performed on the sequenced race 0 strain (carrying the *Avr5* gene) and a race 5 strain (lacking a functional *Avr5* gene) during infection of susceptible tomato. An expressed transcript sequence comparison on highly expressed genes encoding small, secreted, cysteine-rich proteins between the two strains revealed two polymorphic candidate *Avr5* genes in race 5, of which one caused avirulence of race 5 on Cf-5 tomato plants following complementation with the corresponding wild-type allele from race 0 (66). Again, Avr5 is a small, secreted, cysteine-rich protein; races overcoming the *Cf-5* gene in nearly all cases lacked the *Avr5* gene, and in only one case was a mutant Avr5 protein produced.

All cloned *Avr* genes of *C. fulvum* encode small, secreted proteins with an even number of cysteine residues that are recognized by matching Cf proteins. Therefore, we hypothesized that additional Avrs might be identified that induce an HR after recognition by *Cf*-like genes present in wild *Solanum* species. Five extracellular proteins (Ecps) from *C. fulvum* could be purified from AFs, and their encoding genes were cloned by PhD student Richard Laugé and colleagues (55). Injection of these Ecps in wild *Solanum* species indeed caused a clear HR in several of them, suggesting that they carry functional *Cf* genes (*Cf-Ecps*). Even some *Nicotiana* species responded with an HR after injection with Ecps and Avrs. Because some of the *Ecp* genes appeared to encode important virulence factors, their matching *Cf-Ecp* genes may be durable (18, 56). PhD student Maarten de Kock in collaboration with Pim Lindhout has tried to clone the *Cf-Ecp2* gene. They identified two candidate genes, but which of the two was functional could not be determined with certainty (18). The idea of searching for small, secreted proteins that induce an HR in wild species of crop plants was followed up by many researchers in the research community to identify new *R* genes against different pathogens. For the screening of HR-inducing activity of candidate *Avr* genes, often PVX or other viruses were used as expression vectors. At present, with many genome sequences available from different pathogens, high-throughput screens can be set up for hundreds of potential *Avr* genes to identify new matching *R* genes. This method has been successfully applied to identify new *R* genes in wild *Solanum* species directed against *Avr* genes of *Phytophthora infestans* (89).

We wondered whether recognition of Avrs and Ecps from *C. fulvum* by plants like tobacco could explain why they are nonhosts of this fungus. Alternatively, the HR induced in nonhost plants may suggest that pathogens of these plants secrete functional homologs of Avrs and Ecps.

Virulence Functions of *Avr* Genes

It took a long time to discover the primary functions of the Avr proteins of *C. fulvum*. As their primary function is to facilitate infection (virulence factors), Avrs are now called effectors. Finding the avirulence function of an effector was much easier than finding its virulence function. Some fungi may produce as many as 100 effectors. If one assumes that all 100 effectors contribute equally to virulence, then the contribution of one effector is only 1%. To experimentally demonstrate a difference in virulence of only 1% is difficult. Looking for structural and functional homologs of effectors in other pathogens became feasible when more genome sequences became available. Of all *C. fulvum* effectors identified, we know the primary function of only three.

The primary function of Avr4 was discovered by PhD student Harrold van den Burg by comparative protein domain searches in data banks (93, 95), which showed the presence of a functional chitin-binding domain in the Avr4 protein. Avr4 binds to chitin, which is related to its virulence function. Avr4 protects chitin in fungal hyphae against hydrolysis by plant chitinases (92, 103). Much later, in the research group of Bart Thomma, postdoc Melvin Bolton and PhD student Ronnie de Jonge discovered the biological function of Ecp6, which was initially isolated and cloned by Matthieu Joosten and postdoc Jack Vossen (2, 17). This effector also binds chitin but does not protect against plant chitinases. It scavenges chitin oligomers released by chitinases in the apoplast during infection, and prevents them from inducing basal defense responses facilitating the fungus to colonize the apoplast (16, 78). PhD students John van 't Klooster and Peter van Esse studied the virulence function of the Avr2 effector (77, 104) and showed that it inhibits the tomato cysteine protease Rcr3 to prevent it from hydrolyzing fungal proteins required for virulence. For this work, a protease activity profiling assay was developed by Renier van der Hoorn, who was a postdoc in our lab at that time (101). In collaboration with the lab of Jonathan Jones, my research group established that the interaction of Avr2 with Rcr3 triggers Cf-2-dependent HR and resistance to *C. fulvum* (77).

The Era of Sequenced Fungal Genomes

Until the late 1990s, the *Avr* and *Ecp* genes of *C. fulvum* were orphan genes. Initially, no homologs were found in sequenced eukaryotic genomes, but once multiple sequenced genomes from fungal plant pathogens became available, several fungi appeared to carry homologs. *C. fulvum* belongs to the *Dothideomycetes*, and several members of this class appeared to carry homologs of *Avr* and *Ecp* genes, including pathogens of very different host species, such as the wheat pathogen *Zymoseptoria graminicola* and the banana pathogen *Mycosphaerella fijiensis*. Postdoc Ioannis Stergiopoulos carried out this research and found functional homologs of *Avr4*, *Ecp2*, and *Ecp6* in these and other fungi (2, 81). It raised the question of whether these proteins would exhibit core functions in these pathogens inside and outside their host plants. The genome of *C. fulvum* was sequenced relatively late. Several times we submitted a proposal to the JGI-DOE to sequence *C. fulvum*, but we were never successful. Eventually, the *C. fulvum* genome was sequenced in Wageningen (22). We were surprised to see that its closest relative appeared to be *Dothistroma septosporum*, a pathogen of pine, studied by Rosie Bradshaw at Massey University. I enjoyed a short sabbatical in her laboratory in 2011. We set up a successful collaboration that resulted in several joint publications (4, 9, 22). Although both fungi grow extracellularly in close contact with host mesophyll cells, *C. fulvum* is

considered a biotroph, whereas *D. septosporum* is a hemibiotroph. Their genomes have a similar set of genes, but the genome size of *C. fulvum* is twice that of *D. septosporum*, which is mainly due to the high repeat content. Many genes encoding secreted proteins are unique to each species and the repeat-rich areas in *C. fulvum* are enriched for these species-specific genes. *D. septosporum* carries the highest number of *C. fulvum* effector homologs identified in any fungus so far. Strikingly, genes involved in production of the toxin dothistromin, a virulence factor for *D. septosporum*, are conserved in *C. fulvum*, but they are hardly expressed in planta. Also surprising was the fact that *C. fulvum* has catalogs of genes encoding enzymes for the production of secondary metabolites (SMs) and carbohydrate-degrading enzymes that are more similar to those of necrotrophs and hemibiotrophs than to biotrophs. However, many of these genes are not expressed in planta or are pseudogenized. Likely, the two closely related plant pathogens have a common ancestral host but have since adapted to different hosts and lifestyles by a combination of differentiated gene content, pseudogenization, and gene regulation. A clear example of adaptation of *C. fulvum* to tomato was the identification by PhD student Bilal Ökmen of the *CfTom1* gene encoding tomatinase, an enzyme that degrades fungitoxic tomatine into the less fungitoxic tomatidine (70). *CfTom1* is required for full virulence of *C. fulvum* on tomato.

The gene clusters encoding SMs in *C. fulvum* were studied by postdoc Jérôme Collemare (11). *C. fulvum* contains six SM gene clusters that are conserved in other fungal species. It has the potential to produce elsinochrome and cercosporin toxins, but the corresponding core genes are not expressed during infection of tomato. Only two core genes, the polyketide synthase *PKS6* and the nonribosomal peptide synthetase *NPS9* show expression in planta but at significantly lower levels than in vitro. Only the SM cladofulvin could be detected in vitro, which is produced by *PKS6*. The biosynthetic pathway of cladofulvin was dissected by PhD student Scott Griffiths and postdoc Jérôme Collemare (39a). Cladofulvin does not play a role in virulence on tomato, but might protect the conidia of *C. fulvum* against abiotic stresses such as UV light and cold (39a).

Peculiarities of the *Cladosporium fulvum* Genome

PhD student Ate van der Burgt, in collaboration with Jérôme Collemare discovered particular spliceosomal introns in the genome of *C. fulvum* that share high sequence similarity and have since been coined introner-like elements (ILEs) (98). ILEs have multiplied in unrelated genes of 14 fungal genomes identified so far and account for the majority of intron gains in these fungi (10). ILEs contain all characteristics of regular spliceosomal introns (RSIs), but are longer and harbor more stable secondary structures. However, dating of multiplication of ILEs in *Z. graminicola* and related species showed that they degenerate in sequence and length within 100,000 years to eventually become indistinguishable from RSIs (98). So far, ILEs have been identified in *Dothideomycetes* species (10), and the ILE content appears unique to each species, suggesting independent multiplication events. Four genes were identified, each containing two gained ILEs. By analyzing intron positions in orthologs of these four genes in other fungal species, three ILEs were found to have been inserted within a 15-bp window of an RSI and are a result of independent parallel gains (10). Parallel intron gain appears to be a phenomenon that has been highly underestimated in ILE-containing fungi, and likely in the whole fungal kingdom.

The high repeat content is another peculiarity of the *C. fulvum* genome. It might cause genome instability, but may also cause high flexibility, allowing quick adaptation to tomato cultivars carrying new *Cf* genes. Indeed, all effector genes of *C. fulvum* identified so far are located in repeat-rich regions with many transposable elements, which might cause their birth and death (22). In total, five different types of mutations were observed in *C. fulvum* effectors that led to avoidance of recognition by different matching Cf proteins (48, 64, 66, 91, 110): (*a*) mutation in an effector

gene causing a stop codon leading to a truncated effector protein (*Avr2*), (*b*) mutations in an effector gene leading to the production of an effector protein with one or a few different amino acids (*Avr4* and *Avr4E*), (*c*) mutations in an effector gene leading to the production of an unstable effector protein (*Avr4*), (*d*) loss of an effector gene from the genome leading to absence of effector protein production (*Avr2*, *Avr5*, *Avr4E*, and *Avr9*), or (*e*) insertion of a transposon in an effector gene preventing production of a functional effector protein (*Avr2*).

Cf Immune Receptors and Their Interaction with Avr Proteins

Along with the cloning of the *Cf-9* gene by transposon tagging, as discussed above (45), the lab of Jonathan Jones also cloned the *Cf-2*, *Cf-4*, and *Cf-5* genes (31, 32, 87). The *Cf-4E* gene was cloned at the University of Amsterdam by Frank Takken (86). All *Cf* genes encode receptor-like proteins known as receptor-like proteins (RLPs). They are integral plant plasma membrane proteins (74), containing an extracellular leucine-rich repeat (LRR), a membrane spanning domain, and a short cytoplasmic tail without signaling capacity (**Figure 4**).

As discussed earlier in our lab, several PhD students have tried to prove direct interaction between Avr and Cf proteins in vitro by using both radiolabeled and biotinylated Avrs, as well as Cf proteins produced in different heterologous expression systems, but without success (52, 63). Only indirect interaction between Avr2 and Cf-2, via Rcr3, has been demonstrated, as mentioned above (77, 107). Perhaps with the exception of Avr4, as we assume that this chitin-binding protein has no virulence target in the host, we expect that most effectors will be indirectly recognized by Cf immune receptors via their host targets according to the guard hypothesis (97). Cf immune receptors lack a domain, such as receptor-like kinases (RLKs), required for downstream defense signaling and therefore are hypothesized to require engagement with RLK coreceptors to activate defense responses. Our lab and that of Jonathan Jones have tried for many years to find proteins

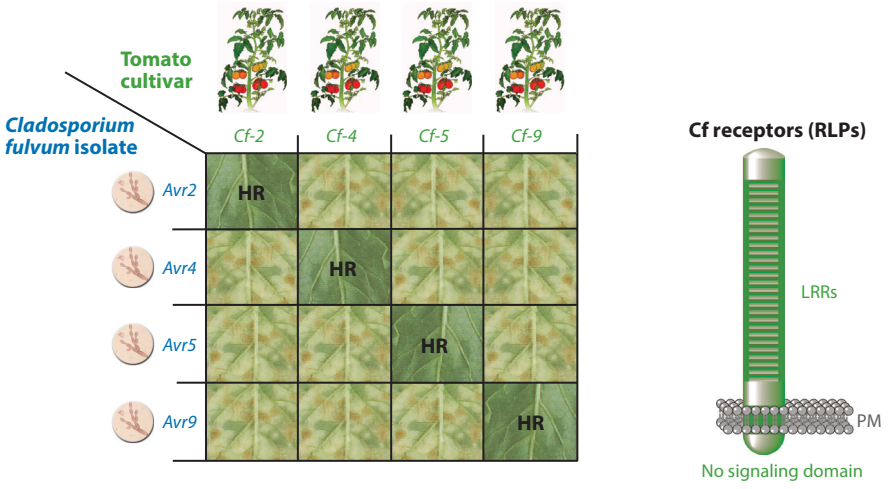

Figure 4

The different Cf immune receptors are LRR-membrane receptor-like proteins without a signaling domain that are involved in recognition of matching *Cladosporium fulvum* Avr proteins. Recognition of matching *C. fulvum* Avr proteins leads to Cf-mediated HR responses and resistance to races of the fungus carrying the matching *Avr* gene. Abbreviations: HR, hypersensitive response; LRRs, leucine-rich repeats; PM, plasma membrane; RLPs, receptor-like proteins.

interacting with Cf immune receptors making use of different yeast two-hybrid screens. Interacting proteins were identified, but they did not appear to be crucial, as silencing of their encoding genes often only weakly compromised HR and resistance (68, 76, 94). Only recently, PhD student Thomas Liebrand in the group of Matthieu Joosten managed to identify a crucial RLK interacting with Cf immune receptors, using a proteomics fishing approach. The RLK, SOBIR1, was isolated from tomato by immunopurification of a functional Cf-4-enhanced green fluorescent protein (eGFP) fusion protein. SOBIR1 appears to be crucial for activity and downstream signaling of different Cf immune receptors and related RLPs, as silencing compromised HR and resistance (61). This was a breakthrough, and now several other labs working on RLPs have confirmed the requirement of SOBIR1 for RLP function. SOBIR1 stabilizes Cf immune receptors and other RLPs and is required for the initiation of downstream signaling through its kinase domain. Very recently, it was discovered that Avr4 promotes the association of the Cf-4 immune receptor, in complex with SOBIR1, with the RLK BAK1/SERK3 to initiate receptor endocytosis and plant resistance (74). Thus, Cf immune receptors are part of a complex that can be activated by fungal effectors and that function very similarly to the well-known cell surface receptor FLS2, which perceives bacterial flagellin.

Avr-Triggered Cf-Mediated Downstream Defense Signaling

Many plant immune receptors and matching effectors have now been cloned and many research groups are studying receptor-mediated downstream defense signaling pathways. I do not go into detail but elaborate only on the efficiency of the plant defense system against so many different pathogens. At the Laboratory of Phytopathology, this is now the focus of Matthieu Joosten's research group. In the past, PhD students Camiel de Jong, Suzan Gabriëls, Iris Stulemeijer, Ahmed Abdel-Haliem, and Desalegn Etalo (PhD student at the Laboratory of Plant Physiology) have all worked on this research topic (15, 37, 39, 84, 109). Joosten's group has identified many Cf-4-activated defense signaling components triggered by the Avr4 effector, making use of transcriptome, proteome, and metabolome analyses. For this purpose, they frequently used the so-called dying seedlings, which are the offspring of a parent that expresses the Cf-4 immune receptor and a parent that expresses the matching Avr4 protein, similar to the Avr9-Cf-9 seedlings exploited by the lab of Jonathan Jones to clone the *Cf-9* gene (45). The seedlings look healthy at 33° C but undergo a systemic HR when the temperature is lowered to 25° C. In this way, changes in the host physiology and biochemistry during initiation of the HR can be studied in a synchronous manner. An interesting nucleotide-binding site (NBS)-LRR protein required for HR signaling mediated by both extra- and intracellular resistance proteins was picked up in this system by PhD student Suzan Gabriëls and was studied in more detail by PhD student Daniela Sueldo (39, 85).

If one considers the huge difference in generation time between a fungal pathogen and its host plant, the plant must employ sophisticated defense mechanisms to defend itself against many fungal pathogens that can quickly adapt to changing environments. The generation time of a fungus is a few days and that of an annual plant is 3–6 months. Taking into account the rate at which pathogens develop mutations in effectors and develop new effectors, one wonders why plants still exist. They must have an efficient, versatile and flexible defense system. It has been reported that some plant immune receptors can work together in receptor complexes active against more than one pathogen. Recently, our colleagues at the Laboratory of Nematology of Wageningen University showed that the Cf-2 immune receptor is also effective against the cyst nematode *Globodera rostochiensis* (62). Like the Avr2 effector of *C. fulvum*, the Gr-VAP1 effector of *G. rostochiensis* inhibits cysteine protease Rcr3, triggering Cf-2-mediated resistance against this nematode. Obviously, Rcr3 seems

to be an important plant defense enzyme to be targeted and inhibited by effectors secreted by different pathogens and pests.

How can plants develop so many highly specific immune receptors? It has been shown that plant immune receptor genes occur in clusters, allowing them to generate new specificities by inter- and intralocus recombination between homologs (54, 100, 113). This is somewhat reminiscent of the mechanisms by which millions of different antibodies are generated in animals by rearranging germ-line DNA segments encoding the variable light and heavy chains (1). PhD students Marco Kruijt and Renier van der Hoorn discovered recombination between homologs of the *Cf-9* receptor genes in wild *Solanum* species in collaboration with the lab of Jonathan Jones (54, 113). Wild tomato species contain numerous homologous *RLP* immune receptor genes, of which only a few have been functionally characterized and shown to function in resistance against different pathogens. Similar RLP immune receptors occur in other plant species and are active against different mainly extracellular pathogens.

Apart from one immune receptor recognizing different effectors, effector-triggered receptor-mediated downstream defense responses overlap and are effective against a broad spectrum of pathogens. These responses include the generation of toxic reactive oxygen species, antimicrobial phytoalexins, and PR proteins, such as chitinases, glucanases, and proteases, often culminating in an HR. Work on PR proteins has remained a recurring object of study in my research group. The cloning of their encoding genes was initiated by Jan van Kan and postdoc Nadia Danhash (13, 105), and PhD student Jos Wubben has worked on their in planta localization (112). Very recently, PhD student Mansoor Karimi Jashni discovered that *Fusarium oxysporum* f. sp. *lycopersici* protects itself against particular plant chitinases by secreting a metallo- and serine protease that by concerted action remove the chitin-binding domain of these enzymes and in this way reduce their antifungal activity (43, 44).

A UNIFYING CONCEPT FOR THE ARMS RACE BETWEEN PATHOGENS AND THEIR HOST PLANTS

One of the achievements in molecular plants pathology of the past two decades is the generation of a unifying concept for pathogen virulence and host resistance (33). The nonspecific elicitors identified in the 1970s and 1980s we now call pathogen-associated molecular patterns (PAMPs). They are mostly invariable compounds present in all microbes and pathogens. Their recognition by pattern recognition receptors (PRRs) of the host leads to PAMP-triggered immunity (PTI), providing basal resistance to all microbes. The first PAMPs described were flagellin from bacteria and chitin from fungi. In order to become successful pathogens, microbes must secrete effectors that suppress PTI. Indeed, numerous effectors secreted by pathogenic fungi have been reported in recent years to suppress PTI, leading to effector-triggered susceptibility (ETS). Through co-evolution, the plant has developed immune receptors and resistance proteins that recognize these effectors, leading to effector-triggered immunity (ETI), a strong type of defense often culminating in an HR. This forms the starting point of the arms race between pathogens and host plants, nicely described as the zig-zag model by Jones & Dangl (47). However, as with most models they form a good basis for discussion but only describe part of the story. As Thomma et al. (88) pointed out, some effectors are not species specific and display a wide distribution among several pathogens, whereas some PAMPs are less conserved or contribute to pathogen virulence. Therefore, the distinctions between PAMPs and effectors, between PRRs and resistance proteins, and between PTI and ETI, are not strict and greatly overlap. The *C. fulvum*–secreted effectors Avr2, Avr4, and Ecp6 all interfere with basal defense in the apoplast in the absence of their matching Cf proteins. Avr2 is species specific, Avr4 also occurs in several related fungal species, and Ecp6

has a wide distribution in many unrelated fungal species. Effectors from other fungal pathogens are translocated into host cells and exhibit their function in the cytoplasm or nucleus. These effectors are recognized by cytoplasmic NBS-LRR immune receptors. As *R* gene–mediated host resistance against apoplastic fungal pathogens was not adequately explained by PTI and ETI, we and others proposed effector-triggered defense (ETD) as an alternative (82). ETD is mediated by *R* genes encoding cell surface–localized RLPs that engage the receptor-like kinase SOBIR1 and, more recently, were shown to also recruit BAK1/SERK3 (74). In contrast to extracellular recognition, ETI is mostly initiated by intracellular detection of pathogen effectors and is usually associated with a fast HR, whereas ETD often triggers host cell death only after an elapsed period of extracellular pathogen growth.

A model describing the arms race between *C. fulvum* and tomato was presented during my farewell address (21) in 2014 (**Figure 5**). It provides a simplified evolutionary scenario that could have occurred during coevolution between *C. fulvum* and wild tomato species. Evolution operates gradually and not in large steps, but, for simplicity, I assume here that it does. In **Figure 5*a***, *C. fulvum* is depicted dwelling in the apoplast of tomato. It is not yet a pathogen, as tomato recognizes its PAMPs, the chitin fragments released from its cell wall, which induce PTI after recognition by plant chitin receptors. The PTI response is not strong, but it is strong enough to keep *C. fulvum* under control. In a continuing evolutionary process, *C. fulvum* developed new weaponry that helped it to grow further. It secreted the Ecp6 effector that scavenges chitin fragments, preventing them from inducing PTI (17, 78). This induces a weak type of ETS, leading to weak disease symptoms. *C. fulvum* colonizes tomato to some extent (**Figure 5*b***). In order to become more virulent, *C. fulvum* produces additional effectors such as Avr2 and Avr4, leading to more significant disease symptoms (92, 103, 104) (**Figure 5*c***). However, in the arms race, tomato fights back by sequentially developing the Cf-Ecp6, Cf-2, and Cf-4 immune receptors linked to coreceptor SOBIR1 (**Figure 5*d***). In this model, development of the Cf-Ecp6 immune receptor is supposed to be the first defense weapon developed by tomato but many more followed, and as many as 100 effectors might be employed by *C. fulvum*, and a similar number of Cf immune receptors may have developed in wild tomato. Eventually, the arms race between *C. fulvum* and tomato, making use of 100 effectors and 100 corresponding Cf immune receptors, becomes true trench warfare, as was also proposed by Renier van der Hoorn (99). We might already have identified the strongest effectors, but there are many more to be discovered with smaller effects. It would take a plant pathologist several careers to discover and functionally analyze all these effectors and their matching immune receptors.

APPLYING MOLECULAR RESEARCH IN DISEASE RESISTANCE BREEDING

Modern crop plants should be equipped with multiple immune receptors against a particular pathogen. With a mutation rate of one in one million nucleotides per *Avr* gene per generation, the chance of overcoming, for example, five *R* genes by five *Avr* genes is estimated to be 10^{21} times smaller than overcoming one *R* gene by one *Avr* gene. Pyramiding of different *R* genes in one cultivar or using multilines each containing different *R* genes in time and space in a crop is predicted to be durable (5, 111). However, even when we have developed tools and methods to effectively prevent plant diseases, this does not always lead to their implementation in practice. Application depends on the attitude of breeders, growers, and consumers. I can illustrate this with two examples.

The first example comes from our own *C. fulvum* research. The five resistance genes *Cf-2*, *Cf-4*, *Cf-4E*, *Cf-5*, and *Cf-9* are very effective against *C. fulvum*. Before the 1970s, only one or two *Cf*

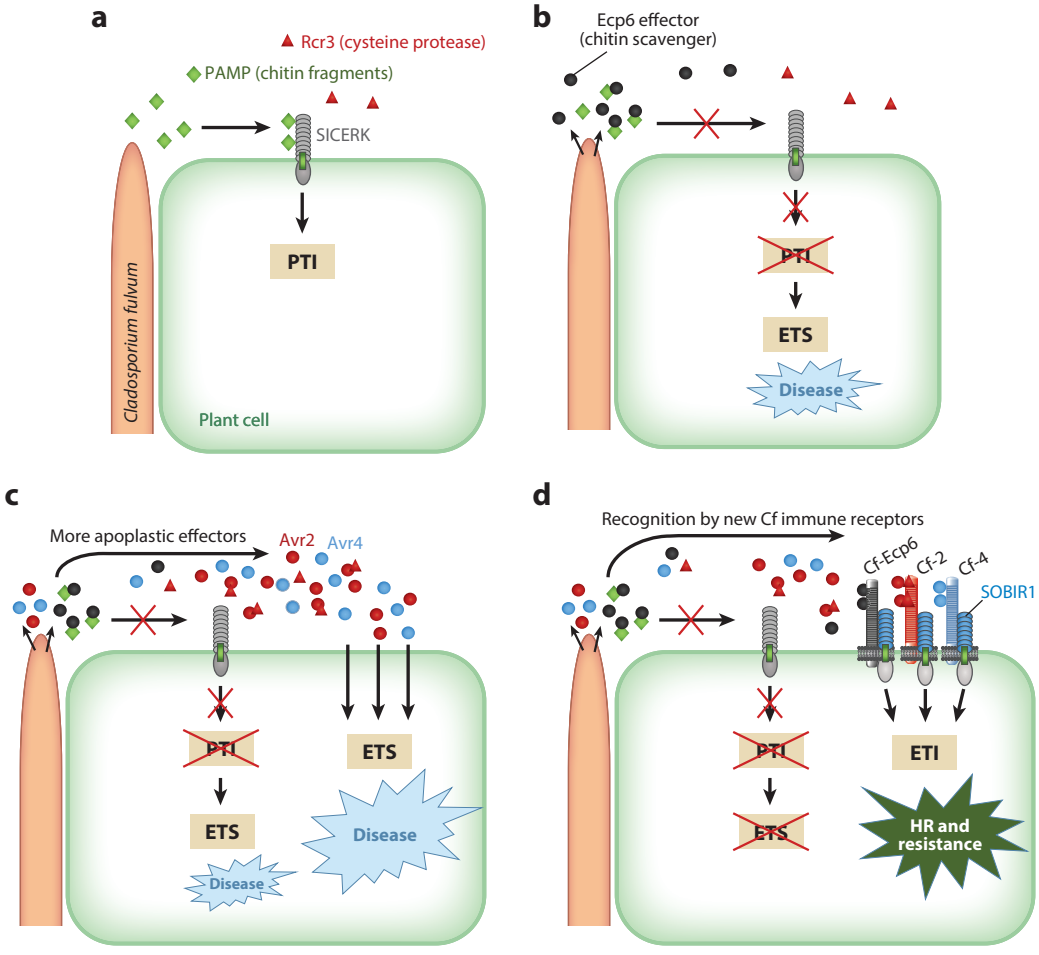

Figure 5

An evolutionary scenario of the arms race between *Cladosporium fulvum* and tomato. (*a*) Chitin fragments from *C. fulvum* are recognized by chitin receptor–inducing PAMP (pathogen-associated molecular pattern)-triggered immunity (PTI). (*b*) *C. fulvum* secretes the Ecp6 effector that scavenges chitin fragments to prevent PTI, leading to a weak form of effector-triggered susceptibility (ETS). (*c*) *C. fulvum* secretes additional effectors (Avr2) targeting apoplastic host targets (cysteine protease Rcr3) and Avr4 (protecting against plant chitinases) to increase virulence. (*d*) Tomato develops new Cf immune receptors to recognize new effectors to induce effector-triggered immunity (ETI). Adapted from Reference 21. Abbreviation: HR, hypersensitive response.

genes were present in commercial tomato cultivars. This led to frequent outbreaks by new races. Since then, most commercial tomato cultivars contain three or more *Cf* genes, a development that has prevented new disease outbreaks for several decades. However, after 2010, new *C. fulvum* outbreaks were reported in greenhouses of organic tomato growers in The Netherlands and neighboring countries. The virulence spectrum of the races was diagnosed, and mainly races that could only overcome the *Cf-2* or *Cf-9* gene were detected. The cultivars did not contain the *Cf-4* and *Cf-5* genes. Why? The *Cf-9* gene was the last *Cf* gene introduced by breeders and gave good protection against all races identified at that time. Most breeders considered this *Cf-9* gene so powerful that it was the only gene introduced in cultivars cultured by organic growers, explaining

why they became heavily infected by races with a simple virulence spectrum. This example shows that breeders and growers always need to be alert, as outbreaks of *C. fulvum* develop quickly when tomato cultivars are not equipped with a sufficient number of different *Cf* genes.

The second example refers to the introduction of genetically modified (GM) disease-resistant plants. Nobody wants to consume food that is contaminated with pesticides. Growing disease-resistant cultivars is an excellent alternative. Decreasing the use of agrochemicals was my biggest motivation to study plant pathology. In some cases, this might require using GM plants when traditional resistance breeding is not possible. GM plants meet much opposition in our society, despite objective information about their safety. With all the knowledge generated in the past decade, we can now generate new resistant genotypes with multiple *R* genes that do not need chemicals to protect them against pathogens. Hopefully, GM disease-resistant plants will eventually become accepted by the public and can be cultured to secure our crops (6, 114).

REFLECTIONS AND A GLIMPSE INTO THE FUTURE

My career has been full of surprises. A personal characteristic that helped me to succeed was to never give up. It is a characteristic that is a prerequisite to be successful in sports. From every defeat one learns by practicing or trying something different. I always enjoyed sports and I still love them, but I am happy that I did not need to earn my living from sports. Yet, it gave me endurance and new inspiration, whether it was playing tennis, golf, or running. I recall playing tennis with my PhD students and postdocs and discussing scientific bottlenecks during the breaks. I am also very fortunate that my PhD supervisor, Johan Dekker, gave me sufficient time to develop a model system. Had I been given only four years to finish my PhD, I would have failed. I am also fortunate to have been surrounded by so many excellent PhD students and postdocs, who all have contributed so much to the research.

Since the discovery of the first fungal *Avr* gene in 1991, numerous *Avr* genes have been isolated from other fungi (80). Extracellular pathogens appear to secrete small, cysteine-rich proteins in the apoplast of their host oomycetes, and other fungal pathogens, such as rusts and mycorrhizal fungi, contain large gene catalogs encoding this type of effectors. Many scientists have now focused their research on effector biology, including some of my own colleagues (Francine Govers working on *Phytophthora infestans*, Bart Thomma on *Verticillium* sp., Jan van Kan on *Botrytis cinerea*, and Matthieu Joosten on effector-triggered Cf-mediated defense signaling pathways). The biggest challenge is to identify the functions of so many effector genes. Knocking out multiple genes at a time can be achieved only for a limited number, as only a few selection markers are available. In some fungi, silencing multiple genes by RNA interference (RNAi) is possible, but one must be aware of the off-target silencing of homologous genes. Analysis of transcription factor–interacting libraries and other protein-protein–interacting libraries of diverse organisms have shown that most proteins participate in complexes and different networks; removing one or more may produce pleiotropic effects. We should realize that what has developed at random during millions of years of evolution cannot be simply reconstituted and understood by knocking out or silencing subsets of genes. Thus, functional analysis of (effector) genes remains a bottleneck in genome research, but it must be done otherwise comparative genomics will not bring us new insights.

Also, homologous genes in different species have often diversified in different directions. A good example is the *C. fulvum* transcription factor CfWor1. This protein is a homolog of FoSge1 in *F. oxysporum* f. sp. *lycopersici*, where it regulates expression of its effector genes; however, this is not the case for CfWor1 in *C. fulvum*, where it acts more as a global transcription factor (69).

Phylogenetically closely related fungal species have often adapted to completely different niches or host plants. New outbreaks of fungal plant diseases might be caused by growing high yielding

crop plants outside of their gene centers. Fungi become pathogenic on new hosts by adaptation, which might require involvement of only a few genes. Crop plants might also have lost important immune receptors by outbreeding to increase yield, which might have made them more vulnerable to potential pathogens. Maintenance of basal defense mechanisms in plants requires energy that cannot be spent on increasing yield. Thus, some fungi may become aggressive under changing environmental conditions, including global warming. They may also be part of a disease complex consisting of different species. Changing environmental conditions might distort the balance in the complex, leading to new disease outbreaks. This might have caused the outbreak of, for example, the black Sigatoka disease of banana caused by *M. fijiensis*, which is part of the Sigatoka disease complex that also includes *Mycosphaerella musicola* and *Mycosphaerella eumusae*.

DISCLOSURE STATEMENT

The author is not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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