Cf-Dependent Early Defense Responses Induced by Avirulence Proteins of the Tomato Pathogen *Cladosporium fulvum*

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Stellingen

1. De sterke afname van het aantal AVR9 bindingsplaatsen bij hoge temperatuur vormt de moleculaire basis van de temperatuurgevoeligheid van *Cf-9/Avr9*-afhankelijke afweerreacties.
Dit proefschrift.

2. De specifiek door AVR4 geïnduceerde accumulatie van fosfatidylzuur in *Cf-4*$^+$-tabakscellen wordt in hoofdzaak gegenereerd via activering van fosfolipase C.
Dit proefschrift.

3. Om een representatief beeld te krijgen van de transcriptionele herprogrammering tijdens de overgevoeligheidsreactie, hadden Durrant en medewerkers tijdens hun differentiële cDNA-AFLP analyse op *Cf-9*$^+$-tabakscellen gebruik moeten maken van een AVR9 concentratie die celdood induceert.
Durrant, W. E., Rowland, O., Piedras, P., Hammond-Kosack, K. E., and Jones, J. D. G. (2000). cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell **12**, 963-977, *Dit proefschrift.*

4. Het is beter om te spreken van signaaltransductie schakelingen dan van signaaltransductie routes.
Genoud, T., Trevino Santa Cruz, M.B., and Métraux, J.P. (2001). Numeric simulation of plant signaling networks. Plant Physiol. **126**, 1430-1437.

5. Een succesvolle verdediging door de plant tijdens een aanval van een pathogeen wordt niet alleen bepaald door het tijdig onderkennen van de aanwezigheid van een pathogeen maar ook door het kiezen van een passende afweerreactie.
Govrin, E.M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea.* Curr. Biol. **10**, 751-757.

6. Aangezien er nog steeds meer urgente wetenschappelijke vraagstellingen binnen de huidige drie dimensies zijn dan er geld is om ze op te lossen, is het voorbarig om 7 miljard dollar uit te geven aan een lineaire superversneller om daarmee een extra dimensie aan te tonen.
Amerikanen willen weer superversneller, De Volkskrant, 2 februari 2002.

7. Een goed wetenschapper verwart het onwaarschijnlijke niet met het onmogelijke. Een goed wetenschappelijk beleid maakt het niet onmogelijk het onwaarschijnlijke te kunnen achterhalen.

8. De wetgeving en beschermingsmaatregelen voortvloeiend uit de "Convention of International Trade in Endangered Species" (CITES) is gebaseerd op dieren en houdt geen rekening met de afwijkende voortplantingsbiologie van planten. Hierdoor bedreigen deze maatregelen het duurzaam vermeerderen en veredelen van bedreigde plantensoorten.
Hansen, E. (2000). *Orchideeënkoorts; Uitgeverij BZZTôH, 's-Gravenhage, ISBN 90-5501-755-8* en bronnen daarin.

Stellingen behorende bij het proefschrift:
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door Camiel F. de Jong, Wageningen, 19 juni 2002

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Proefschrift
ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op woensdag 19 juni 2002
des namiddags te half twee in de Aula

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Voor mijn ouders

List of Abbreviations

Avr	avirulence gene
AVR	avirulence protein
CDPK	calcium-dependent protein kinase
Cf	*Cladosporium fulvum* resistance gene
Cf	the protein encoded by a *Cladosporium fulvum* resistance gene
DAG	diacylglycerol
DGK	diacylglycerol kinase
DGPP	diacylglycerol pyrophosphate
DPI	diphenyleneiodonium chloride
EtAc	ethylacetate
HABS	high affinity binding site
HR	hypersensitive response
IP_3	inositol 1,4,5-trisphosphate
K_d	binding constant
MAPK	mitogen activated protein kinase
PA	phosphatidic acid
PAK	phosphatidic acid kinase
PBut	phosphatidylbutanol
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PIP	phosphatidylinositolphosphate
PIP_2	phosphatidylinositolbisphosphate
PLA	phospholipase A
PLC	phospholipase C
PLD	phospholipase D
PVX	potato virus X
R	resistance gene
ROS	reactive oxygen species
TLC	thin layer chromatography
VIGS	virus-induced gene silencing

Contents

Chapter 1

General Introduction and Outline of the Thesis

Introduction to Phytopathology

Plants capture the sun's electromagnetic energy and transform it into chemical energy by means of photosynthesis. During photosynthesis carbon dioxide and water are converted into sugars. The sugars may be stored or used in combination with various minerals to synthesize amino acids, vitamins and other building blocks of life. In this way plants sustain, directly or indirectly, all heterotrophic forms of life on earth, including humans. In pre-historic times humans hunted and collected plants to support a living. The first traces of agriculture were found in the Middle East and date from 6000 BC when humans started to cultivate barley and wheat together with domestication of animals. As compared to hunting and collecting, agriculture was a more reliable way to secure food supplies. However, very soon afterwards our ancestors must have discovered that they were not the only guests at the table: the carefully planted crops were eaten by all kinds of animals and contracted all kinds of diseases too.

The pathogens causing plant diseases do not only reduce the yield but can also poison the remainder of the harvest due to the production of mycotoxins such as ergot alkaloids (ergotism) and aflatoxin which may cause cancer of the liver. Loss of complete harvests frequently resulted in famines that were seen as a punishment sent by a higher power as a result of human ill behavior. However, the real cause of plant diseases remained obscure. During the ages this view changed little until the light microscope was invented by Anthonie van Leeuwenhoek, and people became aware of the microscopic world surrounding them. The occurrence of microorganisms was explained by the theory of spontaneous generation. The structures found on diseased plants were thought to be the result, rather than the cause of the plant disease. During the 1840s a severe epidemic struck the potato crop in Northern Europe. It caused great losses in this area but especially in Ireland where the disease caused the loss of the total potato crop of the years 1845 and 1846. The ensuing famine resulted in the death of hundred thousands of people and a massive emigration of the Irish population to America (Litton, 1994). The German Von Martius was one of the first to assign the cause of the disease to a "fungus". It was until 1861 that another German, deBary, proved irrevocably that the disease was caused by a microorganism, *Phytophthora infestans* (Agrios, 1997). As so many great discoveries in science, this discovery resulted from the condensation of new ideas and observations made during that time. Notably, it was also in this period that the Frenchman Pasteur demonstrated that fermentation is not a purely chemical process but is mediated by microorganisms and that these organisms do not spontaneously generate but are regenerated and multiply from already existing microorganisms. However, it took years before this notion was widely accepted. Meanwhile, techniques to isolate and grow plant pathogens in the laboratory, were greatly improved by scientists like Brefeld, Petri and Koch (Agrios, 1997). The latter formulated four postulates to which a microorganism must comply to be classified as the cause of a plant disease. It was during this period that the science of phytopathology began to take shape and the first book about this subject, "*Diseases of*

Cultivated Crops, Their Causes and Their Control", was written by Kuhn (1858). At first, the science of phytopathology was largely descriptive and increasing numbers of microorganisms causing plant disease were isolated and identified. Using this information, the development of a disease and environmental factors favoring its development were described and cultural measures could be taken to limit the damage caused by plant diseases. It also provided the tools to study epidemics of plant diseases and find ways to prevent spreading of diseases over other geographical areas. To prevent the spreading of the San José aphid, some states of the USA started to demand a certificate stating that plants or plant parts that were imported in these states were free from that pest. In that period the export of horticultural products from the Netherlands to the USA began to increase, forming the direct motive to establish the 'Phytopathologischen Dienst' (PD) (Plant Protection Service) in the Netherlands by professor Ritzema-Bos in 1899 (Van der Waal et al., 1999).

In the late 1870s, the downy mildew disease of grape caused by the pathogen *Plasmopara viticola* spread through Europe destroying most of the wine crop in its wake (Agrios, 1997). This inconvenience elicited a search for chemicals that could control this disease, especially by the wine loving French. Therefore, it was not surprising that in 1885 the Frenchman Millardet discovered that a mixture of copper sulfate and hydrated lime could effectively control downy mildew of grape. The mixture that became known as Bordeaux mixture, was one of the first fungicides and proved to be successful to control numerous other fungal plant diseases. As chemistry evolved as well, increasing numbers of pesticides were developed. However, the toxicity to the environment and humans was not much of a concern until the book Silent Spring by Rachel Carson was published in 1962. In this book several cases of pollution of the environment by toxic chemicals resulting in death of birds, fish and men were described. Initially, the book was received skeptically but more and more evidence was accumulating about the deleterious effect of pesticides on the environment. This resulted in stricter regulation of the use of agro chemicals. Consequently, methods were developed to reduce the use of pesticides. These included optimizing cultural practices such as crop rotation, removal of plant debris, appropriate fertilization etc. Computer programs using meteorological data were developed that could predict an outbreak of a disease. This allowed farmers to use chemical control when really necessary rather then continuous preventive spraying during the entire growing season (Vanderplank, 1963; Zadoks, 1984). Another approach involves biological control, making use of antagonistic organisms. This technique proved to be especially useful in the control of insect pests but also some promising results are obtained with the use of microorganisms against fungal pathogens (Agrios, 1997).

One very effective and environment-friendly way to control plant disease is the breeding of resistant plant varieties. For this purpose large collections of germplasm of plant species related to the cultivar are collected in the wild and stored in seed banks. Plants are raised from these seeds and tested for resistance to specific pathogens. The resistant wild plants are selected and crossed with the high yielding susceptible crop species. To

preserve the desirable characteristics (yield, fruit size and shape etc.) of the susceptible cultivar several back crosses are performed. The resistant trait of the wild plant is conserved in the backcrosses by selection of resistant individuals finally resulting in a resistant variety very akin to the original susceptible variety. A drawback of this approach is that not only the resistance gene of the wild plant is introduced but often also a great number of other genes that are not desired and may be even disadvantageous. A notorious example is the Dutch potato cultivar Bintje. It is extremely susceptible to potato late blight and requires such large amounts of pesticides to protect it against late blight that it earned the nick name "poison potato". However, the Bintje tubers have the best properties to produce French fries and chips. Unfortunately, efforts to introduce resistance genes from wild potato varieties led to a loss of these desirable characteristics as the crossings disturbed the unique gene combination controlling the characteristics of the Bintje tuber. In the future these drawbacks of traditional plant breeding may be avoided by techniques currently developed in the field of molecular breeding such as marker-assisted breeding.

Plant-Pathogen Interactions

As already outlined in the previous section, plants are constantly challenged by microorganisms. However, most plants stay healthy because the microorganism lacks the "tools" to become pathogenic. This lack of compatibility between the plant and the microorganism is referred to as non-host resistance (Agrios, 1997). However, some microorganisms have the tools to attack a certain range of plant species and become pathogens. Although a pathogen may have all the necessary tools to attack its host, in a natural plant population there will always be some genotypes that are able to recognize and check further development of particular strains of the pathogen. This type of resistance is known as genotype-specific resistance. A hallmark of genotype-specific resistance is the occurrence of a hypersensitive response (HR), involving a local collapse of plant cells at the site of primary infection. Detailed genetic analysis on the *Ustilago tritici*-wheat interaction (Oort, 1944) and the *Melampsora lini*-flax interaction (Flor, 1942; Flor, 1946) revealed that genotype-specific resistance is controlled by a monogenic, dominant trait both in the host plant and the attacking pathogen. The monogenic dominant trait of the plant confers resistance against a specific strain of the pathogen and is designated "resistance gene" (*R* gene), whereas the pathogen counterpart confers avirulence to the specific strain of the pathogen and is referred to as "avirulence gene" (*Avr* gene). This type of interaction follows the so-called gene-for-gene concept.

R Versus *Avr* Genes

In the last decade numerous resistance genes conferring resistance against viruses and organisms such as bacteria, fungi, nematodes and aphids have been cloned. Although these genes confer resistance to a variety of different pathogens, the architecture of their products is remarkably similar (Hammond-Kosack and Jones, 1997). In general, the predicted

proteins contain a leucine-rich repeat (LRR) domain, Pto being a notable exception. The LRR domain is thought to play a role in protein-protein interactions (Kobe and Deisenhofer, 1995). The *R* genes may be classified according to whether the LRR domain of the encoded protein is located in- or outside the cytoplasm. All the *R* gene-encoded proteins with a cytoplasmic LRR domain also contain a nucleotide-binding site (NBS). These proteins can be further classified according to the absence or presence of an additional domain that may be a leucine zipper (LZ) or a Toll/interleukin 1 receptor-like (TIR) domain. The R proteins with an extracellular LRR domain have a membrane-spanning region and can be divided in proteins with or without a cytoplasmic kinase domain. The different structural domains are thought to play a role in signaling. The class of R proteins with extracellular LRRs, but without a cytoplasmic kinase domain, has only a very small cytoplasmic domain without any structural features that may be involved in signaling. To perform this function, additional protein(s) may be required.

In contrast to the structural similarity of *R* gene-encoded proteins, the proteins encoded by *Avr* genes are very diverse and no proper classification can be made according to common functional domains (Laugé and De Wit, 1998; Van 't Slot and Knogge, 2002). To name but two examples, the fully processed AVR9 protein from the tomato pathogen *Cladosporium fulvum* is a small peptide of only 28 amino acid residues (Van den Ackerveken et al., 1993). The peptide is knitted together by 3 cysteine bridges forming a cysteine knot, yielding an extremely stable structure exhibiting some structural homology to carboxy peptidase inhibitors (Vervoort et al., 1997). On the other hand, the fully processed AVR-Pita from the rice pathogen *Magnaporthe grisea* consists of a large 176 amino acid protein having homology to fungal Zn^{2+} proteases (Jia et al., 2000).

The elegant simplicity of the gene-for-gene concept suggests an equally simple biochemical concept that is formulated in the elicitor-receptor model (Gabriel and Rolfe, 1990; Keen, 1990). In this model it is proposed that the *Avr* gene encodes a ligand that binds to a receptor encoded by the *R* gene. The genetics and predicted structural features of most of the R proteins tend to confirm this theory. However, so far only in two cases a direct interaction between an R protein and the corresponding AVR protein has been reported. The first case involves the interaction between Pto, which is a kinase without a LRR domain, and AvrPto (Jia et al., 2000). The second case is the interaction between AVR-Pita described above and the resistance gene product from *Pi-ta*, a predicted cytoplamic protein with a NBS and a leucine-rich carboxyl terminus (Bryan et al., 2000; Jia et al., 2000). In the other cases it is thought that additional components are required to form a functional receptor complex that can harbor AVR and R protein and has to be formed before downstream signal transduction pathways can be triggered (Joosten and De Wit, 1999).

Elicitor-Induced Defense Responses

It is often difficult or even impossible to study elicitor-induced defense responses in intact plants. Consequently, studies on defense responses are often performed with cell suspensions. Cell suspensions consist of relatively undifferentiated cells grown in a liquid nutrient medium. Elicitors are added to cell suspensions and the induced defense responses are recorded.

Upon elicitor perception by plant cells numerous biochemical processes are activated (Felix et al., 1991b). Pioneering work has shown that upon addition of elicitors to tomato cells several defense responses are induced. These include alkalization of the extra-cellular medium and alterations of phosphorylation status of proteins, induction of ethylene biosynthesis and phenylalanine ammonia-lyase activity. These responses could be inhibited by protein kinase inhibitors such as K-252a and staurosporine and mimicked by the phosphatase inhibitor calyculin A (Felix et al., 1991a; Felix et al., 1991b; Felix et al., 1993; Felix et al., 1994; Grosskopf et al., 1990). Specific defense responses could also be induced upon elicitation of parsley (*Petroselinum crispum*) with crude elicitor protein preparations isolated from the mycelium of *Phytophthora sojae*, with a purified 42-kDA glycoprotein from the same pathogen or with a 13-amino acid oligopeptide fragment (Pep-13) derived from this glycoprotein. Elicitor treatment resulted in production of reactive oxygen species (ROS), influx of Ca^{2+} and H^+, efflux of K^+ and Cl^-, PR gene induction, ethylene and phytoalexin production (Dangl et al., 1987; Jabs et al., 1997; Nürnberger et al., 1994; Parker et al., 1991; Somssich et al., 1989). Exclusion of Ca^{2+} from the medium or treatment with Ca^{2+} ion channel blockers could inhibit these elicitor-induced defense responses. Beside ion fluxes over the plasma membrane, O_2^- but not H_2O_2 was essential for phytoalexin production in this system.

Elicitor treatment of tobacco cells resulted in activation of a Mitogen Activated Protein Kinase (MAPK) as demonstrated by an *in gel* kinase assay (Suzuki and Shinshi, 1995). MAPK are serine/threonine protein kinases that are part of signaling cascades. Beside MAPKs, these cascades consist of MAPK kinases (MKKs) and MAPKK kinases (MKKKs) that amplify incoming signaling events and allow branching or crosstalk between signaling pathways.

Changes in phospholipid metabolism and increases in inositol 1,4,5-triphosphate (IP_3) upon elicitor treatment of cultured plant cells have also been reported (Legendre et al., 1993; Walton et al., 1993). IP_3 and diacylglycerol (DAG) are generated via hydrolysis of phosphoinositol 4,5 bisphosphate (PIP_2) by phospholipase C. Treatment with the PLC inhibitor neomycin blocked the elicitor-induced oxidative burst in soybean cells indicating a role for phospholipid signaling in plant defense (Legendre et al., 1993).

Infiltration of tomato cotyledons with apoplastic fluid containing race-specific elicitors from *Cladosporium fulvum* resulted in the production of ethylene and salicylic acid (Hammond-Kosack et al., 1996). In an incompatible interaction between the plant and the pathogen, the above described responses lead to changes in gene expression and subsequent

production of anti-microbial proteins, phytoalexins and reinforcement of the cell wall of cells adjacent to the site of primary infection (Dixon et al., 1994). Changes in gene expression also orchestrate the final execution of the HR resulting in death of the cells at the site of primary infection.

The *Cladosporium fulvum*-Tomato Interaction

The fungus *Cladosporium fulvum* (syn. *Fulvia fulva*) is the causal agent of leaf mold of tomato (*Lycopersicon* ssp) and has a biotrophic lifestyle. During humid conditions in the greenhouse, conidia of *C. fulvum* germinate on the leaf surface and the produced runner hyphae enter the leaf via stomata. In a compatible interaction the fungus colonizes the intercellular spaces but does not penetrate the mesophyll cells and does not form feeding structures like haustoria (De Wit, 1977). Approximately ten days after penetration, conidiophores start to emerge from the stomata and produce the asexual spores. The *C. fulvum*-tomato interaction strictly obeys the gene-for-gene concept. Several near-isogenic lines (NILs) of the tomato cultivar MoneyMaker (MM), harboring individual resistance genes against *C. fulvum* (*Cf* genes) (Tichelaar, 1984) are available, as well as a large collection of *C. fulvum* strains that are avirulent on tomato lines carrying specific *Cf* genes.

AVRs from *C. fulvum*

Since *C. fulvum* grows strictly in the apoplastic space, the various AVR proteins produced by the different races of the fungus (so-called race-specific elicitors) are also confined to that space and can be isolated from apoplastic fluid obtained from a compatible interaction (De Wit and Spikman, 1982). To isolate the AVR of interest, a strain of *C. fulvum* expressing that AVR is inoculated on tomato plants that do not express the corresponding resistance gene. The AVR is produced by the fungus during the ensuing compatible interaction and can be subsequently collected by isolating apoplastic fluid from this interaction. Upon purification of a specific AVR protein, its presence is monitored by injection of partly purified fractions into leaves of a tomato plant expressing the *R* gene corresponding to the AVR protein of interest. When the AVR protein is present, *Cf*-mediated necrosis will appear in the injected areas one or two days after injection. Using this approach, AVR4 (Joosten et al., 1994), and AVR9 (Scholtens-Toma and De Wit, 1988) have been purified. Once the protein is purified the corresponding gene can be cloned by reverse genetics.

Avr4 and *Avr9* have been cloned from *C. fulvum* and encode small, cysteine-rich proteins (Joosten and De Wit, 1999). To perform biochemical studies, these elicitors are produced in large quantities by the *Pichia pastoris* heterologous expression system (Invitrogen, USA) (Van den Burg et al., 2001) or chemical synthesis, respectively (Mahé et al., 1998). The last approach is only feasible for small peptides such as AVR9.

Numerous races of *C. fulvum* exist that are able to overcome resistance mounted by one or more specific *Cf* genes. This can be the result of deletions or point mutations either

resulting in complete absence of the encoded AVR or by the production of unstable forms of AVR4 that are rapidly degraded, and subsequently allowing the fungus to elude the plant defense response (Joosten and De Wit, 1999). Often, the loss of the AVR proteins appears not to result in a notable fitness penalty for the pathogen. As a result, resistance mounted by *Cf* genes recognizing AVR proteins that are not essential to the pathogen is not expected to be durable (Laugé et al., 1998).

Besides race-specific elicitors, other small proteins are also produced during the compatible interaction and are designated Extra Cellular Proteins (ECPs). To date, ECP1, ECP2, ECP3, ECP4 and ECP5 have been purified from apoplastic fluids and except for ECP3, their corresponding cDNAs and genes have been cloned (Joosten and De Wit, 1999). In contrast to race-specific elicitors, these proteins are produced by all strains of *C. fulvum* during the compatible interaction, suggesting that they play an important role in *C. fulvum* pathogenicity. Indeed, disruption of *Ecp1* and *Ecp2* resulted in reduced virulence of *C. fulvum* demonstrating that at least ECP1 and ECP2 are virulence factors (Laugé et al., 1997). Because of their importance for the fungus, resistance based on recognition of essential virulence factors by the host is expected to be durable as loss of these factors will result in a fitness penalty for the pathogen. Therefore, wild tomato accessions were screened for their ability to respond with a HR to any of the ECPs. Strikingly, for all five individual ECPs corresponding responsive wild tomato accessions were found that responded with a HR upon perception of a specific ECP. (Laugé et al., 1998) (Joosten and De Wit, 1999).

The *Cf* Genes from Tomato

For research purposes, individual *Cf* genes from wild tomato species were crossed into the tomato cultivar MoneyMaker (MM) harboring no resistance genes against *C. fulvum* (MM-Cf0). By repeatedly performing back crosses, the near-isogenic lines (NILs) MM-Cf2, MM-Cf4, MM-Cf5 and MM-Cf9 were obtained (Tichelaar, 1984). Of the *Cf* genes, *Cf-9* was the first to be isolated. The gene was cloned by transposon tagging on the basis of loss of AVR9 recognition in *Cf-9*-tagged plants (Jones et al., 1994). The other three *Cf* genes were isolated by map-based cloning and subsequent complementation for resistance against specific strains of *C. fulvum* by transferring a cosmid containing the candidate *Cf* gene to MM-Cf0 plants (Dixon et al., 1998; Dixon et al., 1996; Thomas et al., 1997).

The *Cf* genes belong to the class of *R* genes that encode membrane proteins with a predicted extra-cellular LRR domain and a small cytosolic part without any apparent domains involved in signaling (Jones et al., 1994; Thomas et al., 1997). The architecture of the Cf proteins is very similar although extensive variation in the number of LRRs occurs among the various Cf proteins.

Genomic Organization of *Cf* Genes in Tomato

Mapping studies revealed that *Cf-4* and *Cf-9* are located on the short arm of chromosome 1 of tomato and are closely linked. Both genes are part of a cluster of *Cf* homologues, designated *Hcr9s,* for homologue of *Cladosporium fulvum* resistance gene *Cf-9*. In MM-Cf9 plants a cluster containing 5 *Hcr9*s (*Hcr9-9A* to *Hcr9-9E*) was introgressed from the original wild *Lycopersicon* species. The functional *Cf-9* gene is *Hcr9-9C*, as only this homologue confers the ability to recognize AVR9 (Jones et al., 1994). In MM-Cf4 plants a cluster from a different wild *Lycopersicon* species containing other *Hcr9*s was introgressed. This cluster also contains five homologues, designated *Hcr9-4A* to *Hcr9-4E*, of which *Hcr9-4D* confers AVR4 recognition (Thomas et al., 1997).

Cf-2 and *Cf-5* map at identical locations on the short arm of chromosome 6. The cluster of *Cf* genes in MM-Cf2 comprises three *Hcr2*s of which two represent functional resistance genes (*Cf-2.1* and *Cf-2.2*) against strains of *C. fulvum* expressing *Avr2*. The third homologue, *Hcr2-2A,* does not confer resistance to *C. fulvum* (Dixon et al., 1996). The *Hcr2-5* cluster in MM-Cf5 contains 4 homologues of which *Hcr2-5C* is the functional *Cf-5* gene itself (Dixon et al., 1998).

The presence of different *Cf* gene clusters at the same locus in the genome in individual plants suggests a tendency for chromosomal mispairing and unequal crossing over to occur after crossing. Indeed, detailed analysis of the *Hcr9* family members showed that extensive sequence exchange has occurred between the *Hcr9*s. These recombination events may play a role in the natural process of evolution to generate new recognitional specificities (Parniske et al., 1997).

AVR Perception by Cf Tomato Plants

Injection of AVR9 in tomato leaves will only result in HR when the *Cf-9* gene is present, suggesting that Cf-9 is directly involved in AVR9 perception via a simple direct ligand-receptor interaction. Binding studies performed with ^{125}I-radiolabelled AVR9 demonstrated that a high affinity binding site (HABS) for AVR9 is present on plasma membranes isolated from tomato plants (Kooman-Gersmann et al., 1996). Furthermore, a strong correlation between binding affinity for the HABS and necrosis-inducing activity of AVR9 mutant peptides was observed (Kooman-Gersmann et al., 1998). However, the AVR9 binding site is also present in tomato plants that do not contain *Cf-9*, indicating that the HABS is not Cf-9 itself. Furthermore, the AVR9 HABS is present in all solanaceous species tested, such as potato and tobacco (Kooman-Gersmann et al., 1996). Although they are not a host for *C. fulvum*, transformation of potato and tobacco with the *Cf-9* gene confers the ability to recognize AVR9 to these species (Hammond-Kosack et al., 1998). It is thought that Cf-9 interacts with the AVR9 HABS upon AVR9 perception to activate downstream signaling pathways. The mechanism of binding is still unknown and is still subject to research (Joosten and De Wit, 1999).

Cf/Avr-Mediated Late Defense Responses

Having introduced the mayor players in the *C. fulvum*-tomato interaction, being Cf proteins of the host and the AVR proteins of the pathogen, events that ensue after direct or indirect interaction between these proteins are described.

The final outcome of the *Cf/Avr*-mediated defense response is the hypersensitive response (HR), which consists of a local collapse of the cells at the primary site of infection. After spraying tomato plants with a suspension of conidia of *C. fulvum*, accumulation of pathogensis-related (PR) proteins occurs in the plant, both in a compatible and an incompatible interaction (De Wit and Van der Meer, 1986; Joosten and De Wit, 1989). However, in an incompatible interaction the induction of transcription of genes encoding the classical PR proteins is faster and corresponds with an earlier accumulation of PR proteins as compared to the compatible interaction (Danhash et al., 1993; Joosten and De Wit, 1989; Van Kan et al., 1992). Acidic PR proteins such as chitinases and glucanases are localized in the apoplast and are thought to play a role in degradation of the hyphal wall. During HR basic PR proteins located in the vacuole, are released and may add to inhibition of fungal outgrowth. Other responses of the plant aimed at stopping fungal growth include the formation of cell wall appositions, callose formation at the site of hyphal contact with the plant cells (De Wit, 1977; Higgins, 1982; Lazarovits and Higgins, 1976) and accumulation of phytoalexins (De Wit and Kodde, 1981).

Early *Cf/Avr*-Mediated Defense Responses

After injection of apoplastic fluid containing AVR2 or AVR9 in the cotyledons of tomato seedlings expressing the corresponding *Cf* gene, several early defense responses were observed (Hammond-Kosack et al., 1996; May et al., 1996). For example, levels of reactive oxygen species (ROS) were detected 2 to 4 hr after injection. ROS are thought to have intrinsic anti-fungal activity, but also contribute indirectly to the defense response by induction of reinforcement of the walls of cells surrounding the primary infection site. ROS also contribute to the induction of expression of defense-related genes, and the execution of HR (Doke et al., 1996; Lamb and Dixon, 1997). At 2 to 4 hr after injection of the elicitor, lipid peroxidation occurs, and levels of glutathione start to increase from 2 to 8 hr after injection. Around 8 hr after injection significant electrolyte leakage, which is a sign of membrane damage, was reported. Lipid peroxidation, electrolyte leakage and increased levels of glutathione are indicators of severe oxidative stress (May and Leaver, 1993). Within 24 hr after injection, a transient increase in the levels of ethylene and free salicylic acid (SA), involved in the activation of systemic acquired resistance, was observed.

The use of cell suspensions in studies on early plant defense responses has great advantages over injection of AVRs into leaves. Cell suspensions can be grown in carefully controlled conditions whereas intact plants are grown in less controlled conditions in the greenhouse. Cell suspensions also represent a system that is much more amenable to synchronization of the defense response after elicitor treatment, compared to treatment of

whole leaves. This is especially important when studying very early responses such as oxidative burst, medium alkalization and protein phosphorylation. Consequently, we wanted to use tomato cell suspensions derived from tomato plants containing individual *Cf* genes to study AVR-induced early defense responses as already described in the section *"Elicitor-Induced Defense Responses"*. Although aspecific elicitors induced an oxidative burst and medium alkalization in tomato cell suspensions harboring the *Cf-9* gene, these cell suspensions failed to respond to AVR9 (Honée et al., 1998). This lack of responsiveness to AVR9 is probably developmentally regulated and greatly hampered studies on AVR9-induced early responses in tomato cell suspensions (Honée et al., 1998). However, the laboratory of Dr. Jonathan Jones, The Sainsbury Laboratory, Norwich UK, discovered that tobacco cell suspensions transgenic for *Cf-9* do respond to AVR9 (Piedras et al., 1998). Consquently, we made tobacco plants transgenic for *Cf-4* or *Cf-9*. Cell suspensions were started from AVR4 or AVR9 responsive plants. The induced cell suspensions were subsequently selected for their responsiveness towards the respective AVRs. When these cell suspensions became available, the studies on *Cf/Avr*-mediated early defense responses in cell suspensions became possible. This was crucial for studies on AVR-induced early defense responses and signal transduction described in this thesis.

Outline of the Thesis

In the introduction (**Chapter 1**) the state of the art has been described at the time I started my experimental work described in this thesis. The following chapters show the progress made on early defense responses induced in tobacco by well-characterized AVR4 and AVR9 peptides and mutants made thereof.

Chapter 2 describes the nature of specific AVR9-induced responses in *Cf-9*$^+$-tobacco cell suspensions. The work was performed with both *Cf-9*$^+$-tobacco cell suspensions initiated by the author and a *Cf-9*$^+$-tobacco cell suspension that was provided by Dr. Jonathan Jones, The Sainsbury Laboratory, Norwich, UK. Upon treatment with AVR9, *Cf-9*$^+$-tobacco cell suspensions responded with medium alkalization, an oxidative burst and the activation of a MAP kinase. We found that AVR9-induced cell death occurs when *Cf-9*$^+$-tobacco cell suspensions are treated with AVR9 concentrations that induce HR after injection in Cf9 tomato and in *Cf-9*$^+$-tobacco leaves. In addition to their ability to induce HR, various mutant AVR9 peptides were also compared for their ability to induce medium alkalization and an oxidative burst in tobacco cell suspensions. In *Cf-9*$^+$-tobacco cell suspensions, the mutant AVR9 peptides showed differential biological activities comparable to those observed *in planta*, indicating that *Cf-9*$^+$-tobacco cell suspensions, at least in part, reflect the responses observed in intact plants. Meanwhile, *Cf-4*$^+$-tobacco plants were generated and from these plants also cell suspensions were initiated.

The work presented in **Chapter 3** was performed in close collaboration with Dr. Ana Laxalt and Dr. Teun Munnik, Swammerdam Institute for Life Sciences, Department of Plant Physiology, University of Amsterdam. After treatment of *Cf-4*$^+$-tobacco cell suspensions with AVR4, levels of the second messenger phosphatidic acid (PA) increased dramatically. This increase was observed within two minutes after AVR4 treatment and appears to be one of the fastest responses induced upon specific elicitor perception. Both a differential labeling strategy and studies employing pharmacological inhibitors indicated that the AVR4-induced increase of PA levels originates from the activation of phospholipase C (PLC). We showed that inhibitors of PLC blocked AVR4-induced responses, such as medium alkalization, oxidative burst and MAP kinase activation, indicating that PLC activation is required for these responses to occur.

During my studies described in chapter 2, I discovered that *Cf/Avr*-mediated defense responses are temperature sensitive. The temperature-sensitivity of *Cf/Avr*-mediated defense responses is described in **Chapter 4**. In this chapter we show that seedlings expressing both a *Cf* and the corresponding *Avr* gene rapidly die after germination at 20°C, as a result of systemic necrosis, but are rescued by incubating them at 33°C. However, after lowering the temperature to 20°C, defense-related gene expression was rapidly and synchronously induced, eventually resulting in systemic HR and death of the initially rescued seedlings. In *Cf*$^+$-tobacco cell suspensions AVR-induced defense-related gene expression and medium alkalization was also suppressed at 33°C. We showed that the cell suspensions slowly lost their ability to respond with medium alkalization upon AVR

treatment after transfer from 20°C to 33°C. In contrast, this ability was quickly regained after transfer from 33°C to 15°C. We finally demonstrated that the amount of AVR9 high affinity binding sites present in microsomal fractions isolated from cell suspensions incubated at 33°C, was decreased with 80% as compared to cells incubated at 20°C. These data indicate that temperature-sensitivity of at least AVR9-induced defense responses resides at the level of elicitor perception.

In the summarizing discussion (**Chapter 5**) the results described in this thesis are discussed including unpublished data. A model of *Cf/Avr*-mediated defense responses is proposed comprising the findings described in this thesis and data presented by other workers in the field.

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Chapter 2

Early Defence Responses Induced by AVR9 and Mutant Analogues in Tobacco Cell Suspensions Expressing the *Cf-9* Resistance Gene

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Abstract

The interaction between the fungal leaf pathogen *Cladosporium fulvum* and its only host, tomato, fits the gene-for-gene model. In tomato, the *Cf-9* resistance gene product mediates specific recognition of the fungal avirulence gene product AVR9, resulting in a hypersensitive response and resistance. Cf9 tomato leaves respond with necrosis after injection with AVR9, whereas Cf9 tomato cell suspensions do not show defense responses after treatment with AVR9.

Here we report on early defense responses induced in *Cf-9* transgenic tobacco leaves and *Cf-9* transgenic tobacco cell suspensions after treatment with synthesized AVR9 and mutant analogues R08K, F10A and F21A. The necrosis-inducing activity of the AVR9 peptides increased in the order F21A, F10A, AVR9, R08K. An oxidative burst took place at a much lower AVR9 peptide concentration as compared to medium alkalization and necrosis. Interestingly, the mutant peptide F21A failed to induce necrosis and medium alkalization but did induce an oxidative burst. In all assays, the relative differential activities of the AVR9 peptides were similar to those observed in Cf9 tomato leaves. Both AVR9 and F21A activated a MAP kinase in *Cf-9* transgenic tobacco cell suspensions. AVR9 also induced specific cell death in these suspensions. The relation between the induction of early defense responses and necrosis is discussed.

Introduction

Upon recognition of an avirulent strain of a pathogen by a plant various defense responses are induced, including the reinforcement of cell walls, production of reactive oxygen species, phytoalexins and anti-microbial proteins (Dixon et al., 1994). The defense response often culminates in a hypersensitive response (HR): a local collapse of plant cells around the site of infection. Prior to the actual defense response several events that are proposed to play a role in defense signaling occur. These include in- and effluxes of ions, protein phosphorylation and production of ethylene and nitric oxide (Delledonne et al., 1998; Hammond-Kosack et al., 1996; May et al., 1996). When entering the plant, the pathogen releases compounds of various nature of which some allow recognition of the pathogen by the plant (Laugé et al., 1998). These elicitor molecules may be non-race- or race-specific. Non-race-specific elicitors can originate from the cell wall of the pathogen or the plant and are released by cell wall-degrading enzymes produced upon infection. Race-specific elicitors usually are small proteins secreted by the pathogen (De Wit, 1995). A race-specific elicitor is by definition encoded by an avirulence (*Avr*) gene, of which recognition is mediated by the product of the matching plant resistance (*R*) gene. This is the molecular basis of the gene-for-gene model (Flor, 1942).

The interaction between the leaf pathogen *Cladosporium fulvum* and its only host tomato, (*Lycopersicon esculentum*), is a well-described model system to study plant-pathogen interactions (Joosten and De Wit, 1999). *C. fulvum* is a non-obligate biotrophic fungus that enters the plant through stomata and colonizes the intercellular spaces between

mesophyl cells. During the interaction the fungus excretes various proteins, which are either involved in virulence, such as the extra cellular protein 2 (ECP2) (Laugé et al., 1997), or have a yet unknown function such as AVR9 (Scholtens-Toma and De Wit, 1988) or AVR4 (Joosten et al., 1994). These proteins are specifically recognized by tomato plants possessing the matching resistance gene *Cf-ECP2*, *Cf-9* or *Cf-4*, respectively (Joosten et al., 1994; Laugé et al., 1998; Van den Ackerveken et al., 1992). The best-characterized elicitor of *C. fulvum* is the cysteine-rich peptide AVR9. Secreted by the fungus as a pre-protein, it is proteolitically processed into a mature peptide of 28 amino acids (Van den Ackerveken et al., 1993). The three-dimensional structure of the mature protein has been elucidated by ^{1}H-NMR spectroscopy (Mahé et al., 1998; Vervoort et al., 1997). The six cysteine residues present in the AVR9 peptide are all involved in disulphide bridges, interconnecting three antiparallel β-strands resulting in a cysteine-knot, which is extremely stable. The tomato cultivar Moneymaker, into which the *Cf-9* resistance gene has been introgressed (MM-Cf9) (Tichelaar, 1984), recognizes the AVR9 peptide and is resistant to races of *C. fulvum* expressing *Avr9*.

Inoculation of tomato plants carrying the *Cf-9* resistance gene with recombinant potato virus X (PVX) (Chapman et al., 1992) expressing AVR9, resulted in the development of severe systemic necrosis and subsequent death of the plant, whereas tomato plants lacking the *Cf-9* resistance gene only showed mosaic symptoms (Hammond-Kosack et al., 1995; Kooman-Gersmann et al., 1997). PVX was subsequently used to test mutagenized AVR9 peptides of which each of the amino acid residues was exchanged one by one by alanine (alanine scan), in order to determine which amino acid residues are important for the necrosis-inducing activity (NIA) of AVR9 in MM-Cf9 tomato plants. This scan identified mutant AVR9 peptides that showed either higher, equal, lower, or no NIA as compared to wild-type AVR9 (AVR9) (Kooman-Gersmann et al., 1997). Exchanging phenylalanine at position 10 by alanine yielded a mutant peptide (F10A) that showed lower NIA as compared to AVR9, while exchanging phenylalanine at position 21 yielded a peptide (F21A) that did not show NIA. Exchanging arginine on position 8 by the homologous amino acid lysine resulted in a mutant (R08K), which showed higher NIA as compared to AVR9. The peptides F10A, F21A, R08K and AVR9 have also been chemically synthesized (Mahé et al., 1998). The differences in NIA, as determined by injection of the various synthetic peptides into MM-Cf9 leaves, were similar to the differences observed by employing the PVX system. However, F21A showed chlorosis but no necrosis upon injection of a concentration as high as 32 μM into MM-Cf9 leaves (Kooman-Gersmann et al., 1998).

Recognition of AVR9 results in HR and several associated defense responses (May et al., 1996). To study these responses in more detail, cell suspensions were generated as they represent a homogenous and convenient model system. Cell suspensions derived from MM-Cf9 plants, however, failed to respond to AVR9, possibly due to developmental regulation of one of the components involved in the signal transduction pathway that mediates the AVR9-induced host defense responses (Honée et al., 1998). Strikingly, cell suspensions

derived from transgenic tobacco plants (*Nicotiana tabacum* cv Petit Havana SR1) expressing the *Cf-9* gene (*Cf-9*$^+$-tobacco) clearly show the characteristic defense responses, such as medium alkalization and the generation of an oxidative burst upon AVR9 treatment (Piedras et al., 1998; Romeis et al., 1999).

Here, we have used *Cf-9*$^+$-tobacco cell suspensions to study the intensity and specificity of various defense responses after treatment with AVR9 and its mutant analogues. Furthermore, we compared these responses with the NIA of the various AVR9 peptides in both MM-Cf9 tomato and *Cf-9*$^+$-tobacco leaves. Surprisingly, the oxidative burst is induced at peptide concentrations which are approximately 100 times lower than the concentration required to induce necrosis in leaves, whereas medium alkalization is induced at concentrations similar to those required to induce necrosis. The ability of the various AVR9 peptides to induce necrosis in leaves, was comparable to their ability to induce medium alkalization and oxidative burst. Strikingly, F21A, which does not induce necrosis upon injection in leaves, also does not induce medium alkalization in tobacco cell suspensions whereas it does elicit an oxidative burst. Furthermore, we observed that *Cf-9*-mediated recognition of AVR9 results in specific cell death in *Cf-9*$^+$-tobacco cell suspensions.

Results

Determination of the Necrosis-Inducing Activity of Mutant AVR9 Peptides in Tomato and Tobacco Plants

To determine whether the necrosis-inducing activity (NIA) of the various mutant AVR9 peptides in *Cf-9*$^+$-tobacco correlated with the NIA observed in MM-Cf9 tomato, dilution series of the peptides were injected in both MM-Cf9 tomato and *Cf-9*$^+$-tobacco leaves. Visual determination of the extent of necrosis induced two days after injection of the peptides, revealed no significant differences in responsiveness to the various peptides between *Cf-9*$^+$-tobacco and MM-Cf9 tomato (Table 1).

Table 1. Necrosis-Inducing Activities (NIA)[a] of AVR9 and the Mutant Peptides R08K, F10A and F21A in Tomato (MM-Cf9) and *Cf-9*$^+$-Tobacco (SR1-Cf9) Leaves, Injected at Different Concentrations

Peptide	Plant	Concentration of peptide (μM)								
		0.0032	0.016	0.032	0.16	0.32	1.6	3.2	16	32
AVR9	MM-Cf9	-	-	-	+/-	+	++	++	++	++
	SR1-Cf9	-	-	-	+/-	+	++	++	++	++
R08K	MM-Cf9	-	-	-	+	+	++	++	++	++
	SR1-Cf9	-	-	-	+	+	++	++	++	++
F10A	MM-Cf9	-	-	-	-	-	+/-	+	++	++
	SR1-Cf9	-	-	-	-	-	+/-	+	++	++
F21A	MM-Cf9	-	-	-	-	-	-	-	-	-
	SR1-Cf9	-	-	-	-	-	-	-	-	-

[a]: The amount of necrosis was determined visually. Concentrations showing no NIA are designated (-), chlorosis to little necrosis (+/-), necrosis (+), spreading necrosis (++).

When the NIA of the various AVR9 mutant peptides was compared to the activity of AVR9 as found in MM-Cf9 tomato, in *Cf-9*$^{+}$-tobacco the R08K mutant peptide appeared more active, whereas F10A was less active. F21A did not show NIA up to concentrations of 32 μM (Table 1). At this concentration, however, chlorosis was induced in both species.

Medium Alkalization

A typical response observed after challenge of a cell suspension with an elicitor is alkalization of the culture medium caused by influx of protons from the extra-cellular medium. This response was reproducibly observed when *Cf-9*$^{+}$-tobacco cell suspensions were challenged with AVR9, whereas wild-type tobacco cell suspensions showed no response upon challenge with AVR9 (data not shown). Treatment of *Cf-9*$^{+}$-tobacco cell suspensions with a concentration range of the various AVR9 peptides resulted in various degrees of alkalization which could be quantified and used to determine dose-response curves for the various AVR9 peptides (Figure 1). Typically, within seconds after challenge with AVR9 peptide the pH increased slightly, whereas after 2 to 4 min a steady increase of the pH was observed. After approximately 10 min, the pH became more or less stable or increased only slightly (see insert Figure1). It was not possible to determine the exact onset of medium alkalization, as adding water to the cell suspensions also resulted in a low, albeit transient alkalization of the medium (data not shown). The amplitude and slope of the pH curves recorded after challenge with the various AVR9 peptides proved to be concentration- and peptide-dependent. The slope of the recorded curve became steeper with increasing concentration of the various peptides and was used to determine dose-response curves (see insert Figure 1).

All the mutants induced medium alkalization except F21A, which failed to induce this response. Treatment with the mutant AVR9 peptide F10A, which has a lower NIA then AVR9 (Table 1), gave reduced alkalization as compared to AVR9. However, the mutant peptide R08K, that has higher NIA then AVR9 (Table 1), showed medium alkalization similar to that of AVR9. The mutant peptide F21A that only induces chlorosis but no necrosis in the plant (Table 1), did not induce medium alkalization up to a concentration of 8.1 μM.

Oxidative Burst

Upon treatment of *Cf-9*$^{+}$-tobacco cells with various AVR9 peptides, a significant decrease in pyranin fluorescence was observed. The fluorescent pyranin is readily degraded by H_2O_2 produced by challenged plant cells. The decrease in fluorescence was recorded for various concentrations of each of the AVR9 peptides (see insert Figure 2A). The onset of pyranin degradation was both peptide- and concentration-dependent. The responses to different concentrations of the various peptides were recorded and used to determine dose-response curves (Figure 2A). In this assay the activity of the mutant peptides decreased in the order: R08K, AVR9, F10A and F21A.

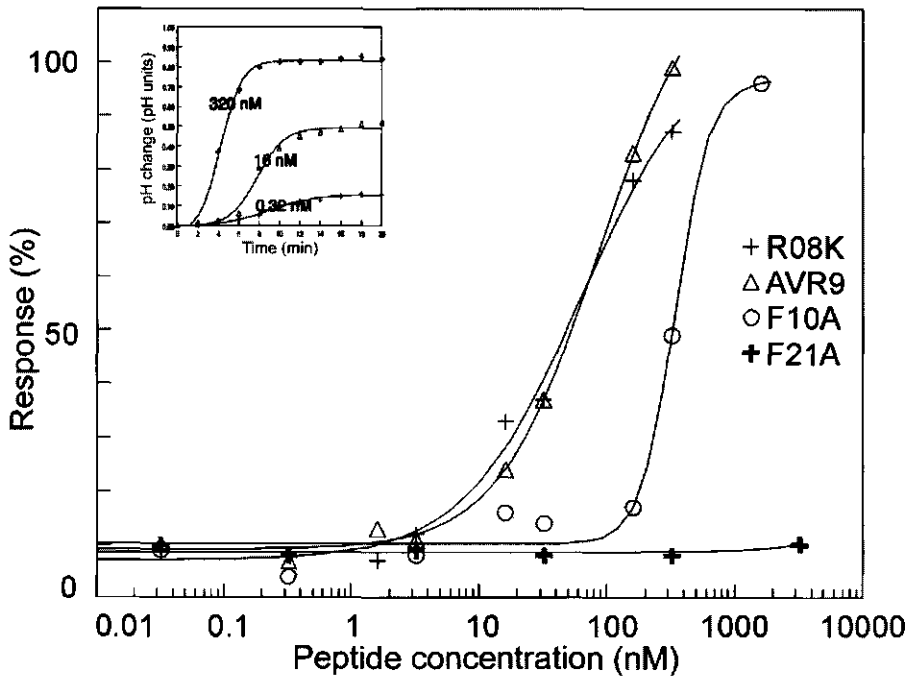

Figure 1. Dose-Response Curves of Medium Alkalization Determined for various AVR9 Peptides. *Cf-9^{+}-transgenic tobacco cells were treated with various AVR9 peptides, medium alkalization was recorded and the slope of the curves was determined. Full medium alkalization activity, resulting from treatment with 0.32 μM AVR9 was set to 100%; the slope was expressed as percentage of the slope of full medium alkalization activity. Insert: Typical pH shift curves at three different concentrations of the R08K mutant peptide. The recorded curves show the increase in pH of the extra-cellular medium.*

Adding peptide at concentrations that resulted in a maximum response, the earliest onset of the oxidative burst could be observed after ca. 200 seconds (Figure 2B). This appeared to be the minimum time required to induce an oxidative burst for the R08K, AVR9 and F10A peptides. Due to the limited availability of this peptide, the concentration of F21A could not be increased further to test whether this time point would also hold for this mutant peptide (Figure 2B). Typically, the difference which is minimally required to induce an oxidative burst and the concentration of the peptide that induces a full response, was about a factor hundred (See Figure 2A). For the F21A peptide, however, increasing the minimum peptide concentration for an oxidative burst a hundred fold, only resulted in the elicitation of 60% of the full response (Figure 2A).

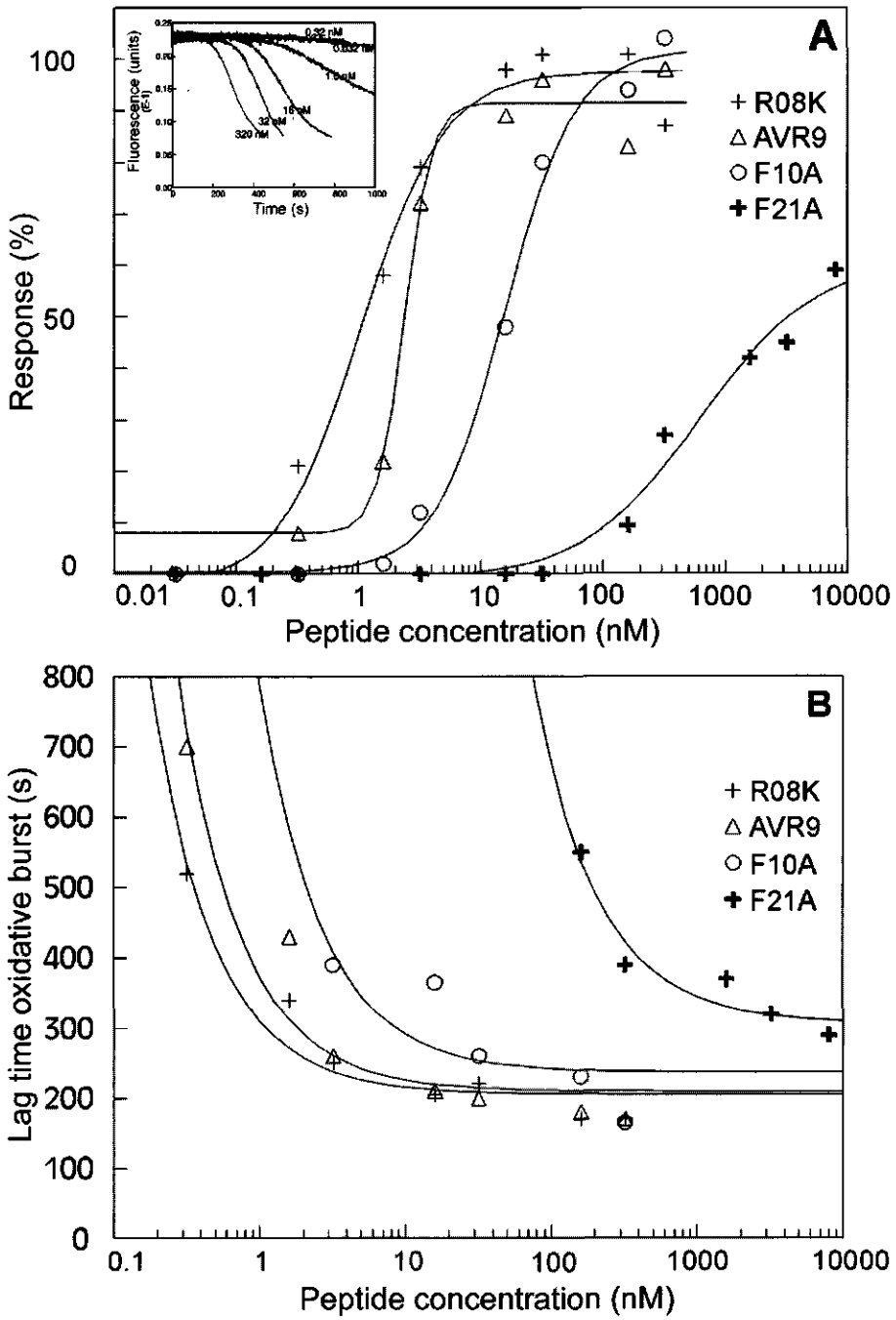

Figure 2. Analysis of the Oxidative Burst in *Cf-9*$^{+}$-Tobacco Cell Suspensions after Treatment with various AVR9 Peptides.

Figure 2 (Continued) *(A) Dose-response curves of the oxidative burst determined for the various AVR9 peptides. Cf-9$^+$-transgenic tobacco cells were treated with the various AVR9 peptides, the oxidative burst was recorded and the slope of the curves was determined. Full oxidative burst activity, resulting from treatment with 0.32 μM AVR9, was set to 100%. The slope of the various curves was expressed as percentage of the slope of full oxidative burst activity.*
Insert: Typical oxidative burst curves for various concentrations of the mutant peptide R08K. The recorded curves show the decrease in fluorescence, due to degradation of pyranin by the oxidative burst.
(B) Time elapsed between addition of the various AVR9 peptides and the onset of the oxidative burst. Different concentrations of the various AVR9 peptides were added to Cf-9$^+$-tobacco cell suspensions and the decrease in fluorescence of pyranin was recorded. The time of onset of the oxidative burst, was visible as a decrease in fluorescence, and plotted against the concentration of the various peptides.

AVR9-Induced MAP Kinase Activation

MAP kinases have shown to be involved in several stress responses including *Cf-9*-mediated recognition of AVR9 (Hirt, 1997; Romeis et al., 1999). To determine whether MAP kinases are also activated upon treatment with the AVR9 and F21A peptides, they were added to *Cf-9*$^+$-tobacco cell suspensions at a final concentration of 0.32 μM. At different time points after adding these peptides samples were collected, cell free extracts were prepared and MAP kinase activity was determined by the in-gel kinase assay.

In the water-treated negative control, the in-gel kinase assay revealed two bands of approximately 46 kD (Figure 3). The intensity of these bands varied somewhat between different time points but did not change significantly in time. Upon AVR9 treatment, however, a third band, just below the 46 kD molecular weight marker, appeared within 10 min (Figure 3). The intensity of this band increased for approximately 10 min and then decreased but was still visible 30 min after treatment with AVR9. Adding the mutant peptide F21A did only result in a minor increase in intensity of the third band.

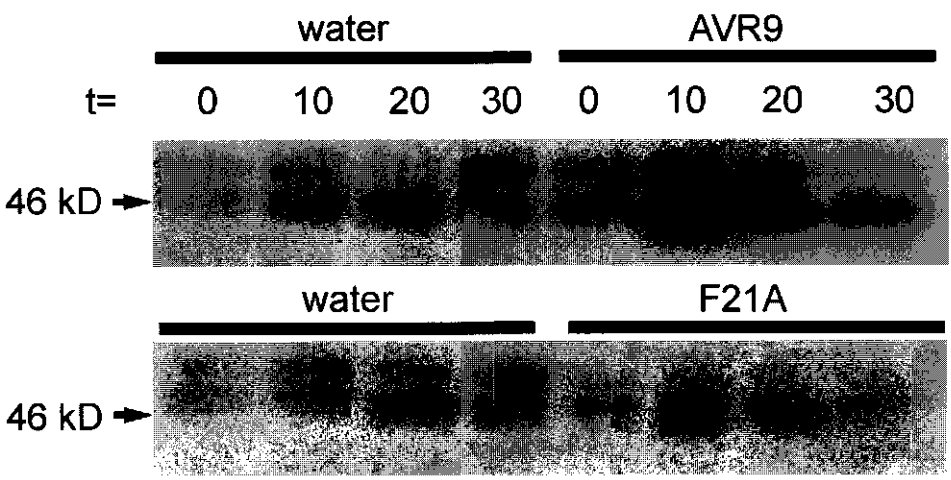

Figure 3. MAP Kinase Activation by AVR9. *Cells were treated with water, AVR9 or the mutant AVR9 peptide F21A to a final concentration of 0.32 μM. Samples were collected at 0, 10, 20 and 30 min after adding the elicitors. Each lane was loaded with 20 μg of protein.*

AVR9-Induced Cell Death

At a macroscopic level, *Cf-9*$^+$-tobacco cell suspensions treated with AVR9 turned brown while the water-treated control did not show significant browning within 24 hr (Figure 4). Furthermore, AVR9-treated *Cf-9*$^+$-tobacco cell suspensions were arrested in growth, as determined by measuring the packed cell volume (data not shown), whereas the water-treated control grew normally. To check both wild-type and *Cf-9*$^+$-tobacco cell suspensions for the appearance of cell death, cells were stained with fluorescein diacetate (FDA) and propidium iodide and examined by fluorescence microscopy, 18 hr after challenge with either water or 3.2 μM AVR9. When compared to the water-treated control, *Cf-9*$^+$ cell suspensions challenged with AVR9 showed a significant increase in red fluorescent cells of which the DNA was stained by the propidium iodide, indicating that they are dead (Figure 4). This response was not observed in the wild-type tobacco cell suspension. Wild-type and the *Cf-9*$^+$ cell suspensions that had been treated with water contained around 10% percent of dead cells (Table 2). A similar percentage of dead cells was observed in the wild-type, AVR9-treated tobacco cell suspension. However, approximately 75% of the AVR9-treated *Cf-9*$^+$-tobacco cells were stained by propidium iodide, indicating a dramatic induction of cell death by AVR9 (Table 2).

Table 2. AVR9-Induced Cell Death in Tobacco Cell Suspensions

cell suspension:	treatment:	percentage of dead cells[a]:
wild-type	water	8.5 ± 4.8
wild-type	AVR9	7.1 ± 4.4
Cf-9$^+$	water	11.2 ± 8.5
Cf-9$^+$	AVR9	73.0 ± 19.4

[a]: Wild-type and *Cf-9*$^+$-tobacco cell suspensions were either treated with water or with 3.2 μM AVR9. Cell death was determined 18 hr after treatment.

Discussion

The race-specific elicitor AVR9 induces medium alkalization, oxidative burst, MAP kinase activation and cell death in cell suspension cultures of tobacco that express the *Cf-9* resistance gene. MM-Cf9 tomato leaves, injected with AVR9 mutant peptides F21A, F10A and R08K showed different NIAs (Kooman-Gersmann et al., 1997). We showed that injection of these peptides into *Cf-9*$^+$ tobacco leaves resulted in comparable differences in NIA, while no difference in sensitivity between MM-Cf9 and *Cf-9*$^+$ tobacco was observed. Injection of the mutant peptide F21A did not result in necrosis but only induced chlorosis at concentrations up to 32 μM.

To assay for the different biochemical responses induced by AVR9 and the various mutant peptides, *Cf-9*$^+$-tobacco cell suspensions were used. Comparison of the different AVR9 mutant peptides in the medium alkalization assay showed that R08K has the same activity as AVR9, whereas F10A has a lower activity compared to AVR9 (Figure 1). The mutant peptide F21A failed to induce medium alkalization up to a concentration of 8.1 μM (Figure 1).

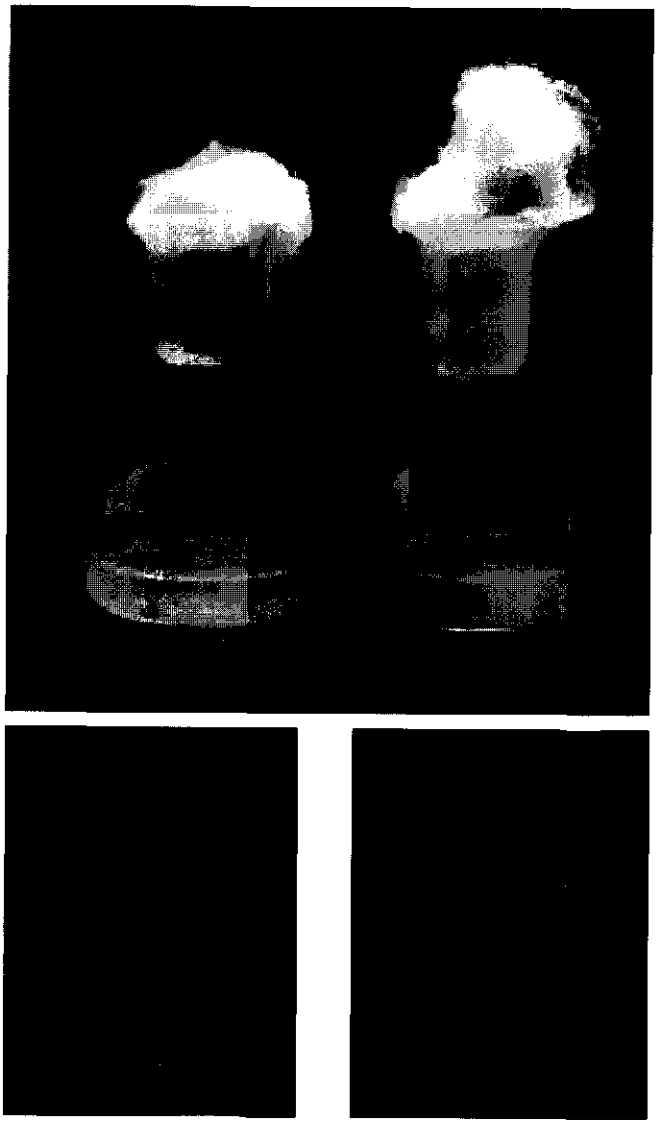

Figure 4. AVR9-Induced Cell Death.
Cf-9^{7}-tobacco cell suspensions were challenged with either water or 3.2 μM AVR9. Upper panel: AVR9 treatment results in a brown coloration of the suspension (right flask) as compared to the water-treated control (left flask). The photograph was taken 12 hr after adding water or AVR9.
Lower panels: Cells stained with both FDA and propidium iodide, 48 hr after AVR9 or water treatment. The AVR9-treated cells show both green and red fluorescent cells (the nucleus of dead cells is stained by propidium iodide, visible as dots in the cells) (right panel), indicating the presence of both living and dead cells, whereas the water-treated control only shows bright green fluorescent cells indicating cells are alive. See also cover of this thesis.

In the oxidative burst assay the activity of R08K was found to be higher and that of F10A lower as compared to AVR9 (Figure 2A). The mutant peptide F21A induces an oxidative burst, although no full response was observed up to a concentration of 8.1 μM. The inability of F21A to induce necrosis might be related to the fact that this mutant is not able to induce medium alkalization in suspension cultured *Cf-9*$^{+}$-tobacco cells.

It has been reported that diphenylene iodonium and catalase inhibit the oxidative burst in tobacco cells but do not compromise cell death (Yano et al., 1999). In agreement with this report our data indicate that the oxidative burst alone is not sufficient to elicit AVR9-induced necrosis in tobacco. The AVR9 peptides that provoked medium alkalization in addition to an oxidative burst also induced necrosis.

Surprisingly, an oxidative burst could still be observed in *Cf-9*$^{+}$-tobacco cell suspensions at peptide concentrations approximately 100 times lower than the minimal concentration required to induce necrosis in leaves. The minimal concentration of AVR9 peptide required to induce medium alkalization is in the same order of magnitude as the concentrations of AVR9 peptide required to induce necrosis in leaves. These data suggest that medium alkalization, which is the outcome of ion fluxes over the plasma membrane, is not required for the induction of the oxidative burst but is possibly involved in the generation of necrosis.

The difference between the peptide concentration required to elicit a response in the oxidative burst assay and the peptide concentration required in the medium alkalization assay, might also be due to the different conditions under which both assays were performed. The alkalization assays were carried out in medium in which the cells had been growing for 4 days, whereas the oxidative burst assays were carried out in an optimized assay buffer (See Methods). Efforts to measure medium alkalization in the oxidative burst assay buffer were not successful due to the high buffering capacity of this buffer. Although lowering the buffer capacity of the oxidative burst assay buffer allowed detection of medium alkalization, this was only the case for peptide concentrations that also elicited a response in the original medium alkalization assay. This indicates that the difference between the peptide concentration required to elicit an oxidative burst and the peptide concentration required to elicit medium alkalization, cannot be attributed to differences in sensitivity of both assays. However, in an oxidative burst assay buffer with lowered buffer capacity the pH did not reach a stable level, thereby compromising accurate measurements to obtain dose-response curves for medium alkalization assays in this buffer.

A direct correlation between the lag time of the oxidative burst, the concentration of the various AVR9 peptides and their relative activities in the oxidative burst assay was observed. At higher peptide concentrations, the onset of the oxidative burst occurred faster. A less active AVR9 mutant peptide showed a later onset of the oxidative burst as compared to the same concentration of a more active AVR9 peptide (Figure 2B). The minimum lag time was ca. 200 seconds for F10A, AVR9 and R08K, whereas the lag time of F21A could not be determined. These data indicate that at sufficiently high concentrations, the diffusion of AVR9 through the cell wall, binding to its receptor, activation of signal transduction

events, activation of the production of reactive oxygen species and finally the breakdown of the fluorescent probe, all take place within 200 seconds.

As F21A induced an oxidative burst but no medium alkalization, we tested this mutant for the ability to activate MAP kinases at a concentration where it was able to induce an oxidative burst. In the in-gel kinase assay, using myelin basic protein as a substrate, activation of one MAP kinase was observed after challenge of *Cf-9*$^+$-tobacco cells with both AVR9 and F21A. Activation of this kinase occurred within 10 min after adding of the peptide and already showed a decrease in activity after 20 min (Figure 3). Although to much lower levels, F21A was also able to activate this MAP kinase, suggesting that MAP kinase activation is independent of medium alkalization.

Upon addition of sterile AVR9 at a concentration of 3.2 μM, *Cf-9*$^+$ tobacco cell suspensions accumulated phenolic compounds (Figure 4). A change in color was observed 4 hr after adding the elicitor, which became more intense in time (Figure 4, upper panel). Furthermore, growth of the AVR9-treated cells was inhibited compared to the water control as determined by measuring the packed cell volume. This observation was confirmed by the observed massive AVR9-specific cell death in the *Cf-9*$^+$-tobacco cells. Examination of cells stained with propidium iodide did not reveal disintegration of the nucleus into typical programmed cell death-associated nuclear bodies (Wang et al., 1996). Although AVR9-induced oxidative burst, medium alkalization and MAP kinase activation has been reported before, (Piedras et al., 1998; Romeis et al., 1999) this is the first report about AVR9-induced cell death in cell suspensions. This observation allows more detailed research on the nature of AVR9-induced necrosis, as cell suspensions are more convenient for biochemical and physiological studies than leaves. No responses resulting from flooding and wounding of the leaf are activated in cell suspensions whereas adding pharmacological agents can be done more accurately.

Methods

Selection and Cultivation of Tobacco Plants Expressing the Cf-9 *Resistance Gene*

Seeds from *Nicotiana tabacum* cv Petit Havana SR1, transformed with pMOG1096 containing the *Cf-9* resistance gene (Kamoun et al., 1999), were provided by Zeneca Mogen (Leiden, The Netherlands). After surface-sterilization in 1% NaOCl solution for 1 hr, seeds were rinsed with sterile water and transferred to pots containing solid Murashige-Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 10 g/L sucrose and 100 mg/L kanamycin. The pots were incubated at 25°C with a 16 hr light and an 8 hr dark regime. Prior to transfer of the kanamycin-resistant plants to the greenhouse, explants were taken for callus induction. Plants were cultivated under greenhouse conditions and leaves were injected with about 100 μL of synthetic AVR9 at a concentration of 0.65 μM (Mahé et al., 1998), to select for AVR9-responding plants. Plants showing necrosis upon AVR9 injection were selfed in order to obtain lines homozygous for *Cf-9* (*Cf-9*$^+$).

Callus Induction and Maintenance

Explants taken from the *in vitro* grown kanamycin-resistant tobacco plants, were transferred to solid Linsmaier-Skoog (LS) medium (Linsmaier and Skoog, 1965), supplemented with 40 g/L sucrose, 2 mg/L 1-naphtaleneacetic acid (NAA), 0.2 mg/L kinetin and 100 mg/L kanamycin and incubated at 25°C in the dark. The resulting callus was subcultured every four weeks to the same medium. Callus derived from kanamycin-resistant plants that did not show necrosis upon injection of AVR9 into the leaves of the corresponding greenhouse-grown plants was discarded. Callus derived from plants responding with necrosis was used to generate *Cf-9*$^{+}$ cell suspensions.

Generation, Cultivation and Selection of Cell Suspensions

Pieces of callus were transferred to 300 mL Erlenmeyer flasks containing 50 mL of liquid LS medium supplemented with 30 g/L sucrose, 2 mg/L NAA and 0.2 mg/L kinetin. Flasks were incubated in the dark on a gyratory shaker at 120 rpm, 25°C. Testing of cell suspensions for medium alkalization (see below) upon challenge with synthetic AVR9 at a final concentration of 100 ng/mL suspension, resulted in the identification of one responding cell suspension. Every 7 days, cells growing in the log phase were transferred to fresh liquid LS medium. An additional *Cf-9*$^{+}$-tobacco cell suspension and a wild-type cell suspension were a gift of Dr. J. Jones (Sainsbury Laboratory, John Innes Centre, Norwich UK). The latter were grown on liquid MS medium, supplemented with B5 vitamin mixture (Gamborg et al., 1968), 1 mg/mL 2,4-dichlorophenoxyacetic acid and 0.1 mg/mL kinetin and also subcultured on weekly intervals. Cell suspensions that were grown for 4 days after subculturing were used in all experiments.

Determination of the Necrosis-Inducing Activity of Mutant AVR9 Peptides in Tomato and Tobacco Plants

Using PVX as an expression vector, Kooman-Gersmann and co-workers (Kooman-Gersmann et al., 1997) identified and isolated several AVR9 mutant peptides showing intermediate (F10A) or no (F21A) necrosis-inducing activity (NIA) in MM-Cf9 tomato plants when compared to AVR9. One mutant peptide (R08K) was identified that showed a higher NIA when compared to AVR9. These AVR9 mutant peptides were also chemically synthesized (Mahé et al., 1998). To determine whether the various AVR9 peptides induced similar responses in both MM-Cf9 tomato plants and *Cf-9*$^{+}$-tobacco, a series of different concentrations of the peptides were injected into leaves of both species.

Medium Alkalization

Aliquots of 7.5 mL cell suspension, representing around 1 gram of cells, were transferred to 25 mL Erlenmeyer flasks and allowed to equilibrate for 3 hr on a rotary shaker at 220 rpm at room temperature (RT). Upon challenging the cells with AVR9 peptide, the extra cellular pH was recorded by immersion of a mini pH electrode (InLab 423, Mettler Toledo) in the medium while shaking. The slopes from the resulting pH curves and the dose-response

curves for the different AVR9 peptides were determined as described for the oxidative burst assay (see below).

Oxidative Burst Assay

To prepare cells for the oxidative burst assay, cells present in suspensions were pelleted at 450g for 8 min and washed with 100 mL filter-sterilized assay buffer (5 mM MES/NaOH pH 5.7, 175 mM mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$) and allowed to equilibrate for 20 min on a gyratory shaker at 25°C, 120 rpm. After repeating this procedure three times, 2.5 g of cells were resuspended in 50 mL assay buffer in an open 300 mL Erlenmeyer flask and allowed to equilibrate for three hours at RT on a gyratory shaker at 120 rpm.

For the oxidative burst assay, 1.5 mL of the equilibrated cell suspension was transferred to a 3 mL quartz cuvette and 3 µL (1 mg/mL) of the fluorescent probe pyranin was added (Apostol et al., 1989). The cells were gently stirred with a magnetic rod in the cuvette and after the probe was homogeneously distributed throughout the cuvette, AVR9 peptide was added. Directly after addition of AVR9 peptide, the quenching of pyranin fluorescence due to the production of H_2O_2 by the cells, was recorded at an excitation wavelength of 405 nm and emission wavelength of 512 nm. The curve resulting from the AVR9-induced quenching of pyranin showed a linear part of which the slope was used to quantify the oxidative burst activity.

To determine dose-response curves, cells were challenged with different concentrations of wild-type and mutant AVR9 peptides. The AVR9 peptides were added from stock solutions in water to yield the desired final concentration in the cuvette. For each concentration of AVR9 peptide, the slope of the linear part of the quenching curve was determined. The oxidative burst activity resulting from challenge with AVR9 (0.32 µM) was set at 100%. Activities determined for the various AVR9 peptides and concentrations were expressed as a percentage of this maximum response.

Efforts to assay for the oxidative burst in the original culture media were not successful, probably due to high concentrations of transition metal ions present in the medium. These ions catalyze the decomposition of hydrogen peroxide produced by the cells during the oxidative burst, into water and oxygen. On the other hand, assaying for medium alkalization in the oxidative burst assay buffer is not possible due to the high pH buffering capacity of this assay buffer.

MAP Kinase Activation Assay

Aliquots of 10 mL *Cf-9*$^+$-tobacco cell suspension were transferred to 25 mL Erlenmeyer flasks and allowed to equilibrate for 3 hr on a rotary shaker at 220 rpm at RT. Medium alkalization was recorded to check for responsiveness of the cells. Cells were either challenged with AVR9 or the mutant peptide F21A, both at a final concentration of 0.32 µM or with water. At different time points after challenge with elicitor, cells were collected by filtration over a Büchner filter, washed with water, frozen in liquid nitrogen and stored at -80°C.

Cells were ground in a mortar, pre-cooled with liquid nitrogen in the presence of sand and extraction buffer (Romeis et al., 1999). The resulting homogenate was filtered through Miracloth™ and cell debris was removed by centrifugation at 16,000 x g for 20 min. The supernatant was desalted on a NAP-5 column (Amersham/Pharmacia), equilibrated with elution buffer (Romeis et al., 1999). The eluate was divided in aliquots of 100 µl, frozen in liquid nitrogen and stored at -80°C. The protein content of the different samples was determined according to the method described by Bradford (Bradford, 1976).

For the in gel kinase assay, 20 µg of total protein per lane was separated on a 10% SDS-polyacrylamide gel containing 50 µg/mL myelin basic protein (MBP; Sigma). After denaturing and renaturing the gel as described by Suzuki. (Suzuki and Shinshi, 1995), two gels were phosphorylated at RT in 50 mL equilibration buffer containing 25 µM ATP and 5.6 MBq γ-^{32}P-ATP. After 30 min the gel was transferred to 5% trichloroacetic acid and 1% NaP_2O_7. Unincorporated γ-^{32}P-ATP was removed by extensive washing with the stopping solution until no radioactivity in the washings could be detected. A Kodak X-OMAT film was exposed to the dried gel.

AVR9-Induced Cell Death

Aliquots of 7.5 mL cell suspension were transferred to sterile 25 mL Erlenmeyer flasks. After adding 75 µl filter sterile synthetic AVR9, yielding a final concentration of 3.2 µM AVR9, or sterile water, the suspensions were incubated in the dark on a rotary shaker at 220 rpm, 25°C. At different time points, one drop of cell suspension was transferred to an Eppendorf vial containing 1 mL of water to which 10 µL of fluorescein diacetate (FDA) stock solution (0.5 mg/mL) and 20 µL propidium iodide stock solution (1 mg/mL) were added to assay for cell death. FDA solution was freshly prepared by diluting a 1 mg/mL FDA stock in acetone with water. FDA is hydrolyzed in living cells to fluorescein, which accumulates in viable cells (Oparka and Read, 1994). Dead or dying cells with a leaky cell membrane do not accumulate fluorescein. Propidium iodide fluoresces red upon binding to DNA after passing the leaky cell membrane of dead cells. This compound is not able to pass the intact cell membrane of viable cells and consequently does not stain their DNA (Oparka and Read, 1994). Using both dyes simultaneously, a fluorescence microscope equipped with a FITC filter (Leitz Laborlux S) reveals both green fluorescent viable cells and red fluorescent dead cells simultaneous. At 30 random fields dead and living cells were counted and the percentage of cell death was determined.

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Chapter 3

Cf-4/Avr4-Dependent Defense Signaling Is Mediated by the Lipid Second Messenger PA

This chapter will be published by Camiel F. de Jong, Ana Laxalt, Wilco Ligterink, Pierre J.G.M. de Wit, Matthieu H.A.J. Joosten and Teun Munnik

Abstract

The *Cf-4* resistance gene of tomato confers resistance to strains of the fungus *Cladosporium fulvum* expressing the corresponding avirulence gene *Avr4*, encoding the AVR4 elicitor protein. One of the earliest induced defense responses after elicitor perception is an oxidative burst. The signaling events activating the oxidative burst are not clear. Using transgenic tobacco cell suspensions expressing the *Cf-4* resistance gene as a model system, phospholipid signaling pathways were studied by pre-labeling the cells with $^{32}P_i$ and assaying for the formation of lipid signals after challenge with the matching AVR4 elicitor. The most dramatic response within minutes was an increase in ^{32}P-phosphatidic acid (PA), and its metabolic product diacylglycerol pyrophosphate (DGPP). The AVR4-induced PA and DGPP accumulation was time- and dose-dependent, whereas the non-matching elicitor, AVR9, did not trigger these responses. A transphosphatidylation assay and a differential ^{32}P-labeling protocol were used to determine the respective contributions of phospholipase D (PLD) and diacylglycerol (DAG) kinase activity to AVR4-induced PA accumulation. The results clearly demonstrated that most PA was produced from DAG, which is in most cases generated by phospholipase C (PLC) activation. Neomycin and U73122, which are inhibitors of PLC, effectively prevented AVR4-induced PA accumulation. In addition, U73122 also blocked the AVR4-induced oxidative burst. The NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI) also blocked the AVR4-induced oxidative burst, but did not prevent PA accumulation, suggesting that the PA response is upstream of the oxidative burst. Conversely, when cells were treated with a water-soluble, synthetic PA analogue, an oxidative burst was induced. Together, these results provide evidence for PA being a second messenger invoked by *Cf-4*-mediated recognition of the race-specific elicitor AVR4 to activate downstream defense responses, such as the oxidative burst.

Introduction

In their struggle to survive, plants have evolved mechanisms to defend themselves against a range of pathogens, including viruses, bacteria, fungi and insects. Recognition is mediated by molecules, called elicitors, that are produced by the pathogens. Elicitors can be relatively unspecific, such as cell wall fragments, or highly specific proteins encoded by avirulence (*Avr*) genes produced by the pathogen (Laugé et al., 1998). One of the most effective defense responses mounted by the plant after pathogen recognition is the hypersensitive response (HR), a collapse of cells at the site of primary infection, visible as necrosis. Other defense responses include the production of signal molecules such as ethylene and salicylic acid and the accumulation of pathogenesis-related proteins, phytoalexins and the reinforcement of cells walls (Hammond-Kosack and Jones, 1996).

One of the earliest plant defense responses is the oxidative burst, the production of reactive oxygen species (ROS) within minutes after elicitor perception. The production of ROS is remarkably similar to the oxidative burst produced after antigen perception by macrophages, cells that belong to the mammalian innate immune system. In macrophages,

ROS are produced by a plasma membrane-bound NADPH oxidase complex and play a role in killing of pathogens and tumor cells. Recently, it was shown that the Arabidopsis homologues *AtrbohD* and *AtrbohF* of the mammalian *gp91*^{phox}, that encode the catalytic subunit of NADPH oxidase, are indeed required for the accumulation of ROS (Torres et al., 2002). The activated complex produces O_2^-, that is rapidly converted into H_2O_2 by superoxide dismutase (SOD). Besides being able to directly kill the invading pathogen and by containing it through reinforcement of plant tissue by cross-linking of proteins present in the cell wall, these ROS are also thought to play a role in signaling. In parsley cells, O_2^- has been shown to be essential for defense gene activation and phytoalexin accumulation (Jabs et al., 1997). ROS are also implicated in systemic acquired resistance after pathogen perception (Alvarez et al., 1998). ROS were shown to be necessary but not sufficient to induce a HR and recent evidence showed that the ratio between nitric oxide (NO) and H_2O_2 levels after pathogen perception determines whether a HR will take place (Delledonne et al., 2001).

The elicitor-induced oxidative burst is blocked by the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), whereas inhibition of the Ca^{2+} influx was also shown to block this response (Jabs et al., 1997; Keller et al., 1998; Lamb and Dixon, 1997; Scheel, 1998). Furthermore, pharmacological studies demonstrated that inhibitors of protein kinases blocked, whereas inhibitors of phosphatases induced the oxidative burst, suggesting the requirement of protein kinases to activate the NADPH oxidase (Jabs et al., 1997; Lamb and Dixon, 1997; Levine et al., 1994; Piedras et al., 1998). In mammalian systems, both protein kinases and phospholipid signaling are implicated in activation of NADPH oxidase (Erickson et al., 1999; Lennartz, 1999; McPhail et al., 1999; Waite et al., 1997). Lipid signaling cascades have also been identified after perception of aspecific elictors by plants (see reviews by Chapman, (1998) and Munnik et al., (1998a). The key enzymes relevant to this report are phospholipase C (PLC), phospholipase D (PLD) and diacylglycerol (DAG) kinase (DGK), because they are directly or indirectly involved in production of the lipid second messenger phosphatidic acid (PA) (Munnik, 2001).

PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol 1,4,5-trisphosphate (IP_3) and DAG. PLC has been invoked as a signaling enzyme in tissues of different plant species responding to aspecific pathogen elicitors, including pea epicotyl tissue (Toyoda et al., 1992; Toyoda et al., 1993) cell suspensions of tobacco (Kamada and Muto, 1994), soybean (Legendre et al., 1993), lucerne (Walton et al., 1993) and tomato (Van der Luit et al., 2000). While IP_3 has been shown to release Ca^{2+} from intracellular stores in plants, the function of DAG is not clear (Munnik et al., 1998a). In animal cells, DAG can activate certain members of the protein kinase C family but plant genomes seem to lack such genes. In practice however, when PLC is activated, the resulting DAG is rapidly phosphorylated by DGK, resulting in generation of PA. Accumulating evidence strongly suggests that PA is a second messenger in its own right in plant signal transduction pathways (Munnik, 2001). PA is biologically active in some plant systems and currently its target molecules are being identified. In addition, PA

is also produced when the second signaling enzyme, PLD, is activated to hydrolyze a structural membrane lipid such as phosphatidylcholine. When tomato suspension cultures were challenged with the aspecific elicitor xylanase, PLD was activated within minutes (Van der Luit et al., 2000), while the expression of the *PLDβ1* gene increased 9-fold within 2 hr (Laxalt et al., 2001).

In all these cases, the accumulation of PA is transient. PA is converted into diacylglycerol pyrophosphate (DGPP) by phosphatidic acid kinase (PAK). This conversion is thought to be part of a mechanism involved in attenuation of the PA signal. DGPP is a sensitive reporter of PA signaling, because it is hardly detectable in non-stimulated cells. It is formed irrespective whether PA originates from PLC or PLD activity. Consequently, DGPP accumulation has been observed after treatment of plant cells with non-specific elicitors (Van der Luit et al., 2000), nod factors (Den Hartogh et al., 2001), osmotic stress (Munnik et al., 2000; Pical et al., 1999), and UV light (Pierrugues et al., 2001). Whether DGPP itself is a second messenger in its own right remains to be determined.

Here we report about the accumulation of PA after treatment of tobacco cells with a race-specific elicitor of the fungal tomato pathogen Cladosporium. The tomato-Cladosporium interaction obeys the gene-for-gene concept as formulated by Flor, (1942). During an incompatible interaction, a Cladosporium *Avr* gene product is perceived via the corresponding tomato *R* gene (*Cf*) product, resulting in a HR conferring complete resistance against the fungus. From this interaction, several *Cf* genes and their corresponding *Avr* genes have been cloned. The best studied *Cf/Avr* gene pairs are *Cf-4/Avr4* (Thomas et al., 1997; Joosten et al., 1994), and *Cf-9/Avr9* (Jones et al., 1994; Van Kan et al., 1991).

As tomato cell suspensions are not responsive to AVRs (Honée et al., 1998), responsive Cf^+-tobacco cell suspensions (Piedras et al., 1998) were used instead to study early AVR-induced defense responses. *Cf*-mediated recognition of the matching avirulence factor results in several responses thought to be involved in defense signaling. These responses include ion fluxes over the plasma membrane (Blatt et al., 1999), activation of two MAP kinases (Romeis et al., 1999) and a calcium-dependent protein kinase (CDPK) (Romeis et al., 2000). Also the production of ethylene, ROS and the expression of genes encoding pathogenesis-related proteins have been reported (De Jong et al., 2000; Hammond-Kosack et al., 1996; May et al., 1996; Piedras et al., 1998). Here we have tested whether phospholipid signaling also plays a role in defense signaling upon challenging tobacco suspension cultures expressing $Cf\text{-}4^+$ with the race-specific elicitor AVR4. We report a rapid AVR4-induced accumulation of PA, followed by its conversion into DGPP. We found that the majority of this PA originates from the combined activities of PLC and DGK and we showed that PA is involved in the activation of the oxidative burst. The role of PA in AVR4-induced defense signaling in *Cf-4*-containing tobacco cells is discussed.

Results

AVR4 Triggers a Rapid and Dose-Dependent PA response in Cf-4⁺-Tobacco Cells

To study phospholipid signaling during the *Cf-4/Avr4*-mediated defense response, suspension-cultured *Cf-4*⁺-tobacco cells were incubated with $^{32}P_i$ for 3 hr to allow incorporation of label into structural phospholipids. After treatment with different concentrations of AVR4 or the non-matching elicitor AVR9 as a control, lipids were extracted and analyzed by ethylacetate-thin layer chromatography (TLC). As shown in Figure 1A, AVR4 treatment triggered a dose-dependent increase in PA. Chromatographing the same samples in an alkaline TLC system demonstrated that diacylglycerol pyrophosphate (DGPP), the phosphorylated conversion product of PA, also increased in a similar manner (Figure 1C). AVR9 did not trigger either of these responses (Figures 1B and 1D), demonstrating the specificity of the response.

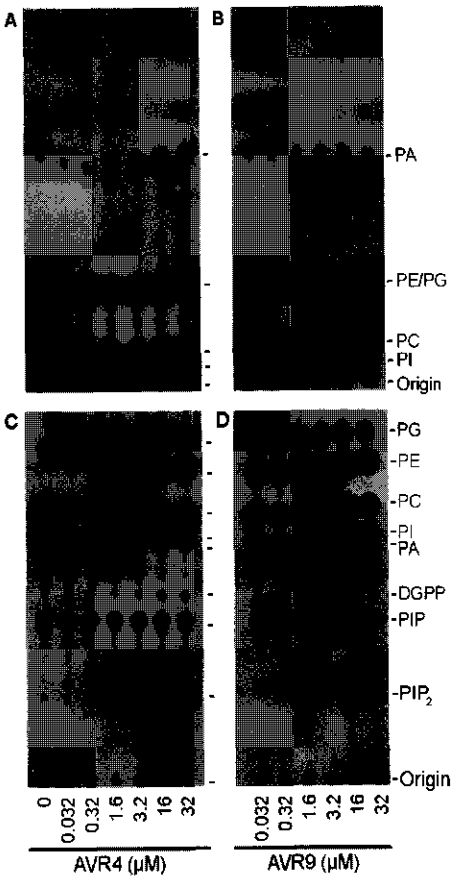


Figure 1. AVR4 Specifically Triggers the Accumulation of PA and DGPP in *Cf-4*⁺-Tobacco Cells in a Dose-Dependent Manner.
Cf-4⁺-tobacco cells were labeled for 3 hr with $^{32}P_i$ and subsequently treated for 15 min with different concentrations of AVR4 or AVR9. Phospholipids were extracted, separated by an ethylacetate (EtAc-TLC) or alkaline TLC system and visualized by autoradiography. The PA response was best visualized by EtAc-TLC and the DGPP response by alkaline TLC.
***(A)** Lipid profile of Cf-4⁺-tobacco cells after treatment with AVR4, analysis by EtAc-TLC*
***(B)** Lipid profile of Cf-4⁺-tobacco cells after treatment with AVR9, analysis by EtAc-TLC*
***(C)** Lipid profile of Cf-4⁺-tobacco cells after treatment with AVR4, analysis by alkaline TLC*
***(D)** Lipid profile of Cf-4⁺-tobacco cells after treatment with AVR9, analysis by alkaline TLC*

Quantification of the TLC spots by phospho-imaging confirmed the dose-dependent increases in PA and DGPP levels (Figure 2). In general, when compared to treatment with

AVR9, 32 µM AVR4 induced a 5-fold increase in both PA and DGPP. AVR4 did not induce PA accumulation in tobacco cell suspensions expressing *Cf-9*, demonstrating that AVR4-induced PA accumulation is dependent on the presence of the *Cf-4* gene (data not shown).

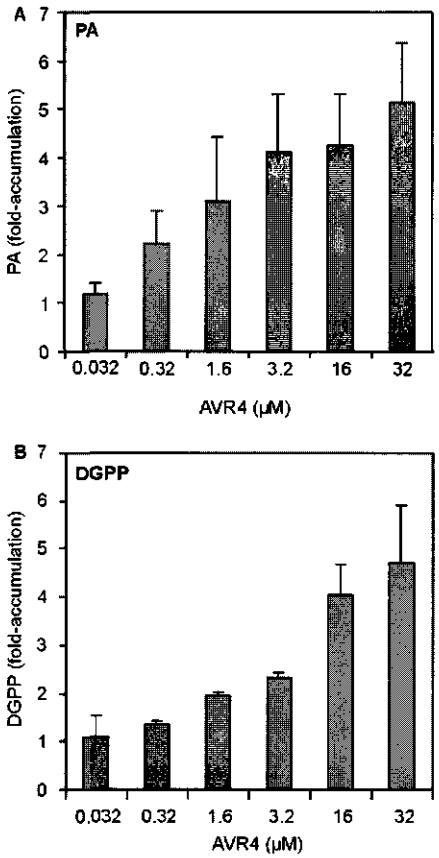


Figure 2. Quantification of PA and DGPP Accumulation in Time after Treatment of *Cf-4*$^+$-Tobacco Cells with Different Concentrations of AVR4.

(A) The fold increase of PA levels in AVR4-treated Cf-4$^+$-tobacco cells with regard to PA levels in the AVR9-treated control. Data are represented as the means of four independent experiments with the standard deviations indicated.

(B) The fold increase of DGPP levels in AVR4-treated Cf-4$^+$-tobacco cells with regard to DGPP levels in the AVR9-treated control. Data are represented as the means of four independent experiments with the standard deviations indicated.

To investigate the timing of PA and DGPP accumulation, cells were treated with 3.2 µM AVR4 over different periods of time. Treatment with AVR9 was again used as a control. Samples were analyzed using both TLC systems. As shown in Figure 3, treatment with AVR4 resulted in an increase of PA level within 2 to 4 min, whereas the PA level in the AVR9-treated controls remained constant (compare Figures 3A and 3B). The level of DGPP increased slightly later, between 4 and 8 min after addition of AVR4, while AVR9 had no effect on the DGPP level (compare Figures 3C and 3D). The delay between the PA and DGPP responses probably reflects the conversion of PA to DGPP (Munnik et al., 1996).

Figure 3. PA and DGPP Rapidly Accumulate Upon Treatment of *Cf-4*$^+$-Tobacco Cells with AVR4.

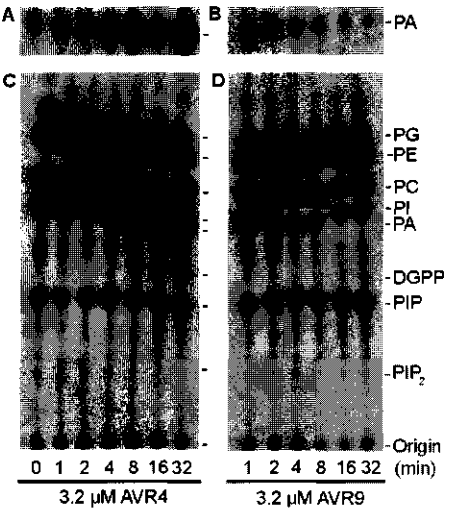
Cf-4$^+$*-tobacco cells were labeled with* $^{32}P_i$ *for 3 hr and subsequently treated with 3.2 μM AVR4 or AVR9. At t=0, 1, 2, 4, 8, 16 and 32 min, cells were harvested and their phospholipids extracted and separated.*

(A) *AVR4 treatment results in PA accumulation between 2 and 4 min after treatment of Cf-4*$^+$*-tobacco cells with AVR4, analysis by EtAc-TLC.*

(B) *AVR9-treatment does not result in PA accumulation, analysis by EtAc-TLC.*

(C) *AVR4 treatment results in DGPP accumulation between 4 and 8 min after treatment of Cf-4*$^+$*-tobacco cells with AVR4, analysis by alkaline TLC.*

(D) *AVR9-treatment, does not result in DGPP accumulation, analysis by alkaline TLC.*



These results demonstrate that AVR4 specifically induces PA and DGPP formation upon treatment of tobacco cells expressing the *Cf-4* gene and the kinetics of their formation illustrate that this is one of the fastest responses measured for this system.

AVR4-Induced PA Accumulation Is Generated by DGK

In general, PA is generated mainly via PLC and/or PLD pathways (Munnik, 2001). In order to determine which pathway is induced by AVR4, a differential $^{32}P_i$-labeling protocol was used which has been described earlier in more detail using the green algae *Chlamydomonas* (Munnik, 2001; Munnik et al., 1998b). In brief, the method is based on the fact that $^{32}P_i$ is only slowly incorporated into structural phospholipids, which are the substrate for PLD, but is quickly incorporated into the ATP pool, from which DAG is phosphorylated by diacylglycerol kinase (DGK) to ^{32}P-PA (^{32}P-PA_{DGK}). In contrast, the PA derived from PLD activity (PA_{PLD}) will only be radio-labeled when its precursor, a structural phospholipid, is already radio-labeled which, however, requires relatively long $^{32}P_i$-labeling times. Consequently, a short labeling period strongly favors the incorporation of radiolabel in ^{32}P-PA_{DGK} rather than in ^{32}P-PA_{PLD}. Conversely, long labeling times favor incorporation of radiolabel in ^{32}P-PA_{PLD}.

First, the incorporation of ^{32}P into *Cf-4*$^+$ tobacco cells was studied to determine the lipid labeling kinetics. As shown in Figure 4A, the structural lipids phosphoinositol (PI), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), which are labeled during *de novo* synthesis, were more slowly labeled than the minor lipids phosphatidylinositol phosphate (PIP) and PA, which directly receive their label from ^{32}P-ATP. Accordingly, cells were labeled for only 2 min instead of 3 hr prior to treatment with AVR4 or AVR9. AVR4 triggered a rapid increase in the level of ^{32}P-PA before significant labeling of the structural lipids occurred (Figure 4B) proving that radioactivity is incorporated in PA from ^{32}P-ATP via DGK.

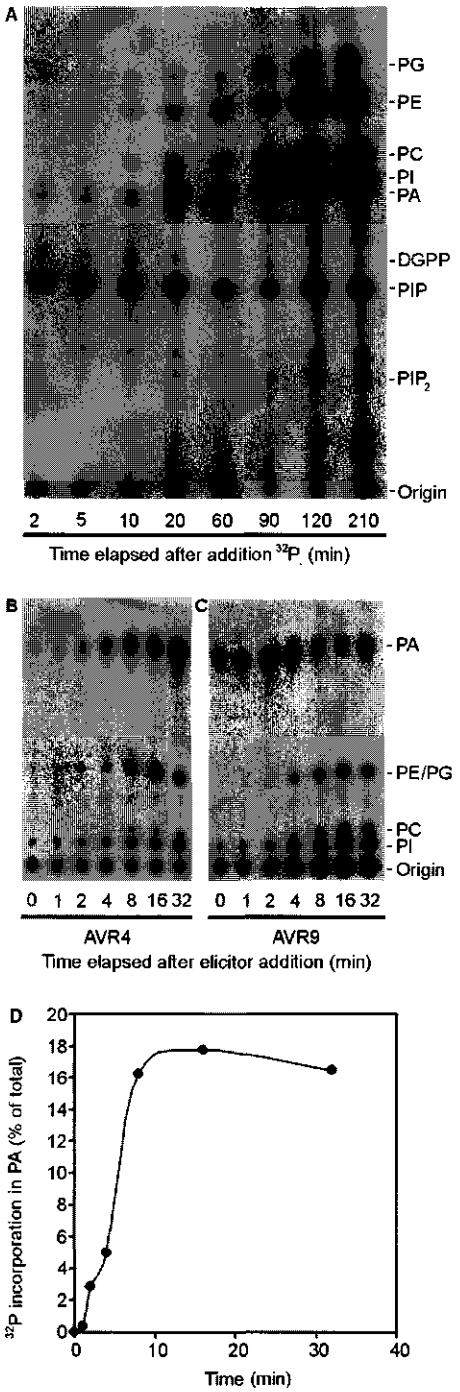


Figure 4. The AVR4-Induced PA Response Is Predominantly Generated via Phosphorylation of DAG.

To distinguish between PLD- and DGK-generated PA, a differential ^{32}P labeling protocol was used.

***(A)** Cells were labeled with $^{32}P_i$ for different times and the extracted phospholipids were analyzed on alkaline TLC to visualize the accumulation of radioactivity in the various phospholipids.*

***(B)** $^{32}P_i$ was added to Cf-4$^+$-tobacco cells, 2 min prior to treatment with 3.2 μM AVR4. Cells were harvested at t=0, 1, 2, 4, 8, 16 and 32 min after elicitor treatment and the extracted phospholipids were analyzed by EtAc-TLC analysis.*

***(C)** As in **(B)** but after treatment with 3.2 μM AVR9.*

***(D)** Using phospho-imaging, the percentage of radiolabel incorporated in ^{32}P-PA with regard to total incorporated label into all phospholipids was calculated for both AVR4- and AVR9-treated cells. Subtracting the percentage of radio-activity incorporated in PA from AVR9-treated control cells from that of AVR4-treated cells, yielded the net AVR4-induced PA accumulation.*

In AVR9-treated cells no significant increases in PA accumulation were observed (Figure 4C). In figure 4D the AVR4-induced PA accumulation subtracted by the increase in PA

accumulation in the AVR9-treated cells is presented, yielding the net AVR4-induced PA accumulation. This curve illustrates rather dramatically that AVR4 induces PA accumulation via DGK activity with a lag time of only 2 min.

In order to test whether PLD also contributes to the elicitor-activated PA formation, the ability of this enzyme to transfer the phosphatidyl group of its substrate to a primary alcohol, such as 1-butanol, is used. The production of phosphatidylbutanol (PBut) can then be used as a relative measure of PLD activity (Munnik, 2001; Munnik et al., 1995). PLD activity was therefore tested in cells that were pre-labeled with $^{32}P_i$ for 3 to 16 hr to ensure accumulation of radiolabel into structural lipids. The cells were subsequently treated with AVR4 or AVR9 for different periods of time in the presence of 0.5 % (v/v) 1-butanol. No clear increase in PBut formation was detected (results not shown) confirming that AVR4-induced PA accumulation mainly results from phosphorylation of DAG.

AVR4-Induced PA Accumulation Is Blocked by PLC Inhibitors

The AVR4-induced PA accumulation resulting from an increase in DGK activity could reflect an increase in the concentration of the substrate of DGK, for example due to the activation of PLC that hydrolyzes phosphoinositol 4,5 bisphosphate (PIP_2) to produce DAG. In support of this, we regularly observed a decrease in levels of PIP_2 after AVR4 treatment. However, this effect was inconsistent because it was small and there was little radioactivity incorporated in PIP_2 (results not shown). We therefore used the PLC inhibitors neomycin and U73122 to test whether they reduced DAG production and as a result also reduced the accumulation of PA.

To test for the involvement of PLC in AVR4-induced PA accumulation, *Cf-4*$^+$-tobacco cells were pre-incubated for 2 min with $^{32}P_i$, in the presence of neomycin or U73122. Either AVR4 or AVR9 were subsequently added and the cells were extracted for TLC analysis after 8 min. As shown in Figure 5A, neomycin inhibited the AVR4-induced PA accumulation in a dose-dependent manner. As expected, the AVR9 control did not show any PA accumulation and PA levels were not affected by the inhibitor (Figure 5B).

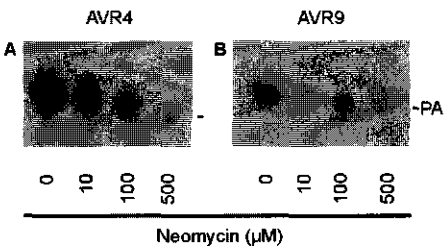


Figure 5. AVR4-Induced PA Accumulation in *Cf-4*$^+$-Tobacco Cells Is Blocked by the PLC Inhibitor Neomycin.

$^{32}P_i$, and neomycin to a final concentration of 0, 10, 100 or 500 µM were added 2 min prior to elicitation. Cells were then treated for 8 min with 3.2 µM AVR4 or AVR9, after which the lipids were analyzed by EtAc-TLC. Only the relevant part of the autoradiogram is shown.

(A) ^{32}P-PA accumulation in AVR4-treated Cf-4$^+$-tobacco cells.

(B) ^{32}P-PA accumulation in AVR9-treated Cf-4$^+$-tobacco cells.

Since the uptake of radioactivity and incorporation into lipids was not disturbed by these inhibitors, cell viability appeared unaffected. This was confirmed by fluorescein diacetate/propidium iodine double staining as described previously (Oparka and Read, 1994) (results not shown). The PLC inhibitor U73122 also inhibited the AVR4-induced PA-response, whereas its less active analogue (U73343) did not (data not shown). The inhibition was approximately 80% using a concentration of 10 μM U73122. Since both PLC inhibitors effectively repressed the AVR4-induced PA accumulation in *Cf-4*$^+$ tobacco cells, the production of DAG as a result of PLC activation appears to be the major cause of the increase in PA levels.

AVR4-Induced PLC Signaling in Relation to the Oxidative Burst

Because AVR4 induces PA production within 2 min after addition of this elicitor to the cells, PA is likely to be part of a signaling pathway. If that is the case, inhibition of AVR4-induced PA accumulation should affect AVR4-induced down-stream responses, rather than the other way round. Consequently, we tested whether the PLC inhibitors blocked the AVR4-induced oxidative burst just as they repressed PA accumulation. Cells were pre-treated with different concentrations of inhibitors, after which AVR4 was added and the formation of ROS was measured by the quenching of pyranin (see Methods). Inhibition of the AVR4-induced oxidative burst by U73122 or its less active analogue U73343 is detected as a reduction in quenching of pyranin fluorescence (Figure 6). The oxidative burst was inhibited in a dose-dependent manner by U73122 but not by its less active analogue. Unfortunately, neomycin interfered with the assay, precluding its usefulness.

It was previously demonstrated that diphenyleneiodonium chloride (DPI), an irreversible inhibitor of the oxidative burst, completely blocked this response induced by AVR9 in *Cf-9*$^+$-cells (Piedras et al., 1998). Similarly, we found that the AVR4-induced oxidative burst in *Cf-4*$^+$-cells was completely inhibited by a 2 min pre-treatment with 1 or 10 μM DPI (data not shown). If AVR4-induced PA signaling is upstream of the oxidative burst then DPI should have no effect on AVR4-induced PA accumulation. To test this, cells were labeled for 2 min with $^{32}P_i$ in the presence of 1 or 10 μM DPI, prior to treatment with AVR4 or AVR9. After 8 minutes the lipids were extracted and chromatographed. The autoradiogram showed that DPI had no effect on the induction of PA accumulation (Figure 7) indicating that PLC activation and PA production occurs upstream of the oxidative burst.

Synthetic, Short-Chain PA Induces a Small and Transient Oxidative Burst

If PA signaling is upstream of the oxidative burst, it should be possible to induce this response by adding exogenous PA. There are limitations to administering native PA, because it is a lipid that is relatively insoluble in water. However, synthetic analogues with short fatty acid chains are more soluble and can sometimes mimic responses that are downstream of PLC and PLD signaling.

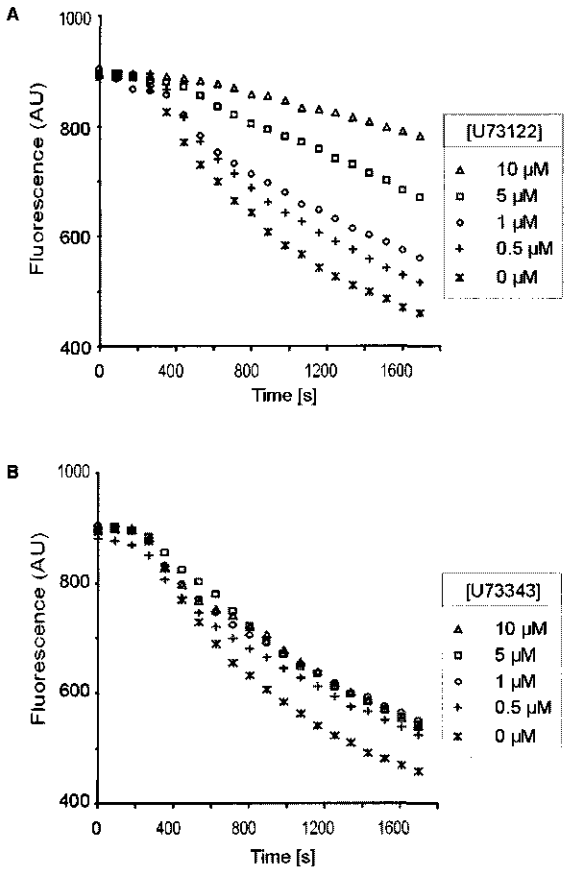


Figure 6. AVR4-Induced Oxidative Burst Is Blocked by the PLC Inhibitor U73122.
Cells were treated with different concentrations (0, 0.5, 1, 5 or 10 µM) of the PLC inhibitor U73122 or its less active analogue U73343, 2 min prior to AVR4 treatment. The reduction of fluorescence caused by the oxidative burst was recorded immediately after addition of AVR4 to the cells.
(A) AVR4-induced oxidative burst in the presence of different concentrations of the PLC inhibitor, U73122
(B) AVR4-induced oxidative burst in the presence of different concentrations of the control compound, U73343

To investigate whether the cells are responsive to exogenous PA, the effect of a water-soluble, synthetic PA analogue (PA_{syn})(see Methods) on the oxidative burst was tested.

As shown in Figure 8A, a small, transient, dose-dependent oxidative burst was detected immediately after addition of PA_{syn}, whereas the AVR4-induced oxidative burst showed a distinct lag but lasted longer (Figure 8A). Furthermore, addition of the PA_{syn} to a final concentration of 500 µM, resulted in some cell death (data not shown). A water-soluble, synthetic analogue of the structural phospholipid PC (PC_{syn})(see Methods) was used as a control and was found to be completely inactive (Figure 8B). Although the oxidative burst results in a decrease in fluorescence due to degradation of the pyranin probe, a small increase was sometimes found after addition of the lipids to the cells. Whether this was the result of a physiochemical or a biological process (e.g. the production of phenolics) is not known. We showed that addition of PA_{syn} to *Cf-4*$^{+}$-tobacco cells induced a small but transient oxidative burst. Conversely, when AVR4-induced PA accumulation was inhibited, the AVR4-induced oxidative burst was also blocked. Inhibition of the oxidative burst did not inhibit AVR4-induced PA accumulation. These data indicate that PLC signaling is located upstream of the AVR4-induced oxidative burst.

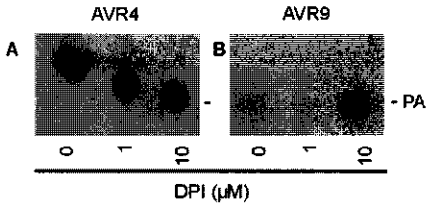


Figure 7. AVR4-Induced PA Accumulation in *Cf-4*$^+$-Tobacco Cells Is not Inhibited by the NADPH Oxidase Inhibitor DPI.
DPI was added to the cells to a final concentration of 0, 1 or 10 μM, together with $^{32}P_i$, 2 min prior to treatment with 3.2 μM of AVR4 or AVR9. Cells were harvested 8 min later for EtAc-TLC analysis of the phospholipids. Only the relevant part of the autoradiogram is shown.
*(**A**) PA levels in AVR4-treated Cf-4$^+$-tobacco cells in the presence of different concentrations of DPI.*
*(**B**) PA levels in AVR9-treated Cf-4$^+$-tobacco cells in the presence of different concentrations of DPI.*

Discussion

PA Accumulation Is Specifically Induced by AVR4 in Tobacco Cells Expressing Cf-4

We have shown that AVR4 specifically induces the accumulation of PA in *Cf-4*$^+$-tobacco cells. The effect was detectable within 2 min after elicitation and clearly preceded other rapid responses, such as the oxidative burst (Figure 6). The effect is *Cf-4*-specific and dose-dependent. Since PA has an established role as a second messenger, the speed and specificity of the response confirmed that PA is also acting as a signaling component in *Cf-4/Avr4*-mediated activation of defense responses. This is supported by the rapid conversion of PA to DGPP, which only occurs under conditions that activate cell signaling (Munnik, 2001). We presume this PA metabolism is part of an attenuation mechanism that eventually returns PA to pre-stimulation concentrations, but we cannot exclude the possibility that DGPP itself is a signaling molecule.

AVR4-Induced PA Accumulation Is Generated via DGK

The second messenger PA can be synthesized by two enzymes, PLD and DGK. Upon *Cf-4*-mediated recognition of AVR4, an increase in PLD-based transphosphatidylation with 1-butanol was not clearly detected. Yet we readily detected such an increase when the same cells were osmotically stressed (data not shown; (Munnik et al., 2000), demonstrating that PLDs involved in signaling are present in our experimental system but were not activated by AVR4. However, this does not exclude a role for PLD, because some members of this class of enzymes do not transphosphatidylate. We emphasize this point because PLDα has recently been implicated in superoxide production in Arabidopsis (Sang et al., 2001). In contrast to PLD activity, an AVR4-induced increase in DGK activity was readily demonstrated. We pre-labeled cells for just 2 min, thereby creating conditions that strongly favor the incorporation, and consequently the visualization of ^{32}P-PA$_{DGK}$, but severely limit the production of ^{32}P-PA$_{PLD}$. When AVR4 was added, the accumulation of ^{32}P-PA was more pronounced than under long labeling conditions, which allow ^{32}P-PA$_{PLD}$ detection. Conversely, labeling times as long as 16 hr did not clearly reveal ^{32}PBut accumulation in the presence of 1-butanol nor did it enhance incorporation of ^{32}P into PA after AVR4

treatment (results not shown). These data clearly indicate that DGK is the major if not the only direct contributor to PA accumulation.

The increase in DGK activity may be either explained by AVR4-induced biochemical activation of the enzyme or simply by an increase in concentration of its substrate, DAG. Our data support the latter without detracting from the former.

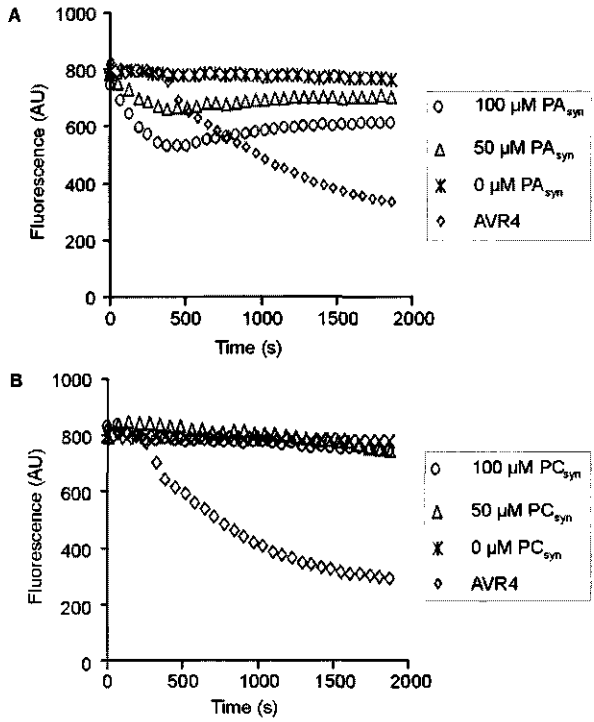


Figure 8. PA_{syn} Induces an Oxidative Burst in *Cf-4*$^+$- Tobacco Cells.

Cells were treated with different concentrations of PA_{syn}, or PC_{syn}, as described in methods. Directly after addition of the lipids or the AVR4 control treatment, quenching of pyranin fluorescence was recorded as a measure of the oxidative burst.

(A) Oxidative burst after treatment with different concentrations of PA_{syn} or 0.32 µM AVR4.

(B) Oxidative burst after treatment with different concentrations of PC_{syn} or 0.32 µM AVR4.

PLC Inhibitors Block AVR4-Induced PA Accumulation

The most common contributor to the accumulation of DAG involved in signal transduction, is PLC. The best way to demonstrate PLC activity is by monitoring the turnover of its substrate, PIP_2. Unfortunately, the extremely low levels and rapid turnover of PIP_2 in higher plants often render this approach impractical (Munnik et al., 1998a; Munnik et al., 1998b). Consequently, despite indications for AVR4-induced depletion of PIP_2 levels in *Cf-4*$^+$-tobacco cells, the limited detection of ^{32}P-PIP_2 did not allow us to register a consistent effect. Therefore, we used different inhibitors to confirm AVR4-induced PLC activity. The results were convincing, because both the PLC inhibitors neomycin and U73122 blocked PA production in a dose-dependent manner. Thus, AVR4 seems to activate PLC and the DAG that it produces is immediately converted to PA by DGK. Being an important intermediate in lipid biosynthesis, DAG is normally present in high concentrations in the plant cell. However, this metabolic DAG pool is normally neither available for signaling nor is it subject to rapid fluctuations in size, thereby excluding that increases in PA levels

are a reflection of fluctuations in levels of the metabolic DAG pool of the cell. In support of this, all the autoradiograms we have presented demonstrate that general phospholipid synthesis was completely unaffected by either AVR4 treatment or other experimental conditions applied to the cells, indicating that the metabolic DAG pool was stable for the duration of our experiments. These data confirm that AVR4-induced PA accumulation is due to a specific signaling event, rather than a reflection of fluctuations in phospholipid metabolism caused by experimental conditions.

A Role for PA in Activation of the AVR4-Induced Oxidative Burst

PA has two general effects in cells. First, due to its negative charge, it affects membrane surface properties, influencing membrane curvature (Munnik, 2001). In this way, PA could play a role in vesicle trafficking and secretion. Second, it acts as a specific membrane-docking site for proteins with a PA-binding domain. Such a domain has been identified in the small mammalian G-protein Raf-1 and is conserved in the plant homologue CTR1 (Munnik, 2001). Upon binding of PA, signaling proteins are locally concentrated and the assembly of signaling complexes is promoted, or alternatively, the conformation of the protein is affected and consequently, its activity. Specific PA targets are being identified in plants and animals (reviewed in Munnik, (2001)). Of particular significance for this study are the reports that PA activates an NADPH oxidase complex in neutrophils (Erickson et al., 1999; McPhail et al., 1999; Waite et al., 1997). A PA-dependent protein kinase triggers the assembly of this complex at the plasma membrane, where it generates an oxidative burst. Plant homologues that form the NADPH complex have been identified (Torres et al., 2002; Torres et al., 1998; Xing et al., 1997), but their dependence on PA has not been tested. Similarly, PA has been shown to activate a CDPK (Farmer and Choi, 1999) and a MAPK pathway (Lee et al., 2001).

In analogy with the situation in neutrophils, PA appears to be important for invoking an oxidative burst in tobacco suspension cells. When AVR4-induced PA synthesis is inhibited by neomycin and U73122, the oxidative burst is also inhibited, and when PA_{syn} is added to unelicited cells, an oxidative burst is induced. PA also induces an oxidative burst in Arabidopsis cells (Sang et al., 2001). Compared to an elicitor-induced oxidative burst, the effects of an exogenously added PA_{syn} in tobacco were weak, being about 25% of the AVR4-induced oxidative burst, probably reflecting the use of an unnatural PA species with short-chain fatty acids. Using short-chain PA_{syn} that is more hydrophylic as compared to endogenous PA, was essential as this molecule had to be administered via the water phase. However, this approach has the disadvantage that PA_{syn} added to cell suspensions will partition non-discriminately into hydrophobic structures of the cell. Furthermore, the presence of the short-chain PA_{syn} in the correct membrane leaflet may only weakly mimic the topographic properties of native PA.

Does PA also Play a Role in other AVR4-Induced Downstream Responses?

Because PA_{syn} is biologically active, we also tested its potential for activating the medium alkalization response. However, while the AVR4-induced alkalization and MAP kinase activation were blocked by PLC inhibitors in a dose-dependent way, we could not mimic the response by the addition of PA_{syn} (data not shown). An extra limitation of this treatment is that it represents only one of the second messengers generated by PLC. The other is IP_3, inducing release of Ca^{2+} from intracellular stores. While PLC inhibition down-regulates the effects of both second messengers, adding PA_{syn} only mimics the effects induced by one second messenger. However, it is likely that some downstream responses require input from both second messengers for their activation. In the future, it may be possible to induce the oxidative burst, medium alkalization and MAP kinase activation by administering IP_3 in combination with native PA, in caged forms. This approach allows passage of both second messengers over the plasma membrane and their subsequent controlled release into the interior of the cell.

Cf-9/Avr9-*Mediated Lipid Signalling*

When *Cf-9*$^+$-tobacco cells were treated with AVR9 no, or only a very small increase of PA level was observed (data not shown) although AVR9 did elicit an oxidative burst and medium alkalization in these cells. In a previous study, Piedras et al. (1998) presented evidence for the involvement of phospholipase A_2 (PLA_2) in the *Cf-9/Avr9*-mediated oxidative burst in tobacco. PLA_2 hydrolyzes phospholipids at the *sn-2* position into either arachidonic acid or linoleic acid and lyso-phosphatidic acid (lyso-PA), depending on the substrate. Inhibitors of PLA_2 blocked the AVR9-induced oxidative burst and MAP kinase activation in *Cf-9*$^+$-tobacco cells whereas treatment of the cells with arachidonic or linoleic acid resulted in a small oxidative burst (Piedras et al., 1998; Romeis et al., 1999). Both lipids cannot be detected by autoradiography as these lipids do not carry a phosphate group. Lyso-PA can be detected but no changes in levels of this lipid were observed after either AVR4 treatment of *Cf-4*$^+$-tobacco cells or AVR9 treatment of *Cf-9*$^+$-tobacco cells (data not shown). Currently, tobacco cell suspensions expressing both *Cf-4* and *Cf-9* are being established to study whether differences between *Cf-4*- and *Cf-9*-mediated defense signaling exist.

A Model for Cf-4/AVR4-Induced Defense Signaling

In summary, we have shown that PA plays an important role in the generation of the oxidative burst in plant defense. In human neutrophils, PA is involved in NADPH oxidase activation. In this system PA can be generated via PLC with DAG as an intermediary, or directly via PLD activity see (Erickson et al., 1999) and references therein. We propose the following model for the involvement of PA in the AVR4-induced oxidative burst in *Cf-4*$^+$-tobacco cells (Figure 9).

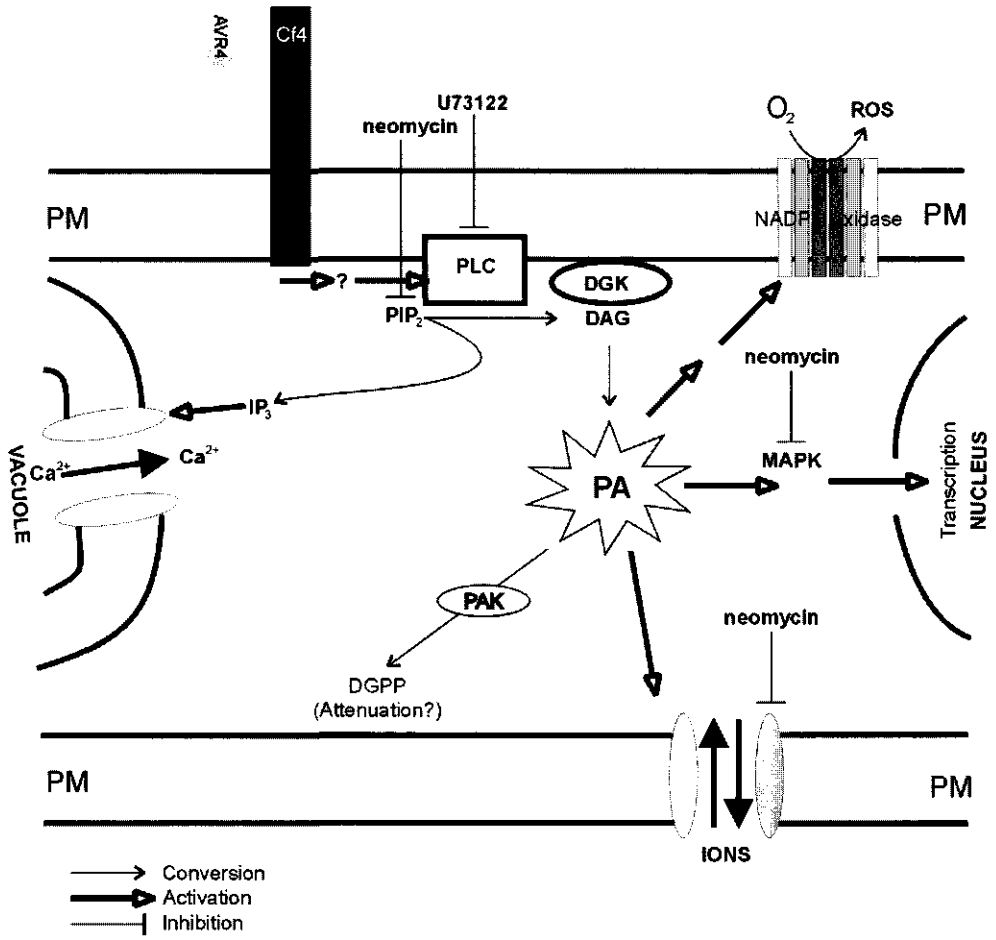


Figure 9. Model for the Role of PA in *Cf-4/Avr4*-mediated Defense Signal Transduction.
Upon AVR4 perception, PLC is activated to hydrolyze PIP_2 into IP_3 and DAG. IP_3 diffuses into the cytosol where it releases Ca^{2+} from intracellular stores, whereas DAG remains in the membrane and is converted by DGK into PA. PA may directly or indirectly activate NADPH oxidase, resulting in the production of ROS. At the same time, PA directly or indirectly, creates the proper conditions in the plasma membrane for other signaling processes to occur, such as ion channel opening, or activation of other components of plant defense signaling like MAP kinases or other protein kinases. (for further details: see text) (PM: plasma membrane).

Upon AVR4 perception by a Cf-4 receptor (complex), PLC is activated and hydrolyzes PIP_2 into DAG and IP_3. IP_3 migrates into the cytosol where it may open calcium channels on the vacuole. DAG is converted into PA by DGK, and PA directly or indirectly activates the NADPH oxidase. Interestingly, in Arabidopsis PLD is involved in activation of the oxidative burst (Sang et al., 2001), suggesting that in plants too redundant signaling pathways for NADPH oxidase exist. In human neutrophils, a PA-activated Protein Kinase (PAPK) has been shown to phosphorylate components of the NADPH oxidase complex (McPhail et al., 1999). In the future, biochemical studies using cell free systems to identify

PA targets will be necessary to unravel how PA activates NADPH oxidase in plants. The inhibition of AVR4-induced medium alkalization and MAP kinase activation by PLC inhibitors suggests additional roles in plant defense signaling for this enzyme. Interestingly, using cDNA-AFLP analysis to identify genes that are differentially expressed during the *Cf-4/Avr4*-mediated defense response, a cDNA fragment with a very high homology to a human PLC was identified. This finding further substantiated the involvement of PLC in *Cf-4/Avr4*-mediated signaling. Studies are currently underway to isolate this gene and characterize its product and subsequently assess its role in AVR4-induced defense responses.

Methods

Preparation of Elicitors

The race-specific elicitor AVR9 of the tomato pathogen *Cladosporium fulvum* was chemically synthesized and the correctly folded product was purified by HPLC according to the procedure described earlier (Mahé et al., 1998). The race-specific elicitor AVR4 of *C. fulvum* was produced in a heterologous *Pichia pastoris* expression system (Invitrogen, USA) and purified from the culture filtrate (Van den Burg et al., 2001).

Cultivation of Cell Suspensions

Cell suspensions from *Cf-4*$^+$- and *Cf-9*$^+$-*N. tabacum* cv "Petit Havana SR1" were cultivated as described previously (De Jong et al., 2000). Briefly, cells were grown in 300 mL Erlenmeyer flasks containing 50 mL MS medium (Murashige and Skoog, 1962), supplemented with Gamborg B5 vitamin mixture (Gamborg et al., 1968), 1 mg/L 2,4-dichlorophenoxyacetic acid and 0.1 mg/L kinetin. The flasks were incubated at 25°C in the dark on a rotary shaker at 110 rpm. Each week, 8 mL aliquots of cells growing in the log phase were transferred to fresh medium. Cells were used 3 or 4 days after sub-culturing.

^{32}P Phospholipid Labeling and Analyses

For labeling experiments, 1.5 mL *Cf-4*$^+$ tobacco cell suspension was transferred to a small Petri-dish (diameter 35 mm) and incubated with 150 µCi carrier-free $^{32}PO_4^{3-}$ (Amersham, 's-Hertogenbosch, The Netherlands) for 1 hr. Ten mL of the remaining cell suspension was used to prepare cell-free medium (CFM) by passing it through a sterilization filter. After 1 hr, the labeled cells were divided into aliquots of 85 µL in 2 mL Eppendorf vials and allowed to label further for another 2 hr unless stated otherwise. Elicitor was added in 85 µL CFM to yield the desired final concentration. For PLD assays, 0.5% (v/v) 1-butanol (final concentration) was included (Munnik et al., 1995).

For short-labeling experiments, 42.5 µL of cell suspension was equilibrated in a 2 mL Eppendorf tube for 2 hr. Two min prior to the addition of elicitor, 20 µL CFM containing 5 µCi $^{32}PO_4^{3-}$, with or without inhibitor, was added to the cells. Elicitor, dissolved in 75 µL CFM in different concentrations, was subsequently added. Incubations were stopped by

adding 20 µL 50% perchloric acid and subsequently freezing in liquid nitrogen. Lipids were extracted by adding 3.75 vol. $CHCl_3$/MeOH/HCl (50:100:1 (v/v/v)) and processed as described before (Munnik et al., 1996).

Lipids were separated on Silica-60 TLC plates (Merck, Darmstadt, Germany) employing an alkaline ($CHCl_3$/MeOH/25% NH_4OH/H_2O; 90:70:4:16 (v/v/v/v)) (Munnik et al., 1994) or an EtAc (EtAc/iso-octane/HCOOH/H_2O;12:2:3:10 (v/v/v/v)) (Munnik et al., 1998b) mobile phase. The alkaline TLC system was used to separate all phospholipids and the EtAc system was specifically used to separate PA from the other phospholipids and for PLD measurements. Radioactivity was visualized by autoradiography and quantified by phospho-imaging (Molecular Dynamics, Sunnyvale, CA, USA). The radioactivity of a lipid was calculated by dividing it by the radioactivity incorporated in all lipids. The fold-increase was calculated by dividing the lipid-radioactivity in the stimulated cells by the lipid-radioactivity in the corresponding control.

Handling of Lipids

The water-soluble, short-chain, PA analogue (PA_{syn}), L-alpha-phosphatidic acid, dioctanoyl C8:0, and the water-soluble, short-chain, PC analogue (PC_{syn}), phosphatidyl choline, didecatanoyl C10:0, were obtained from Sigma (St Louis, Mo). A stock solution was prepared by dissolving 50 mg of the compound in 500 µL $CHCl_3$:HAc (95:5 (v/v)). Prior to use, fresh aquous solutions were prepared by transferring the required amount of stock solution into a glass tube and drying it under a stream of nitrogen for 20 min. The required volume of assay buffer or cell free medium was then added and the tube sonicated in a bath for 1 min, and cooled on ice for 1 min. This procedure was repeated 4 times. The same procedure was followed for PC_{syn}.

Medium Alkalization Assay

Medium alkalization assays were performed as described previously (De Jong et al., 2000). Briefly, 3- or 4 day-old cell suspensions were pooled to obtain the volume required for an experiment. They were then distributed in 7.5 mL aliquots in 25 mL sterile Erlenmeyer flasks and incubated overnight on a rotary shaker at 220 rpm at 20°C. The next day, cells were treated with elicitor and the medium alkalization was recorded using a mini pH electrode (Mettler Toledo, InLab 423).

Oxidative Burst Assay

The oxidative burst assay was performed as described previously (De Jong et al., 2000), except that it was scaled up to 24 wells micro-titer plates. Four days after transfer, *Cf-4*$^+$-tobacco cells were pelleted at 450 *g* for 8 min and resuspended in 50 mL assay buffer (5 mM MES/NaOH, pH 5.7, 175 mM mannitol, 0.5 mM K_2SO_4, 0.5 mM $CaCl_2$) and allowed to equilibrate at 20°C for 20 min on a gyratory shaker (120 rpm). This procedure was repeated three times after which 5 g of cells were re-suspended in 50 mL assay buffer.

Using large orifice pipette tips, aliquots of 250 µL were distributed over 24 wells microtiter plates and allowed to equilibrate overnight at 20°C.

To measure the elicitor-induced oxidative burst, 250 µL assay buffer, supplemented with the elicitor and the fluorescent probe pyranin (Apostol et al., 1989), was added to the cells to produce the desired final concentration of elicitor and a final concentration of 10 µg/mL pyranin. The quenching of pyranin fluorescence due to the production of H_2O_2 was recorded at 1.5 min intervals using an excitation wavelength of 405 nm and emission wavelength of 512 nm in a Perkin Elmer fluorescence spectrophotometer. Inhibitors were added 10 min prior to addition of elicitor and probe.

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Chapter 4

Attenuation of *Cf*-Mediated Defense Responses at Elevated Temperatures Correlates with a Decrease in Elicitor-Binding Sites

This chapter will be published by Camiel F. De Jong, Frank L. W. Takken, Xinzhong Cai, Pierre J. G. M. de Wit and Matthieu H. A. J. Joosten

Abstract

The interaction between the fungal pathogen Cladosporium and its only host tomato, is a well-described gene-for-gene system. Several plant resistance (*Cf*) genes and matching fungal avirulence (*Avr*) genes have been isolated from this pathosystem. The AVR4- and AVR9-induced hypersensitive response (HR) in leaves of tomato plants carrying *Cf-4* or *Cf-9*, respectively, appeared to be suppressed at elevated temperatures. Temperature-controlled experiments revealed that the *Cf*-mediated HR is completely suppressed at 33°C. Tomato seedlings expressing both a *Cf* and the matching *Avr* gene, rapidly die as a result of systemic necrosis at normal temperatures, but are rescued at 33°C. We demonstrate that at 33°C the *Cf/Avr*-mediated induction of defense-related genes is reversibly suppressed. Furthermore, in cell suspensions the AVR-induced medium alkalization response is slowly suppressed upon incubation at 33°C, but is quickly restored after transfer to lower temperatures. A high-affinity binding site (HABS) for AVR9 is present on plasma membranes isolated from Solanaceous plants and has been suggested to act as a co-receptor for AVR9. The amount of AVR9 HABS is 80% reduced in tobacco cell suspensions incubated at 33°C, as compared to cell suspensions incubated at 20°C. Our data indicate that temperature-sensitivity of *Cf*-mediated defense responses resides at the level of perception of the fungal avirulence factors.

Introduction

Plants are constantly challenged by various pathogens like viruses, bacteria, fungi, nematodes and insects. To cope with these foes, plants have developed mechanisms to recognize invading pathogens and subsequently mount active defense responses. In the gene-for-gene model as described by Flor (Flor, 1942), a plant resistance (*R*) gene confers resistance to strains of a pathogen that express the matching avirulence (*Avr*) gene. *R* gene-mediated recognition of an *Avr* gene product results in several biochemical and physiological responses that eventually culminate into a hypersensitive response (HR), resulting in an arrest of growth of the invading pathogen (Dixon et al., 1994; May et al., 1996).

Over the last years, several *Avr*s and their matching *R* genes have been cloned and characterized. *Avr* genes encode a very diverse array of proteins (Bonas and Van den Ackerveken, 1999; Joosten and De Wit, 1999; Laugé and De Wit, 1998; Padgett et al., 1997). In contrast, the majority of *R* genes encodes receptor-like proteins that contain leucine-rich repeats (LRRs), which are thought to be involved in protein-protein or receptor-ligand interactions (Bent, 1996; Hammond-Kosack and Jones, 1997; Jones and Jones, 1997). Overall, R proteins may be divided into three main classes; (i) receptor-like protein kinases, (ii) nucleotide-binding site (NBS)-LRR proteins and (iii) proteins with an extracellular domain containing LRRs and a small cytoplasmic region without any putative signaling domain (Takken and Joosten, 2000). The structure of R proteins suggests a role as receptor for AVRs. It is therefore surprising that to date only in a few cases a direct

interaction between an R protein and an AVR has been described (Jia et al., 2000; Tang et al., 1996).

An intensively studied gene-for-gene system is the interaction between the leaf mold pathogen Cladosporium and its only host, tomato (Joosten and De Wit, 1999). From this pathosystem the matching resistance (*Cf*) and *Avr* gene pairs *Cf-9/Avr9* (Jones et al., 1994; Van Kan et al., 1991), *Cf-4/Avr4* (Joosten et al., 1994; Thomas et al., 1997), *Cf-2/Avr2* (Dixon et al., 1996; Luderer et al., in press) and *Cf-4E /Avr4E* (Takken et al., 1998; Westerink et al., unpublished results), have been characterized. The *Cf* genes are similar to the proteins encoded by the tomato *Ve* genes, which confer resistance against Verticillium species (Kawchuk et al., 2001) and encode membrane-anchored receptor-like proteins that belong to the third class of LRR resistance proteins.

The lack of a cytoplasmic signaling domain suggests that the signaling function might be performed by a third component that interacts with the Cf protein (Joosten and De Wit, 1999). Binding studies performed with ^{125}I-AVR9 on plasma membranes isolated from tomato leaves revealed the presence of a high affinity binding site (HABS) for this peptide (Kooman-Gersmann et al., 1996). Interestingly, the HABS proved to be present on plasma membranes isolated from tomato leaves either lacking or containing *Cf-9*. This indicates that the Cf-9 protein confers the ability to recognize AVR9 but does not represent the HABS itself, and is not required for AVR9 binding to the HABS. In tomato plants containing *Cf-9*, for various mutant AVR9 peptides a clear positive correlation between HR-inducing activity and affinity for the HABS has been found, indicating that the HABS plays an essential role in AVR9 perception (Kooman-Gersmann et al., 1998). Further studies revealed that the HABS was present in all Solanaceous species tested (Kooman-Gersmann et al., 1996). The Cf-9 protein was produced in various heterologous expression systems and was analyzed for AVR9-binding affinity (Luderer et al., 2001). Although various techniques were employed to detect possible binding of AVR9 to Cf-9, no direct interaction between the two proteins could be detected. Transfer of *Cf-9* to other Solanaceous plants that contain the HABS confers the ability to recognize AVR9 (Hammond-Kosack et al., 1998; Van der Hoorn et al., 2000). Together with the lack of a direct binding of AVR9 to Cf-9, this suggests that the HABS may well be the AVR9 co-receptor that interacts with Cf-9 (Jones and Jones, 1997; Joosten and De Wit, 1999).

Cf-9-containing tomato cell suspensions do not respond to AVR9 (Honée et al., 1998). However, AVR9-treated *Cf-9*$^{+}$ transgenic tobacco cell suspensions do show typical defense responses, such as medium alkalization and an oxidative burst. Furthermore, activation of MAP kinases, a calcium-dependent protein kinase (CDPK) and cell death occurred (De Jong et al., 2000; Piedras et al., 1998; Romeis et al., 2000; Romeis et al., 1999). When experiments were performed on the bench rather than in a climate chamber, we observed that during warm days AVR9-induced early defense responses in such transgenic *Cf-9*$^{+}$-tobacco cell suspensions were significantly attenuated. Temperature-sensitivity has also been observed for other *R* genes. A classical example of temperature-sensitivity of resistance responses in Solanaceous plant species is the resistance response to TMV as

conferred by the *N* gene of tobacco (Samuel, 1931). Below 28°C, the *N* gene mediates resistance by specific recognition of the helicase of TMV (Padgett et al., 1997), whereas above this temperature the virus is able to infect the plant systemically. Although numerous examples of temperature-sensitive resistance have been described, the biochemical basis of this phenomenon is far from clear (Dropkin, 1969; Loegering and Giss, 1957; Samuel, 1931)

The availability of various matching *Cf/Avr* combinations and a very sensitive biological system provides us with the tools to study the various steps of the AVR-induced HR, from an event as early as AVR perception until its actual execution. This enabled us to systematically examine the effect of elevated temperatures on individual links in the chain of events leading from AVR perception to HR. Here we demonstrate that tomato seedlings, expressing both an *Avr* of Cladosporium and the matching *Cf* gene, die due to systemic HR at 20°C and are rescued at 33°C. Transfer of rescued seedlings to 20°C quickly results in induction of defense-related genes and subsequent development of systemic HR. Furthermore, AVR-induced medium alkalization in *Cf*-transgenic tobacco cell suspensions was efficiently suppressed at 33°C. Finally, we demonstrate that binding of ^{125}I-AVR9 to microsomal fractions isolated from cell suspensions incubated at 33°C was significantly reduced as compared to cells incubated at 20°C. The implications of these findings are discussed.

Results

AVR4- and AVR9-Induced HR in Tomato Leaflets Is Temperature-Sensitive

To test for temperature-sensitivity of AVR4- and AVR9-induced HR, MM-Cf4 and MM-Cf9 tomato plants were incubated either at 20°C or 33°C for 24 hr and leaflets were subsequently injected with 0.32, 3.2 and 32 µM AVR4 and AVR9, respectively. After injection, plants were incubated for another 48 hr at either 20°C or 33°C and the development of HR was assessed. Figure 1A shows that at 20°C, injection of 3.2 and 32 µM AVR4 induced HR in MM-Cf4 leaflets, whereas injection of 0.32 µM AVR4 did not result in any HR. At 33°C, only a slight chlorosis but no clear necrosis was observed after injection of the AVR4 concentration series. At 20°C, injection of the same concentration series of AVR9 in MM-Cf9 leaflets resulted in HR in all cases, while no HR was observed at 33°C (Figure 1B). However, when the AVR4- and AVR9-injected plants that had been incubated at 33°C were transferred to 20°C, HR developed within 48 hr after transfer (data not shown). This indicates that development of HR is reversibly suppressed at 33°C and demonstrates that the absence of HR at 33°C is not due to instability of the injected elicitors at this temperature.

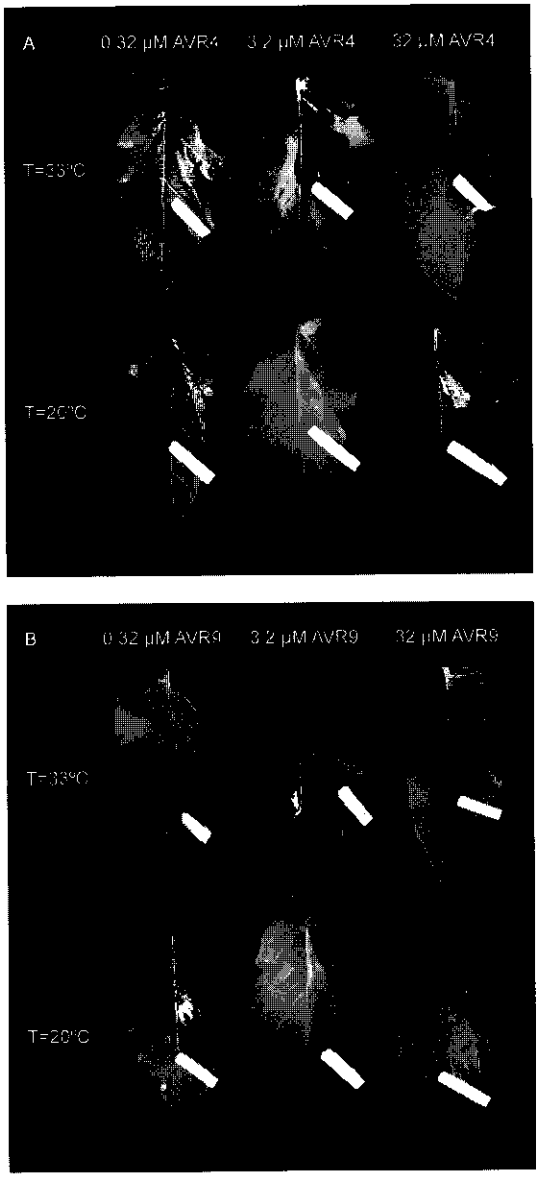

Figure 1. Suppression of the AVR4- and AVR9-Induced Hypersensitive Response at 33°C.

A concentration series of AVR4 and AVR9 was injected into leaves of MM-Cf4 and MM-Cf9 plants, respectively. Plants were incubated for 24 hr at 33°C or at 20°C prior to injection with elicitors. After injection, plants were kept at these temperatures. Photographs were taken 2 days after injection.

(A) Leaflets of MM-Cf4 plants injected with AVR4.

(B) Leaflets of MM-Cf9 plants injected with AVR9.

Tomato Seedlings Expressing Matching Cf *and* Avr *Genes Are Rescued at 33°C*

Under standard greenhouse conditions *Cf-4/Avr4* seeds, obtained from a cross between MM-Cf4 and MM-Cf0 expressing *Avr4* (*Avr4*$^+$ MM-Cf0), and *Cf-9/Avr9* seeds, obtained from a cross between MM-Cf9 and MM-Cf0 expressing *Avr9* (*Avr9*$^+$ MM-Cf0), readily germinate but develop a systemic HR within 2-4 days post emergence of the hypocotyls (Cai et al., 2001; Hammond-Kosack et al., 1994; Thomas et al., 1997).

Cf-4/Avr4 and *Cf-9/Avr9* seeds were sown under standard greenhouse conditions and immediately after emergence of the hypocotyls, the seedlings were transferred to 20°C or 33°C. After three weeks at 20°C, the *Cf-4/Avr4* seedlings had become completely necrotic, whereas the controls, *Avr4*$^+$ MM-Cf0 and MM-Cf4, developed normally (Figure 2A). In contrast, upon incubation at 33°C, the *Cf-4/Avr4* seedlings did not show any HR (Figure 2B). All seedlings grown at 33°C were somewhat retarded in growth as compared to the control seedlings grown at 20°C (data not shown). Transfer of the rescued *Cf-4/Avr4* seedlings from 33°C to 20°C resulted in the development of visible HR symptoms within 12 hr, followed by death of the plants within three days (Figure 2C). Similar results were obtained for tomato seedlings expressing both *Cf-9* and *Avr9* (data not shown).

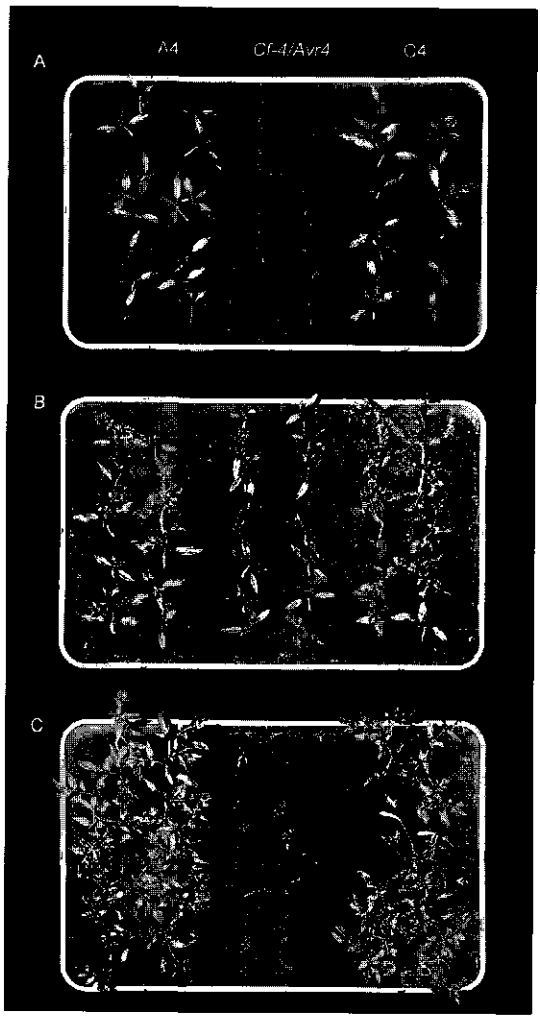

Figure 2. Rescue of MM-Cf4 Seedlings Expressing *Avr4* at 33°C.
Seeds of Avr4$^+$ *MM-Cf0, MM-Cf4 and* Cf-4/Avr4 *were sown under standard greenhouse conditions and after emergence of the hypocotyls, the seedlings were incubated at 20°C or 33°C for three weeks. Seedlings that had been incubated at 33°C were eventually transferred to 20°C.*
(A) *At 20°C,* Cf-4/Avr4 *seedlings die within one week after emergence of the hypocotyls, whereas* Avr4$^+$ *MM-Cf0 (A4) and MM-Cf4 (C4) seedlings develop normally. The photograph was taken three weeks after sowing.*
(B) *At 33°C, all seedlings including the ones expressing both* Cf-4 *and* Avr4*, develop normally. The photograph was taken four weeks after sowing.*
(C) *Upon transfer from 33°C to 20°C,* Cf-4/Avr4 *seedlings develop systemic HR resulting in death within three days after transfer. The photograph was taken 3 days after transfer to 20°C.*

AVR-Induced Expression of Defense-Related Genes Is Reversibly Suppressed at 33°C

The results presented above demonstrate that at 33°C the development of HR in plants expressing both a *Cf* gene and the matching *Avr* gene is suppressed. To test whether the expression of defense-related genes is also affected, the expression level of genes involved in HR (*LeHsr203*), defense signaling (*CDPK*, *NPR1*, *ACCOX*) or defense responses (*P69*, *ChiA*, *ChiB*, *GluA*, *GluB*, *LTP*) (Cai et al., 2001) was determined at different time points after transfer of *Cf/Avr* seedlings from 33°C to 20°C (see Methods). At 33°C (t=0), the expression level of the various classes of genes was similar to that of the control plants. However, within 1 to 8 hr after transfer to 20°C, the expression of *LeHsr203*, *CDPK*, *NPR1*, *ACCOX*, *P69*, *ChiA*, *GluA* and *GluB* was specifically induced in *Cf/Avr* seedlings (Figure 3A). The expression of *LTP* and *ChiB* was induced at later stages. In *Cf-4/Avr4* seedlings, expression of most of the genes appeared to be induced somewhat later compared to *Cf-9/Avr9* seedlings (t=36 hr, Figure 3A). The controls, $Avr4^+$ MM-Cf0, $Avr9^+$ MM-Cf0, MM-Cf4 and MM-Cf9, did not show induction of gene expression after transfer to 20°C (Figure 3A). Thus, the expression of the various defense-related genes is specifically induced upon activation of the HR and not by the temperature shift itself.

AVR- but not INF1-Induced HR Is Completely Suppressed at 33°C in Leaves of Cf-4$^+$- and Cf-9$^+$-Tobacco

To assess whether the induction of HR is also temperature-sensitive in transgenic $Cf\text{-}4^+$- and $Cf\text{-}9^+$-tobacco, these plants were injected with AVR4 and AVR9, respectively. In addition, treatment with the INF1 elicitor of Phytophthora, to which tobacco is responsive (Kamoun et al., 1998), was included as a *Cf*-independent, HR-inducing control. The elicitors were injected using the same concentration range and conditions as described for tomato. At 20°C, the elicitors induced HR at all concentrations tested (Table 1). At 33°C, INF1 still induced HR within 24 hr, whereas the AVR4- and AVR9-induced HR was completely suppressed at 33°C. However, after one week, a HR appeared in the area injected with the highest concentration of AVR4 or AVR9.

In Tobacco Cell Suspensions AVR-Induced Defense Responses Are also Suppressed at 33°C

$Cf\text{-}9^+$-tobacco cell suspensions have been shown to be a very suitable system to study the characteristics of early AVR9-induced defense responses (De Jong et al., 2000; Piedras et al., 1998). To test whether HR-related gene expression is still induced at 33°C, $Cf\text{-}9^+$-tobacco cell suspensions were incubated at 20°C or 33°C for 24 hr and subsequently treated with AVR9 or INF1. RNA gel blot analysis revealed that at 20°C, expression of the HR marker *Hin1* (see Methods) is strongly induced by INF1 or AVR9, within 30 min after addition of the elicitor (Figure 3B). At 33°C, however, no induction of *Hin1* expression is observed upon AVR9 treatment, whereas INF1 still induces expression of this gene, indicating that the cells are viable and are still able to mount a defense response at this

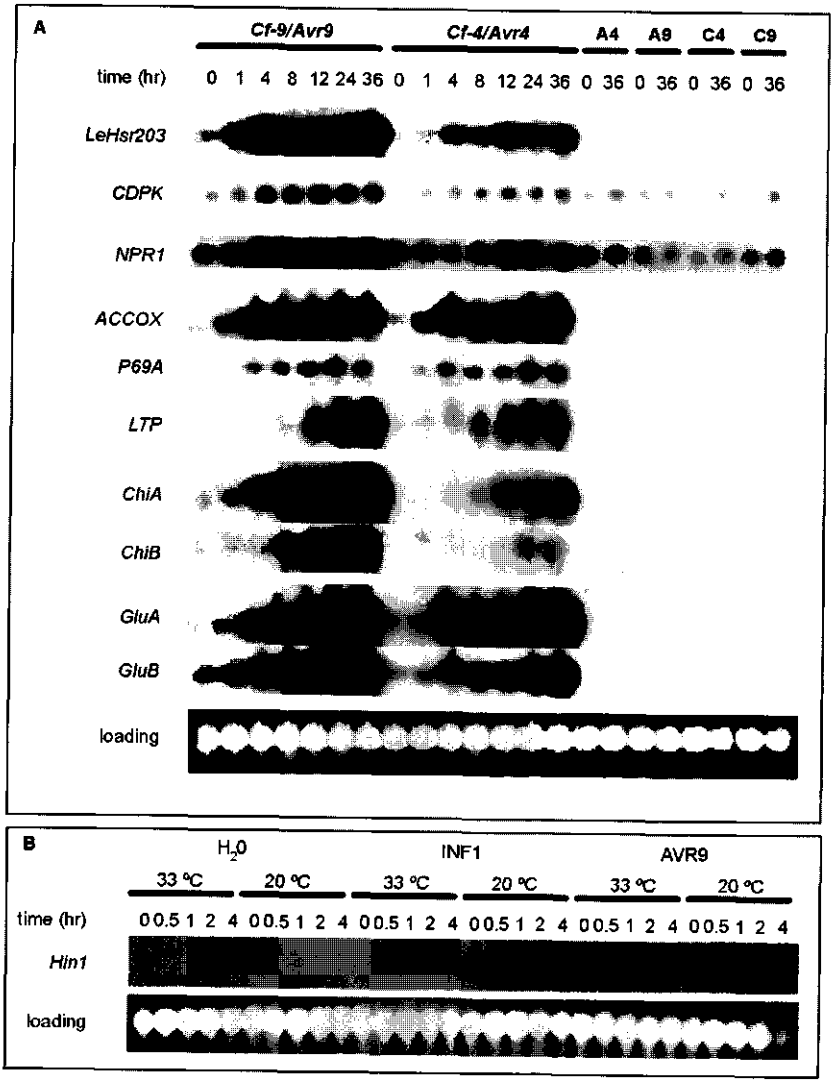

Figure 3. Induction of Defense-Related Gene Expression in Tomato Seedlings and Tobacco Cell Suspensions.

(A) *Tomato seeds of* Cf-9/Avr9, Cf-4/Avr4, Avr4^{+} *MM-Cf0 (A4),* Avr9^{+} *MM-Cf0 (A9), MM-Cf4 (C4) and MM-Cf9 (C9) were planted under normal greenhouse conditions. After emergence of the hypocotyls, the seedlings were incubated at 33°C for two weeks. Seedlings were subsequently transferred to 20°C and RNA was isolated for gel blot analysis at 0, 1, 4, 8, 12, 24 and 36 hr after transfer. The controls, A4, A9, C4 and C9 were only sampled at 0 and 36 hr after transfer. The blots were hybridized with probes derived from defense-related genes as indicated in the left margin (for details see Methods). The panel labeled "loading" shows the ethidium bromide-stained 18S ribosomal RNA.*

(B) *Cf-9^{+}-tobacco cell suspensions were incubated at 33°C or 20°C for 24 hr and were subsequently treated with INF1 or AVR9, both at a final concentration of 0.32 µM, or water. At 0, 0.5, 1, 2 and 4 hr after treatment RNA was isolated, separated on gel and probed with the HR marker* Hin1 *(see Methods). The panel labeled "loading" shows the ethidium bromide-stained18S ribosomal RNA.*

temperature. The tobacco homologue of *LeHsr203* was also tested but this gene was already highly expressed in untreated *Cf-9*$^+$-tobacco cell suspensions at 20°C (data not shown). PR genes, such as *ChiA* and *ChiB*, were also already highly expressed in the control treatments at 20°C. Interestingly, background expression of these genes was attenuated at 33°C (results not shown). Vitality stains (Oparka and Read, 1994) demonstrated that the viability of cells incubated for 24 hr at 33°C was similar to that of cells incubated at 25°C, which is the standard temperature at which the cell suspensions are maintained. Furthermore, the fresh weight of the cells during incubation at 33°C increased progressively, indicating that the cells were healthy and multiplying at this temperature (data not shown).

Table 1. HR Induced in Leaves of *Cf*$^+$-Tobacco Plants After Injection of AVRs and INF1 at 20°C and 33°C at Different Concentrations[a]

	Cf-4$^+$-tobacco				*Cf-9*$^+$-tobacco			
[Elicitor]	AVR4		INF1		AVR9		INF1	
	20°C	33°C	20°C	33°C	20°C	33°C	20°C	33°C
0.32 µM	++	-	++	+	++	-	++	+
3.2 µM	++	-	++	+	++	-	++	+
32 µM	++	-	++	+	++	-	++	+

[a]: *Cf-4*$^+$- and *Cf-9*$^+$-tobacco plants were incubated at 20°C or 33°C for 24 hr prior to injection with 0.32, 3.2 and 32 µM AVR4 or AVR9, respectively, and INF1, at the same concentration range. After injection, plants were incubated at 20°C or 33°C for another 24 hr and necrosis was scored: ++, complete or spreading necrosis; +, patchy necrosis; -, no necrosis.

AVR- but not INF1-Induced Medium Alkalization Is Reversibly Suppressed at 33°C in Cf$^+$*-Tobacco Cell Suspensions*

Alkalization of the culture medium of cell suspensions is one of the earliest responses induced after elicitor treatment (De Jong et al., 2000; Piedras et al., 1998). To extend our studies on the temperature-sensitivity of early defense-related responses, we also generated *Cf-4*$^+$-tobacco cell suspensions. Upon AVR4 treatment, these cells specifically reacted with medium alkalization and an oxidative burst (see Methods). To assess whether AVR-induced medium alkalization is also suppressed at 33°C, *Cf-4*$^+$- and *Cf-9*$^+$-tobacco cell suspensions were incubated for 24 hr at either 25°C or 33°C, after which they were treated with the matching AVR or INF1. At 25°C, treatment with the matching AVR or INF1 results in a rapid increase of the pH of the culture medium (Figures 4A and 4B). At 33°C, however, the AVR-induced response is completely suppressed (Figures 4C and 4D). The increase in pH that is observed after AVR4 treatment (Figure 4C) was also observed in the water-treated control (data not shown). Although reduced, INF1 still induces medium alkalization at 33°C. This observation is in accordance with the response induced in tobacco leaves at 33°C, where INF1 still induces HR, albeit somewhat attenuated.

To assay for recovery of the AVR-induced medium alkalization, cell suspensions that had been incubated for 24 hr at 33°C, were incubated for another 15 hr at 15°C and subsequently treated with the various elicitors. Figures 4E and 4F show that for all elicitor

treatments the medium alkalization response was completely restored, demonstrating that the temperature-sensitive component of the defense-signaling pathway is located upstream of this response.

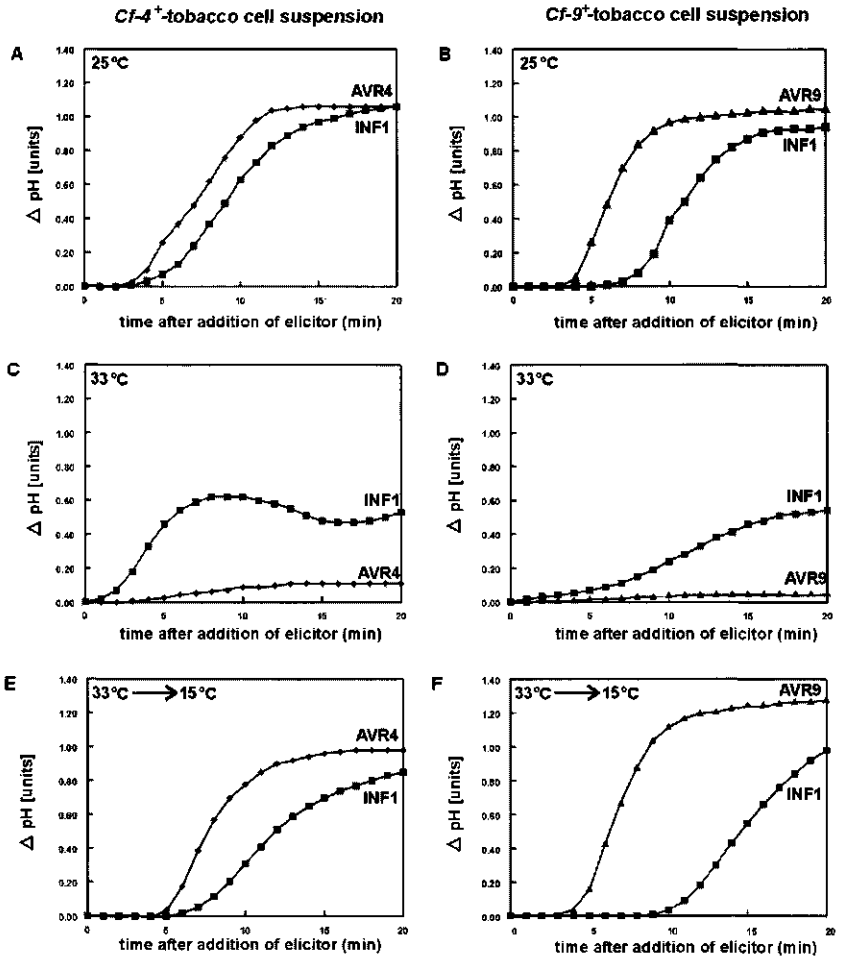

Figure 4. AVR- but not INF1-induced Medium Alkalization in Cf⁺-Tobacco Cell Suspensions Is Reversibly Suppressed at Elevated Temperature. *To assess whether AVR-induced medium alkalization is suppressed at 33°C, Cf-4⁺- and Cf-9⁺-tobacco cell suspensions were incubated at either 25°C or 33°C, three days after sub-culturing. After 24 hr incubation, cell suspensions were treated with the matching AVR (AVR4 (♦) or AVR9 (▲)) and INF1 (■) and the pH of the extracellular medium was recorded. To assay for recovery of the medium alkalization response, cells incubated at 33°C without elicitor treatment were transferred to 15°C. After incubation for 15 hr cells were treated with the matching AVR or INF1 and the pH of the extracellular medium was recorded.*

(A) *AVR4- and INF1-induced response of Cf-4⁺-cells at 25°C.*

(B) *AVR9- and INF1-induced response of Cf-9⁺-cells at 25°C.*

(C) *AVR4- and INF1-induced response of Cf-4⁺-cells after pre-incubation for 24 hr at 33°C.*

(D) *AVR9- and INF1-induced response of Cf-9⁺-cells after pre-incubation for 24 hr at 33°C.*

(E) *AVR4- and INF1-induced response of Cf-4⁺-cells after transfer from 33°C to 15°C and incubation for 15 hr.*

(F) *AVR9- and INF1-induced response of Cf-9⁺-cells after transfer from 33°C to 15°C and incubation for 15 hr.*

Suppression of AVR4- and AVR9-Induced Medium Alkalization at Different Temperatures Reveals Differences in Kinetics between AVR4- and AVR9-Induced Responses

As described above, the AVR4- and AVR9-induced medium alkalization response is completely suppressed after incubation of the cell suspensions for 24 hr at 33°C. To study this phenomenon in more detail, Cf^{+}-tobacco cell suspensions were incubated at different temperatures between 15°C and 37°C and subsequently treated with elicitor (see Methods). Figure 5A shows that upon elevation of the temperature to 33°C, the lag time between elicitor addition and onset of medium alkalization decreased progressively. At this temperature, the lag time was one minute for AVR4 and two minutes for AVR9. Figure 5B shows that the maximal slope of the AVR4-induced medium alkalization curve was reached at 33°C, whereas for AVR9 the fastest response took place at 25°C. Upon elevation of the temperature, the amplitude of the AVR4- and AVR9-induced medium alkalization curve decreases (Figure 5C), an effect which is stronger for AVR9-induced medium alkalization. These data show that, although AVR4- and AVR9-induced medium alkalization is not immediately abolished upon elevation of the temperature, differences in kinetics are apparent when the temperature-sensitivity of AVR4- and AVR9-induced medium alkalization responses are compared.

The Medium Alkalization Response of Tobacco Cell Suspensions Is Slowly Suppressed at 33°C but Recovers Swiftly by Lowering the Temperature to 15°C

The experiments described above show that raising the incubation temperature of tobacco cell suspensions does not result in an acute loss of the medium alkalization response after treatment with the matching elicitor. To determine the time required to fully suppress the medium alkalization response, both *Cf-4*$^{+}$- and *Cf-9*$^{+}$-tobacco cell suspensions were pre-incubated for 15 hr at 15°C and subsequently transferred to 33°C. At different time points after transfer, *Cf-4*$^{+}$- and *Cf-9*$^{+}$- cells were treated with AVR4 and AVR9, respectively, and medium alkalization was monitored. About 20 hr after transfer to 33°C, the AVR4-induced medium alkalization response was fully suppressed, whereas the AVR9-induced response was already fully suppressed within 15 hr after transfer (data not shown). When the cells were pre-incubated at 25°C instead of 15°C, the time required to suppress the AVR4- and AVR9-induced medium alkalization response was reduced to about 14 hr and 11 hr, respectively (data not shown).

To determine the time required to regain the medium alkalization response, *Cf-4*$^{+}$- and *Cf-9*$^{+}$-tobacco cell suspensions cultured at 25°C were pre-incubated for 15 hr at 33°C to reach full suppression of the medium alkalization response. Subsequently, the cells were treated with AVR4, AVR9 or water and incubated at 15°C in a water bath mounted on a gyratory shaker. Immediately after transfer to 15°C, the pH of the extracellular medium was recorded simultaneously for both elicitor- and water-treated cell suspensions. Figure 6 shows that the fluctuations in pH, induced by the rapid change in temperature, had

stabilized after 20 min. At about 45 min after transfer, the pH of the medium started to increase progressively in the elicitor-treated cells, while the pH of the water control remained more or less constant.

Thus, it takes 11 to 14 hr to suppress the medium alkalization response after transfer to 33°C, but only 45 min are required to regain the response upon transfer to 15°C.

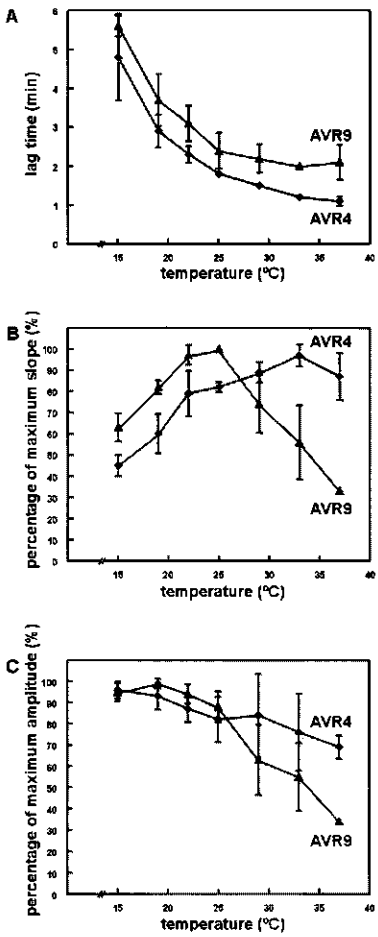

Figure 5. Effect of Temperature on the Lag Time, Slope and Amplitude of the AVR-Induced Medium Alkalization Response of *Cf*$^+$-Tobacco Cell Suspensions.

At 3 days after sub-culturing, Cf$^+$-tobacco cell suspensions were incubated for 15 hr at 15°C. Subsequently, the temperature was stepwise increased to 37°C and after each step cell suspensions were treated with the matching elicitor (AVR4 (♦) or AVR9 (▲)). The graphs represent the mean data points of three independent experiments and the bars indicate their corresponding standard error.

***(A)** Lag times; **(B)** Slopes and **(C)** Amplitudes derived from the curves of the AVR4- and AVR9-induced medium alkalization response of Cf-4$^+$- and Cf-9$^+$-tobacco cell suspensions, respectively, as a function of the incubation temperature.*

Incubation of Tobacco Cells at 33°C Decreases the Amount of High Affinity Binding Sites for AVR9

The experiments described above show that the AVR-induced medium alkalization response of *Cf*-transgenic tobacco cell suspensions is reversibly suppressed at 33°C (see Figure 4). Furthermore, all defense responses in tomato and tobacco leaves, normally induced in *Cf-4/Avr4* and *Cf-9/Avr9* plants, are reversibly suppressed at 33°C (see Figures 1, 2, 3 and 4). These data suggest that at elevated temperatures, AVR-induced signal transduction is blocked at an early step in the pathway.

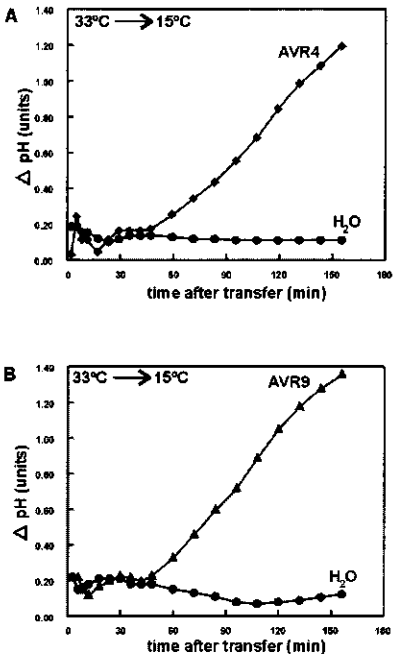

Figure 6. Recovery of the Medium Alkalization Response of *Cf*$^+$-Tobacco Cell Suspensions in Time upon Transfer from 33°C to 15°C.

Cf-4$^+$- and Cf-9$^+$-tobacco cell suspensions were incubated for 15 hr at 33°C at three days after sub-culturing and treated with AVR4 (♦) or AVR9 (▲), respectively, after which the temperature was instantaneously lowered to 15°C. The pH of the extracellular medium was subsequently recorded in time. Control treatments consisted of addition of water (●) before transfer to 15°C.

***(A)** AVR4-induced response of Cf-4$^+$-tobacco cells upon transfer from 33°C to 15°C.*

***(B)** AVR9-induced response of Cf-9$^+$-tobacco cells upon transfer from 33°C to 15°C.*

Earlier work by Kooman-Gersmann et al. (1996 and 1998) has indicated that the high affinity binding site (HABS) for AVR9 is involved in the induction of *Cf-9*-mediated HR. This prompted us to investigate whether AVR9 binding to the HABS is affected at elevated temperatures.

Microsomal fractions were isolated from *Cf-9$^+$*-tobacco cell suspensions that had been incubated at 20°C or 33°C for 24 hr and were used for standard AVR9 binding assays (Kooman-Gersmann et al., 1996). Strikingly, incubation of the cell suspensions at 33°C resulted in a 82% reduction of AVR9 binding when compared to the cells that had been incubated at 20°C (Table 2). To check whether decreased AVR9 binding reflects a decrease in the affinity of the HABS towards AVR9 or a decrease in the actual number of AVR9-binding sites, the binding constant (K_d) was determined. The K_d of the HABS from cells that had been incubated at 33°C, was similar to that of cells incubated at 20°C (Table 2). This K_d was also similar to the K_d reported for the HABS isolated from tobacco leaves (Kooman-Gersmann et al., 1996).

Table 2. Specific Binding of ^{125}I-AVR9 to Microsomal Fractions Isolated from *Cf-9*$^+$-Tobacco Cells Incubated at 20°C or 33°C[a]

Temperature	Binding (pM)	% Binding	K_d (nM)
20°C	31.6 ± 6.6	100	0.033
33°C	5.6 ± 1.5	18	0.051

[a]: Microsomal fractions were isolated from *Cf-9$^+$*-tobacco cell suspensions incubated at 20°C or 33°C for 24 hr. Twelve independent isolations were done for both temperatures. See Methods for further details.

Furthermore, the saturation curves from which the K_ds were determined clearly reflected the significant decrease in the number of binding sites upon incubation at 33°C (data not shown). Consequently, decreased binding of AVR9 does not result from a decreased affinity of the binding site but can most likely be attributed to a decrease in the absolute amount of AVR9-binding sites.

Discussion

It is already known for a long time that *R* gene-mediated resistance against pathogens can be sensitive to elevated temperatures. In recent years, several *R* genes and their corresponding *Avr* genes have been cloned, some of which mediate a temperature-sensitive resistance. However, no systematic studies have been performed to determine the molecular basis underlying the temperature-sensitivity of the HR. Here we show that *Cf-4*- and *Cf-9*-mediated defense responses are temperature-sensitive and that this sensitivity likely resides at the level of elicitor perception. Impaired resistance to Cladosporium at elevated temperatures has no consequences for tomato, because at higher temperatures virulence of Cladosporium is significantly reduced (Small, 1930).

Temperature-Sensitivity of the HR Resides at the Level of Elicitor Perception

Injection of AVR4 and AVR9, at an ambient temperature of 20°C, in leaves of tomato and tobacco plants expressing *Cf-4* or *Cf-9*, respectively, resulted in a HR. This response was completely suppressed at 33°C. Seedlings expressing both an *Avr* and the corresponding *Cf* resistance gene, rapidly die after germination at 20°C but are rescued at 33°C. Defense-related gene expression is suppressed at 33°C, but is rapidly induced after transfer of the rescued plants to lower temperatures. This observation held for genes either involved in HR, defense signaling or defense responses, indicating that the temperature-sensitive component resides early in the pathway leading to the activation of defense responses.

Even a response as early as AVR-induced medium alkalization, taking place within 5 minutes after AVR treatment of Cf^+-tobacco cell suspensions, was suppressed at elevated temperatures. Although both the AVR- as well as INF1-induced responses are temperature-sensitive, individual differences between the responsiveness to various elicitors become apparent at elevated temperatures. The induction of defense responses by INF1 was less temperature-sensitive as compared to the AVR-induced responses (Figure 4), whereas the AVR4- and AVR9-induced medium alkalization response was also differently affected at elevated temperatures (Figure 5). Unlike other elicitors to which tobacco cell suspensions respond, INF1 treatment results in a medium alkalization curve which is very similar to that obtained upon treatment with the AVRs at 20°C (De Jong, unpublished data). Assuming that INF1 and AVR signaling eventually converge into the same signal transduction pathway, the various observations suggest that the temperature-sensitive component of the signaling pathway resides at the level of elicitor perception.

With increasing temperatures, binding of AVR9 to the HABS occurs at a higher rate (Kooman-Gersmann et al., 1996), which is in agreement with a shorter lag time of the medium alkalization response at elevated temperatures. It has been shown that *in vitro* at 37°C, the HABS remains fully stable in isolated membranes over a period of 24 hr (Kooman-Gersmann et al., 1996). However, in cell suspensions, the decrease in AVR9 binding at elevated temperatures without a change in the affinity for AVR9, reflects a decrease of the amount of AVR9 binding sites. An incubation time of 15 hr at 33°C is minimally required to fully suppress AVR9-induced medium alkalization. Upon lowering of the temperature to 15°C, the medium alkalization response was restored within 45 min, indicating that this time span allows for production of sufficient HABS to activate down-stream signal transduction. This period correlates well with the time required for *de novo* protein synthesis.

The stability of the HABS at 37°C *in vitro*, in contrast to the instability of this binding site at 33°C *in vivo*, indicates that *in vivo* the HABS is subject to an active turnover mechanism. Receptor internalization and turnover are well described in mammalian and yeast systems. For cultured mammalian cells it has been estimated that in some cases membrane material equivalent to 50% of the entire cell surface, is internalized every hour. This process plays a role in protein turnover, signaling and adjusting the sensitivity of a cell for a certain ligand (for reviews see Munn (2001) and Waterman and Yarden (2001)). The decrease in the amount of AVR9 high affinity binding sites at elevated temperatures *in vivo*, suggests that either their turnover increases or their *de novo* synthesis decreases. The latter could be the result of an increase in aberrant post-translational protein processing and/or assembly, or temperature-sensitive transcription of the gene(s) encoding the HABS. Possibly, the cell re-directs its resources to meet the requirements of an increased metabolism caused by higher temperatures and consequently economizes on defense, suggesting that temperature-sensitivity of resistance responses may be more common than previously thought. Upon incubation at lower temperatures, defense responses are rapidly restored (Figure 6). In nature, defense responses may be slowly lost at high temperatures during daytime but will be rapidly regained during lower temperatures at night. In this way, the cell efficiently directs its resources without impairing its resistance against pathogens. Re-direction of the cell's resources at elevated temperatures may be accomplished by suppression of certain transcription factors, like the ones that belong to the WRKY group and are involved in regulating plant development and defense. These factors can individually activate transcription of a group of pathogenesis-related genes by binding to W-boxes present in their promoter (Eulgem et al., 2000; Eulgem et al., 1999; Rushton et al., 1996). Interestingly, an unusually large number of W-box sequences were found in the promoter region of receptor-like kinases (RLKs) in Arabidopsis, indicating that WRKY transcription factors may also play a regulatory role in ligand perception (Du and Chen, 2000). Efforts to purify the HABS and isolate the encoding gene(s) are underway. These data will be crucial to gain further insight in the steps affected by elevated temperatures and to study receptor turnover, a subject poorly studied in plants thus far.

The Cf-9 protein is not required for AVR9 binding but is essential to initiate the signal transduction pathways leading to HR. Therefore it would be interesting to determine whether elevated temperatures also have an effect on the Cf-9 protein. However, we have not been able to determine the effect of elevated temperatures on the abundance and/or integrity of the Cf-4 and Cf-9 proteins themselves. Antibodies raised against Cf-9 fail to reveal specific bands in Western blots of microsomal fractions isolated from tobacco cells expressing *Cf-9* under control of its own promoter (unpublished data). Tagged Cf-9 proteins have been over-expressed in tobacco, but in such a system it is not clear whether functional Cf protein or degradation products resulting from over-expression are detected by tag-directed antibodies (Benghezal et al., 2000; Piedras et al., 2000).

The *N* gene of tobacco confers resistance against Tobacco Mosaic Virus (TMV) by mediating recognition of the helicase domain of the TMV replicase proteins at temperatures below 28°C. At higher temperatures, *N*-mediated HR is suppressed and the virus spreads systemically. Mutations made in the TMV replicase gene yielded viruses which could overcome *N*-mediated resistance at temperatures lower than 28°C but higher than 20°C, again suggesting that temperature-sensitivity resides at the level of perception. Whether this phenotype was caused by reduced affinity or reduced stability of the mutant replicase remains to be determined (Erickson et al., 1999; Padgett et al., 1997). Similar results were obtained for the *N'* gene, which mediates recognition of the coat protein of TMV. Mutations made in the coat protein resulted in temperature-sensitive, temperature-insensitive and knockout phenotypes, indicating that in this case stability of the coat protein is affected at higher temperatures (Taraporewala and Culver, 1997). Temperature-sensitive systems are also known from the field of plant development, notably some crosses between different species result in a lethal phenotype that may be rescued at higher temperatures. Recently, Yamada et al. (2000) demonstrated that seedlings and calli resulting from a cross between *Nicotiana suavolens* and *N. tabacum* die due to the systemic initiation of programmed cell death. This lethal phenotype could be overcome by a temperature of 36°C. In this system the direct cause of the temperature-sensitivity is not known.

Application of the Temperature-Sensitivity of Defense Responses in Further Research

The discovery that *Cf/Avr*-induced HR is temperature-sensitive offers great opportunities to study the signal transduction pathway eventually leading to HR and resistance in intact plants. Synchronization of the initiation of the defense response of the plant is a prerequisite to identify the genes that orchestrate the initiation of the HR shortly after elicitor perception. So far, tobacco cell suspensions expressing a *Cf* gene have been the best system available. For example, Durrant et al. (2000) treated *Cf-9*$^{+}$-tobacco cell suspensions with AVR9 and determined differential gene expression employing cDNA-AFLP analysis. Besides genes involved in the defense response, also genes involved in wound responses were identified. As mentioned before, many defense-related genes are constitutively transcribed in suspension cultures, presenting another disadvantage of suspension cultures.

Furthermore, AVR9-induced cell death did not occur in these cell suspensions. Probably the amount of AVR9 that was added to the cells was not sufficient to reach the threshold required for execution of the HR.

We are now able to synchronize the initiation of the HR in intact tomato plants by exploiting the temperature-sensitivity of *Cf*/*Avr*-mediated responses. To this aim, seedlings containing both a *Cf* gene and the matching *Avr* gene are grown at an elevated temperature and subsequently transferred to 20°C, provoking a synchronous and systemic initiation of the HR. We are currently analyzing cDNAs that have been identified by differential cDNA-AFLP analysis of RNA isolated at distinct time-points after the temperature-shift. Differential cDNA-AFLP analysis, in combination with functionality tests of the identified cDNAs by functional genomics, should be an ideal approach to identify the key players that orchestrate the activation of early plant defense responses.

Methods

Preparation of Elicitors

Race-specific elicitor AVR9 of *Cladosporium fulvum* was chemically synthesized and the correctly folded product was purified by HPLC according to the procedure described by Mahé et al. (1998). Race-specific elicitor AVR4 of *C. fulvum* was produced by the yeast *Pichia pastoris* (Invitrogen, USA) and purified from the culture filtrate (Van den Burg et al., 2001). INF1 was purified from culture filtrate of *Phytophthora infestans,* isolate 880069, grown on modified liquid Plich medium at 18°C for 3 weeks (Van der Lee et al., 1997). Briefly, 500 mL of culture filtrate was freeze-dried, dissolved in 20 mL water and dialyzed overnight against water at 4°C. After freeze-drying, the lyophilisate was dissolved in 9 mL of elution buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl) and centrifuged at 13,000g for 15 minutes. The supernatant was loaded on a Sephadex G-50 (Amersham Pharmacia Biotech, Uppsala, Sweden) column (2.6 X 100 cm), equilibrated with elution buffer. The column was eluted at 4°C at a flow rate of 9 mL/hr for 20 hr. The eluate was monitored at 280 nm and 4.5 mL fractions were collected. Samples were taken from the different fractions, based on the UV absorption profile, and analyzed by SDS-PAGE. To assay for biological activity, 0.5 mL aliquots from fractions containing proteins with a molecular mass around 10 kD were desalted on a NAP-5 (Amersham Pharmacia Biotech, Uppsala, Sweden) column, after which they were injected into leaves of *Nicotiana tabacum* cv "Petit Havana SR1" or tomato (*Lycopersicon esculentum*). Fractions inducing necrosis in tobacco but not in tomato were pooled, dialyzed against water and freeze-dried. The lyophilisate was dissolved in 1 mL of 10% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) in water and centrifuged at 13,000 g for 15 min. From the supernatant, 200 µl aliquots were loaded on a ProRPC HR 5/10 reversed-phase column (Amersham Pharmacia Biotech, Uppsala, Sweden) and the column was eluted at a flow rate of 0.3 mL/min, applying a gradient ranging from 10% to 40% ACN in water, containing 0.1% TFA, over a period of 70 min. The eluate was monitored at 280 nm and 0.6 mL fractions were collected.

Based on the UV absorption profile, fractions containing protein were freeze-dried and analyzed both by SDS-PAGE and injection into leaves of tobacco and tomato. Fractions that contained pure INF1 were collected from several runs, pooled and freeze-dried. The lyophilisate was dissolved in 10% ACN in water to a final concentration of 10 mg/mL and stored at -20°C until further use.

Cultivation of Plants

Plants were grown under normal greenhouse conditions, unless stated otherwise. To synchronize germination, tomato seeds were treated with 20% Glorix in water for 20 min and then thoroughly rinsed with water, prior to sowing. Germination took place under normal greenhouse conditions, after which plants were transferred to temperature-controlled incubators (Elbanton, Kerkdriel, The Netherlands) at a 16hr/8hr-light/dark regime.

Crosses between Avr- and Cf-Expressing Tomato Plants

Crosses between *Avr4*- and *Avr9*-expressing Moneymaker (MM)-Cf0 plants and MM-Cf4 and MM-Cf9 plants, respectively, were performed as described earlier (Cai et al., 2001; Hammond-Kosack et al., 1994; Thomas et al., 1997).

Transformation of Tobacco

The *Cf-4* gene was transferred to *N. tabacum* cv "Petit Havana SR1" using the genomic *Cf-4* PstI fragment of 5.8 kB (Thomas et al., 1997), ligated into the pCGN1548 binary vector (McBride and Summerfelt, 1990). Transformation was performed according to standard procedures. Transgenic plants were assayed for AVR4-specific necrosis by expression of *Avr4* in leaf sectors by agroinfiltration (Van der Hoorn et al., 2000). Responding plants were selfed and the resulting seeds were sterilized in 20% Glorix and transferred to MS medium (Murashige and Skoog, 1962), supplemented with 10 g/L sucrose and 100 mg/mL kanamycin. After germination, transformed lines showing a 3:1 segregation for resistance to kanamycin were selected and kanamycin-resistant plants were transferred to the greenhouse. The plants were selfed, after which homozygous ones were identified by sowing the seeds on kanamycin-containing medium. Eventually, seven lines were identified that showed both 3:1 segregation for resistance to kanamycin and AVR4-specific necrosis after *Avr4* agroinfiltration. *Cf-9*$^{+}$ *N. tabacum* cv "Petit Havana SR1" plants have been described previously (De Jong et al., 2000).

Initiation and Cultivation of Tobacco Cell Suspensions

Cell suspensions from *Cf-4*$^{+}$ and *Cf-9*$^{+}$ *N. tabacum* cv "Petit Havana SR1" were initiated and cultivated as described previously (De Jong et al., 2000). Briefly, callus was induced on explants of transgenic tobacco specifically responding to AVR4 or AVR9 and transferred to 300 mL Erlenmeyer flasks, containing 50 mL of liquid MS medium (Murashige and Skoog, 1962), supplemented with Gamborg B5 vitamin mixture (Gamborg et al., 1968), 1 mg/L

2,4-dichlorophenoxyacetic acid and 0.1 mg/L kinetin. The flasks were incubated in the dark at 110 rpm on a rotary shaker at 25°C and the resulting cell suspensions were assayed for AVR4- or AVR9-induced medium alkalization (see below). From responding cell suspensions growing in the log phase, 8 mL aliquots were transferred to fresh medium weekly and cultured at 25°C.

Medium Alkalization and Oxidative Burst Assay

Three days after transfer to fresh medium, several cell suspensions were pooled and mixed to obtain the total volume of cells required for an experiment. The cells were distributed over 25 mL sterile Erlenmeyer flasks, in 7.5 mL aliquots. The flasks were closed with a cotton plug and were pre-incubated on a rotary shaker at 220 rpm at the required temperature. While shaking, medium alkalization was recorded by immersion of a mini pH electrode (InLab 423, Mettler Toledo, Tiel, The Netherlands) into the cell suspension and subsequent addition of elicitor to the medium. Elicitors were added to a final concentration of 0.32 μM, a concentration that elicits a maximal medium alkalization response at 20°C (De Jong et al., 2000). To study the temperature-sensitivity of the medium alkalization response, cell suspensions were pre-incubated for 15 hr at 15°C, after which the temperature was raised stepwise to 19, 22, 25, 29, 33 and 37°C during intervals of 1 hr. At each temperature, medium alkalization induced by the matching elicitor was measured and the lag time of the response, slope and amplitude of the medium alkalization curve were determined (De Jong et al., 2000). The pH electrode was calibrated for each temperature at which the medium alkalization response was measured. The effect of the incubation temperature was quantified by setting the steepest slope of the medium alkalization curve at 100% and expressing the other slopes as a percentage of this.

Oxidative burst assays were performed as described previously (De Jong et al., 2000) and viability of the cells that had been incubated at elevated temperatures was checked as described by Oparka and Read (1994).

RNA Extraction and RNA Gel Blot Analysis

After germination under normal green house conditions, *Cf-4/Avr4* and *Cf-9/Avr9* seedlings and corresponding control seedlings were incubated at 33° for two weeks. The plants were subsequently transferred to 20°C and RNA was isolated at 0, 1, 4, 8, 12, 24 and 36 hr after this temperature-shift. The expression of three classes of defense-related genes was analyzed as described by Cai et al. (2001). Expression levels of the HR-related gene *LeHsr203* (Pontier et al., 1998), and genes encoding proteins involved in defense signaling, such as calcium-dependent protein kinase (*CDPK*) (Ganal et al., 1998; Romeis et al., 2000), *NPR1,* (Cao et al., 1997) and ACC-oxidase (*ACCOX*) (Fluhr and Mattoo, 1996) were determined. Furthermore, the expression levels of genes encoding proteins involved in defense responses, such as *P69A* (Meichtry et al., 1999), lipid transfer protein (*LTP*) (Van Loon and Van Strien, 1999), acidic and basic chitinase (*ChiA* and *ChiB,* respectively) (Danhash et al., 1993) and acidic and basic glucanase (*GluA* and *GluB*, respectively) (Van

Kan et al., 1992) were determined. In tobacco the *Hin1* gene is used as a marker for HR. This gene is highly expressed in incompatible interactions between the plant-pathogenic bacterium *Pseudomonas syringae* and tobacco, but not in compatible interactions (Gopalan et al., 1996). All molecular procedures were performed as described by Sambrook et al. (Sambrook et al., 1989). Total RNA was isolated according to the Extract-A-Plant RNA isolation protocol (Clontech laboratories, Palo Alto, USA). 10 µg of total RNA was separated on a 1.2% agarose gel containing formaldehyde and blotted onto Hybond N^+ membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturers instructions. Hybridization of RNA gel blots was performed overnight with ^{32}P-labelled cDNA probes at 65°C in 0.30 M Na_2HPO_4, 0.14 M NaH_2PO_4, 1 mM EDTA and 7% (w/v) SDS. The cDNA fragments that were used as probes have been described previously (Cai et al., 2001). Membranes were washed at 65°C in 0.5% SSC containing 0.1% SDS and a KODAK X-AR film was exposed to the blot.

Isolation of Microsomal Fractions

In three independent experiments, at three days after sub-culturing, four *Cf-9*$^+$-tobacco cell suspension cultures were incubated for 24 hr either at 33°C or at 20°C. An aliquot of the cell suspensions was subsequently assayed for AVR9-induced medium alkalization and the remaining cells were used to isolate microsomal fractions. Microsomal fractions were isolated as described by Kooman-Gersmann et al. (1996). Briefly, cells were harvested by passing the cell suspension over a Büchner filter, after which they were rinsed three times with water and frozen in liquid nitrogen. Cells were thawed in membrane extraction buffer (10 mM Tris-HCl, pH 7.5, 3 mM EDTA, 0.25 mM sucrose) and homogenized in a Waring blender. The homogenate was squeezed through Miracloth^{TM}, the filtrate was centrifuged at 10,000g for 10 min and the supernatant was subsequently centrifuged at 100,000g for 30 min. The resulting microsomal fraction was resuspended in 10 mM Tris-HCl, pH 7.5 and 0.25 mM sucrose and was divided into 50 µL aliquots that were stored at –80°C until further use. The protein content of the different microsomal fractions was determined with a BCA protein determination kit (Pierce, Rockford, USA) according to the manufacturer's instructions. Binding studies were performed with ^{125}I-radiolabelled AVR9 on microsomal fractions containing 48 µg protein equivalents following the procedure as described earlier (Kooman-Gersmann et al., 1996).

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Chapter 5

General Discussion

Cf$^+$-Tobacco Cell Suspensions: A Suitable System to Study Defense Responses Induced by AVR Proteins of the Tomato Pathogen *Cladosporium fulvum*

Tomato plants expressing resistance (*Cf*) genes against strains of the pathogen *Cladosporium fulvum* expressing one or more of the corresponding avirulence (*Avr*) gene(s) respond with a hypersensitive response (HR) at the site of infection, resulting in resistance. Injection of purified AVR proteins into leaves of tomato and tobacco plants expressing the matching *Cf* genes results in a HR, visible as necrosis of the injected area. To study early defense responses preceding the HR, cell suspensions are far more suitable as compared to whole plants. Consequently, cell suspensions originating from tomato plants expressing *Cf* genes were established. However, it was a long road to discover, and finally accept, that tomato cell suspensions carrying *Cf-9* are not responsive to AVR9. Although aspecific elicitors like the Pmg elicitor from *Phytophthora megasperma* induced an oxidative burst and ion fluxes over the plasma membrane in these cell suspensions, AVR9 did not (Honée et al., 1998). The possibilities to study early defense responses in whole Cf9 tomato and *Cf-9*$^+$-tobacco plants were stretched to the limit when it was discovered that *Cf-9*$^+$*-Nicotiana tabacum* (*Cf-9*$^+$-tobacco) cell suspensions did not only *hold* the promise to study early AVR9 defense responses but did actually *deliver* (Piedras et al., 1998). We have also used tobacco cell suspensions to study AVR-induced defense responses in detail (Chapter 2). It should be kept in mind, however, that they are only a representation for AVR-induced defense responses in tomato plants.

To study and isolate genes underlying the AVR-induced early defense responses, tobacco cell suspensions have a disadvantage. *N. tabacum* is thought to be an allotetraploid species that resulted from hybridization of the two diploid species *N. sylvestris* and *N. tomentosiformis* (Lee et al., 1988). To circumvent this problem, other *Cf*-transgenic diploid *Nicotiana* species might be used for cell suspension studies. We have generated stable diploid *N. benthamiana* transformants homozygous for *Cf-4* and *Cf-9* that are responsive to AVR4 and AVR9, respectively (De Jong and Joosten, unpublished data). Like for *N. tabacum*, cell suspensions are readily generated from *N. benthamiana* (De Jong, unpublished data). Direct isolation of genes or (partial) cDNAs by RT-PCR of AVR-treated *N. benthamiana* cell suspensions has the additional advantage that these sequences can be used directly to assess their function by virus-induced gene silencing (VIGS) in *N. benthamiana* (Baulcombe, 1999). For functional gene knockdowns by silencing, a high homology between the endogenous target sequence and the sequence used to induce silencing is essential. In our lab, silencing systems based on both tobacco rattle virus (TRV) and potato virus X (PVX) are routinely used on *Cf-4*$^+$- and *Cf-9*$^+$*-N. benthamiana* (De Jong, unpublished data). Also, *Cf-4*$^+$- and *Cf-9*$^+$*-N. benthamiana* plants, homozygous for green fluorescent protein (GFP) are available. The GFP function can be used as an internal indicator for timing and localization of gene silencing (De Jong, unpublished data).

Defense Responses Induced by AVR9 and Mutant Analogues in *Cf-9*$^+$-Tobacco Cell Suspensions

Within minutes after addition of AVR9 to *Cf-9*$^+$-tobacco cell suspensions medium alkalization, an oxidative burst and activation of a MAP kinase occur (Chapter 2) (Piedras et al., 1998; Romeis et al., 1999).

Mutant AVR9 peptides were shown to have different necrosis-inducing activities (NIA) upon injection into MM-Cf9 tomato leaves (Kooman-Gersmann et al., 1998). These differential activities were also observed in cell suspensions. Surprisingly, the oxidative burst was induced at 100-fold lower AVR9 peptide concentrations as compared to concentrations that induced medium alkalization or cell death (Chapter 2). An interesting speculation to explain this difference is that after AVR9 perception multiple signaling pathways with a different threshold are activated. The signaling pathway leading to an oxidative burst possibly requires less activated receptor complexes on the cell surface than the pathway leading to medium alkalization and concomitant HR. Such an arrangement has several advantages; at the site of infection, cells are exposed to sufficiently high concentrations of AVR9 and consequently, both an oxidative burst and alkalization of the extra-cellular space are induced and subsequently the cell commits itself to HR. However, distant cells are exposed to lower concentrations of the elicitor that induce an oxidative burst but no alkalization and the cells do not yet commit themselves to HR. The reactive oxygen species (ROS) produced as a result of the oxidative burst might serve as a warning for an incoming attack. ROS have been shown to play an important role in the activation of systemic acquired resistance upon infection of the plant (Alvarez et al., 1998). A similar phenomenon was observed in *Ry*-mediated recognition of the Nla proteinase from potato virus Y (PVY) (Mestre et al., 2000). When plants carrying *Ry* were inoculated with PVY no HR occurred but spreading of the virus was stopped. However, when the Nla proteinase was expressed by agroinfiltration in *Ry*-expressing plants, a clear HR developed. The results were explained by assuming that the virus was stopped and no more accumulation of virus, and consequently Nla proteinase, occurred before the cells committed themselves to HR, while upon transient expression of the Nla proteinase, the cells themselves produced the Nla protein and its production was not stopped by the defense response, thereby committing the cells to HR. This was also observed when *Cf-4*$^+$*-N. benthamiana* plants were inoculated with PVX expressing AVR4. Upon inoculation of the recombinant virus, no HR was observed and plants were completely resistant against this virus. When *Avr4* was transiently expressed in *Cf-4*$^+$*-N. benthamiana* by agroinfiltration, however, a collapse of the infiltrated area was already visible within 8 hr after infiltration (De Jong, unpublished data).

Studies on the nature of AVR9-induced cell death in *Cf-9*$^+$-tobacco cell suspensions did not reveal the various hallmarks of programmed cell death (PCD), such as DNA laddering or disintegration of the nucleus into cell death-associated nuclear bodies (De Jong, unpublished data). Additional studies on AVR-induced cell death were performed on

tomato plants expressing the human anti-apoptotic gene *p35*. *Alternaria alternata* is a necrotrophic pathogen that kills its host by producing *Alternaria alternata* (AAL) toxin. Tomato plants expressing *p35* were reported to be resistant against *A. alternata* f.sp. *lycopersici* by *p35*-mediated inhibition of AAL toxin-induced PCD (Gilchrist, personal communication). However, crossing the *p35* transgene into MM-Cf9 plants did not result in susceptibility to strains of *C. fulvum* expressing AVR9, nor did it compromise the development of necrosis after injection of AVR9 into leaves. Similar results were obtained for plants expressing both *p35* and *Cf-2, Cf-4* or *Cf-5* (De Jong, unpublished data).

Cf/Avr-Mediated Phospholipid Signaling

In chapter 3 we showed that upon AVR4 treatment of *Cf-4*$^+$-tobacco cell suspensions, levels of the second messenger phosphatidic acid (PA) increased dramatically, whereas this response was not observed upon treatment with AVR9. Conversely, AVR4 did not induce PA accumulation in *Cf-9*$^+$-tobacco cells, clearly demonstrating the specificity of this response. AVR4-induced PA accumulation was detected within two minutes after addition of AVR4 to the cells, making it one of the fastest defense signaling responses reported so far. It was shown that AVR4-induced PA accumulation is generated via conversion of diacylglycerol (DAG) by diacylglycerol kinase (DGK). Inhibitors of phospholipase C (PLC) blocked AVR4-induced PA accumulation, indicating that DAG is generated by activation of PLC. PLC inhibitors also blocked the AVR4-induced oxidative burst, medium alkalization and MAP kinase activation demonstrating that these responses are located downstream of PLC. Furthermore, treatment of *Cf-4*$^+$-tobacco cells with a water-soluble synthethic PA analogue (PA_{syn}) induced a small and transient oxidative burst.

During signaling processes PA may also be produced by phospholipase D (PLD). We demonstrated that at least the majority of AVR4-induced PA accumulation resulted from PLC activity. Whether PLD activity also contributed to AVR4-induced PA accumulation could not be established unequivocally.

Recently, PLDα was reported to play a role in activation of the oxidative burst in Arabidopsis (Sang et al., 2001). As we have shown that PA itself may induce an oxidative burst, these data indicate that different pathways may be used to activate the NADPH oxidase generating the oxidative burst. When *Cf-9*$^+$-tobacco cells were treated with AVR9 no, or only a very small increase of PA level was observed although AVR9 did elicit an oxidative burst and medium alkalization in these cells (De Jong, unpublished data). Piedras et al. (1998) presented evidence for the involvement of phospholipase A_2 (PLA_2) in the *Cf-9/Avr9*-mediated oxidative burst in tobacco. PLA_2 hydrolyzes phospholipids at the *sn-2* position, generating either arachidonic acid or linoleic acid and lyso-phosphatidic acid (lyso-PA), depending on the substrate. In neutrophils there is some controversy whether arachidonic acid is a signal to activate NADPH oxidase or an essential co-factor for NADPH activity (Erickson et al., 1999). The AVR9-induced oxidative burst and MAP kinase activation in *Cf-9*$^+$-tobacco cells was blocked by inhibitors of PLA_2 (Piedras et al.,

1998; Romeis et al., 1999). In *Cf4*$^+$-tobacco cells, AVR4-induced medium alkalization, MAP kinase activation and the oxidative burst were blocked by PLC inhibitors. Like we demonstrated for PA, Piedras et al. (1998) showed that both arachidonic acid and linoleic acid induced a small oxidative burst in tobacco cells. Both fatty acids cannot be detected by ^{32}P autoradiography as these fatty acids do not carry a phosphate group. Lyso-PA can be detected, however, but no changes in levels of this lipid were detected after AVR4 treatment of *Cf-4*$^+$-tobacco cells, whereas levels of PA increased dramatically. Overall, these data indicate that different signaling pathways are activated after *Cf-4*- or *Cf-9*-mediated recognition of AVR4 and AVR9, respectively. Although Piedras and co-workers reported possible involvement of PLA_2 in *Cf-9*-dependent defense signaling, we could not detect significant differences in lyso-PA levels after AVR9 treatment of *Cf-9*$^+$-tobacco cells. However, it must be said that using TLC analysis, the levels of lyso-PA detected, were very low. Arachidonic acid or linoleic acid detection by gas chromatography is perhaps more suitable to demonstrate PLA_2 activity and may also reveal which of the two fatty acids is produced in plants. Previously, it has also been reported that arachidonic acid induced defense responses in potato tubers similar to those induced in an incompatible interaction between potato and *Phytophthora infestans* (Tjamos and Kuc, 1982). Whether arachidonic acid acted as an elicitor or induced defense responses by acting as a second messenger remained unclear, however. Currently, tobacco cell suspensions expressing both *Cf-4* and *Cf-9* are being established to address the issue whether indeed different signal transduction pathways are employed for *Cf-4/Avr4*- and *Cf-9/Avr9*-mediated defense responses.

After treatment of *Cf-4*$^+$-tobacco cell suspensions with AVR4, also the levels of diacylglycerol pyrophosphate (DGPP) increased remarkably. This molecule is produced as a result of PA phosphorylation by phosphatidic acid kinase (PAK) in an ATP-dependent reaction. Whether this molecule is a second messenger in its own right or functions to attenuate PA signaling remains to be established (Munnik, 2001). Interestingly, when cells are treated with 10 μM PA_{syn} before adding different concentrations of AVR4, it was expected that the AVR4 response would be amplified. However, dose-response curves revealed that cells pre-treated for 2 minutes with PA_{syn} were less sensitive to AVR4 as compared to non-treated cells (De Jong, unpublished data). The significance of this observation is unclear so far. An interesting speculation is that by PA_{syn} treatment a mechanism involved in attenuation of PA signaling is triggered which diminishes the effect of subsequent treatment with AVR4. It will be interesting to pre-treat cells in a short labeling experiment with PA_{syn} and add AVR4 after different time-points. Ten min after addition of AVR4 the cells can subsequently be analyzed for the accumulation of endogenous PA and DGPP. PA_{syn} will be invisible as it is not labeled but its phosphorylated conversion product ($DGPP_{syn}$) will be detectable and will run differently as compared to endogenous DGPP. Also, the effect of PA_{syn} on AVR4-induced PA accumulation can be monitored. Perhaps such experiments may clarify the significance of DGPP in regulation of

phospholipid signaling and might reveal whether treatment with PA_{syn} leads to activation of an attenuation mechanism for PA signaling.

Temperature-Sensitivity of *Cf/Avr*-Mediated Defense Responses

The virulence of *C. fulvum* itself is significantly reduced at elevated temperatures (Small, 1930). Therefore, it is not surprising that temperature-sensitivity of the AVR-induced HR has not been discovered until recently. The availability of significant amounts of pure AVRs allowed to study defense responses without the requirement of the fungus itself.
We showed that *Cf-4/Avr4-* and *Cf-9/Avr9*-mediated HR is compromised at elevated temperatures in both tomato and tobacco, and that this effect is reversible. Furthermore, we demonstrated that by rescuing tomato seedlings expressing both a *Cf* and matching *Avr* gene by incubating them at 33°C and subsequently activating the HR by transfer to 20°C, the temperature-sensitivity can be used as a switch to synchronize defense-related gene expression. In tobacco cell suspensions we demonstrated that AVR4- and AVR9-induced medium alkalization is slowly lost upon transfer to 33°C but is quickly regained by incubating the cell suspensions again at 15°C. Differences in kinetics of temperature-induced suppression of AVR4- and AVR9-induced medium alkalization were apparent, suggesting that possibly not a shared component of a signal transduction pathway is affected at elevated temperatures, but that two different components specific for either *Cf-4/Avr4-* or *Cf-9/Avr9*-mediated signaling are affected. This hypothesis is further supported by crosses between *Cf-4*$^+$- and *Avr4*$^+$- or *Cf-9*$^+$- and *Avr9*$^+$-*N. benthamiana* plants (De Jong, unpublished data). The seeds resulting from crosses between one *Cf*$^+$-line with different *Avr*$^+$-lines were sown at 33°C. All the *Cf-9/Avr9* seeds germinated and the resulting plants grew vigorously at this temperature. After transfer to 20°C, the seedlings originating from different *Avr9*$^+$-lines died after different time periods, indicating differences in AVR9 expression levels between the various lines. The *Cf-4/Avr4* seeds, however, germinated at 33°C but died soon after germination. Thus, *Cf-4/Avr4 N. benthamiana* seedlings cannot, but *Cf-9/Avr9 N. benthamiana* seedlings can be rescued at 33°C.

Specific components located upstream of the convergence points of signal transduction pathways with different kinetics in temperature-sensitivity, may be present in receptor complexes for the different AVRs. We showed that incubation of *Cf-9*$^+$-tobacco cell suspensions at 33°C resulted in 80% reduction of AVR9 high affinity binding sites present in microsomal fractions as compared to incubation at 20°C. As the affinity of the AVR9-binding site has not changed at both 33°C and 20°C, at equal AVR9 concentrations, the percentage of occupied receptors will be equal at both temperatures. However, at high temperatures the absolute amount of receptors per cell is reduced and consequently also the amount of occupied receptors at a constant AVR9 concentration will be lower. In other words, an elicitor concentration sufficiently high to occupy enough receptor complexes to

pass the threshold for signaling at 20°C, will not trigger a response at 33°C. This could be the molecular mechanism underlying the attenuated responses at elevated temperatures.

Signaling in the *Cf/Avr* Interaction

Events occurring after AVR-perception by a cell expressing the matching *Cf* gene are summarized in a model presented in Figure 1. Both AVR4 and AVR9 are perceived by a receptor (complex) at the plasma membrane. The composition of this complex is not yet clear and is still intensely studied. For AVR9 we have shown that at elevated temperatures the amount of the binding sites decreases. After AVR4 perception, levels of the second messenger PA increase dramatically. This increase is due to the combined activities of PLC and DGK. PLC converts PIP_2 into IP_3 and DAG. IP_3 may open internal calcium channels resulting in an increase in the cytosolic Ca^{2+} concentration. DAG is converted into PA by DGK. Inhibitors of PLC block the AVR4-induced activation of the NADPH oxidase responsible for the oxidative burst, as well as the medium alkalization response and AVR4-induced MAP kinase activation. In Figure 1, medium alkalization activity is represented as a flux of ions over the plasma membrane. At present it is unclear how AVR9 perception by Cf-9 is linked to activation of an NADPH oxidase and medium alkalization. Romeis et al. (1999) demonstrated that upon AVR9 treatment of *Cf-9*$^+$-tobacco cells, two MAP kinases of 46 kD and 48 kD, were activated. These two MAP kinases were identified as wound-induced protein kinase (WIPK) and salicylic acid-induced protein kinase (SIPK). DPI blocked the oxidative burst but did not block activation of either of these kinases. Conversely, the MAP kinase inhibitor PD98059 blocked MAP kinase activity but not the oxidative burst, indicating that both events are independent from each other (Figure 1). Omission of Ca^{2+} from the medium or treatment with external Ca^{2+}-channel blockers inhibited the AVR9-induced oxidative burst and MAP kinase activation, indicating that an influx of Ca^{2+} is essential for activation of these responses. Treatment of cells with general inhibitors of protein kinases blocked the AVR9-induced oxidative burst and MAP kinase activation, whereas both responses were induced by phosphatase inhibitors demonstrating that additional upstream protein kinase activity is required to activate these responses (Piedras et al., 1998; Romeis et al., 1999).

Using a modified in gel kinase assay, (Romeis et al., 2000) also detected a calcium-dependent protein kinase (CDPK) that showed a shift in electrophoretic mobility from 68 kD to 70 kD within 5 min after AVR9 treatment of *Cf-9*$^+$-tobacco cells. This shift was due to a phosphorylation event which converted the kinase from its inactive to its active form and enabled it to phosphorylate histone or the synthetic substrate syntide-2 in the presence of ATP and Ca^{2+}. Histone phosphorylation was inhibited by W-7, a kinase inhibitor, that is known to block CDPK activity. Although W-7 did inhibit CDPK *activity* (phosphorylation of its substrate) it did not block AVR9-induced CDPK *activation* (the AVR9-induced shift

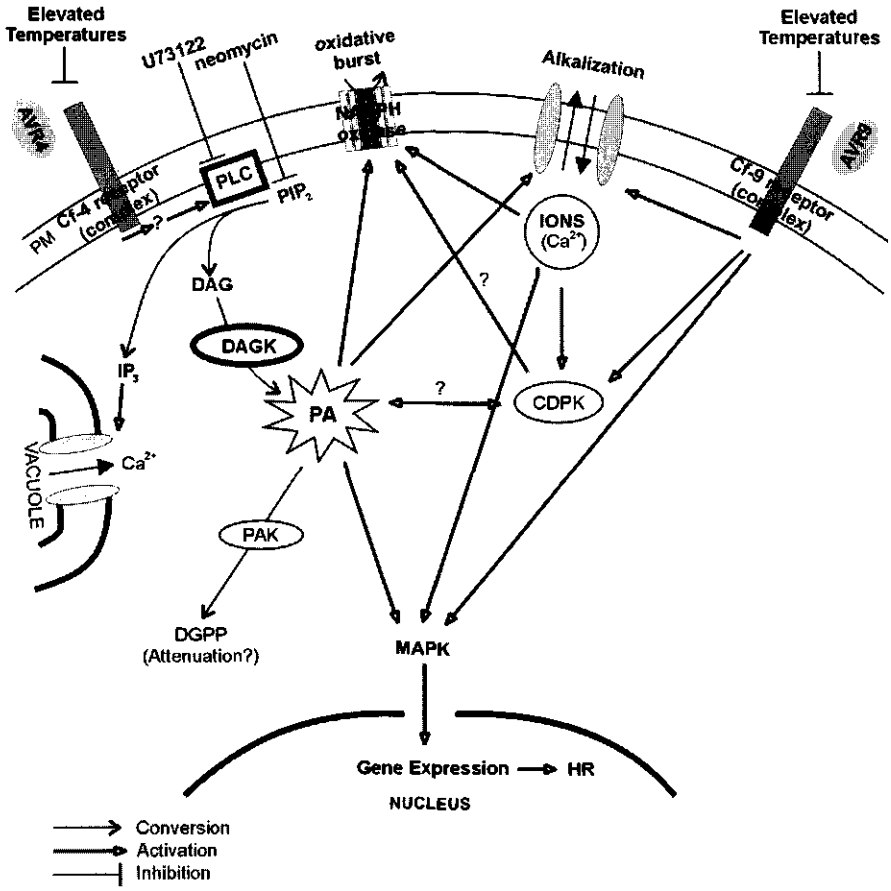

Figure 1. A Model of *Cf/Avr*-Mediated Defense Signaling.
A fraction of a plant cell with plasma membrane (PM), nucleus and vacuole is presented together with a model for Cf-4/Avr4- *and* Cf-9/Avr9-*mediated defense signaling. AVR4 and AVR9 bind to their respective receptor (complexes). At 33°C this process is attenuated due to a decrease in binding sites. Upon AVR4 perception by* Cf-4^{+}*-tobacco cells, phospholipase C (PLC) is activated by an as yet unknown mechanism. PLC hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 may open internal calcium channels. The raise of cytosolic Ca^{2+} concentration may create the proper conditions for activation of additional signaling components, such as calcium-dependent protein kinase (CDPK). DAG is converted by diacylglycerol kinase (DGK) into the second messenger phosphatidic acid (PA). PA may directly activate NADPH oxidase resulting in an oxidative burst. In combination with IP_3 it may induce the medium alkalization response (presented as ion fluxes over the plasma membrane) and activate MAP kinases. Whether PA signaling is also involved in CDPK activation remains to be established. The conversion of PA by phosphatidic acid kinase (PAK) into diacylglycerol pyrophosphate (DGPP) may represent an attenuation mechanism.* Cf-9/Avr9-*mediated medium alkalization, oxidative burst, activation of MAP kinases and CDPK, are induced by a yet unknown pathway. Activation of both MAP kinases and CDPK requires the influx of Ca^{2+}. The MAP kinase pathway may be involved in the activation of defense-related genes. CDPK is either located upstream of the oxidative burst or independent of this response (for further details: see text).*

in electrophoretic mobility). W-7 also blocked AVR9-induced MAP kinase activation and the oxidative burst. Conversely, DPI inhibited the oxidative burst but not CDPK activation.

The MAP kinase inhibitor PD89059 did not inhibit AVR9-induced CDPK activation. These data indicate that CDPK is either independent or located upstream of the oxidative burst or MAP kinase activation. However, Atrboh, the Arabidopsis homologue of the mammalian $Gp91^{phox}$ protein which is the catalytic subunit of the NADPH oxidase complex, contains Ca^{2+}-binding motifs that may be involved in regulation of the plant NADPH oxidase complex (Keller et al., 1998). This should be taken into account when interpreting results obtained with W-7, as this compound also compromises activity of other Ca^{2+}/calmodulin-dependent enzymes.

Recently, two CDPKs (*NtCDPK2* and *NtCDPK3*) were cloned by RT-PCR on RNA isolated from AVR9-treated *Cf-9*$^{+}$-tobacco cell suspensions (Romeis et al., 2001). The transcripts of both genes accumulated after treatment with AVR9 and during osmotic stress. Transient expression of myc-tagged NtCDPK2 in both *N. benthamiana* and *N. tabacum* revealed a rapid conversion of this kinase from the inactive to the active form both after elicitation and osmotic stress. Knockdown of *NtCDPK2* by VIGS suppressed both *Cf-4/Avr4-* and *Cf9/Avr9*-mediated HR in *N. benthamiana,* indicating that *NtCDPK2* or a close homologue, is essential for *Cf-4-* and *Cf-9*-mediated HR.

Perspectives for Studies on *Cf/Avr*-Mediated Signaling

The observation that AVR4 rapidly induces PA accumulation in *Cf-4*$^{+}$-tobacco cells, whereas AVR9 does not in *Cf-9*$^{+}$-tobacco cells, indicates that the Cf-4 and Cf-9 resistance proteins employ different signaling components. To study this difference in more detail, tobacco cell suspensions are currently established that express both resistance genes. The finding that PLA_2 inhibitors affect AVR9-induced downstream responses (Piedras et al., 1998; Romeis et al., 1999) suggests that a closer look to putative AVR9-induced accumulation of PLA_2 conversion products may be worthwhile. The role of PLD in both *Cf-4-* and *Cf-9*-mediated signaling should also be reconsidered, as a form of PLD that does not have the ability to transphosphatidylate structural lipids into phosphatidyl butanol (PBut) may also be activated (Munnik et al., 1998). It will be interesting to relate the already identified MAP kinase- and CDPK signaling to PLC signaling, both by detailed pharmacological studies and gene silencing approaches.

New components in *Cf/Avr*-mediated signal transduction may be identified by a proteomics approach from cell suspensions fed with radio-labeled phosphate prior to elicitation. Proteins can be isolated, separated on two-dimensional gels and analyzed for differentially phosphorylated spots as compared to untreated cells (Peck et al., 2001). The differential spots are isolated and digested and the fragments are analyzed by mass spectroscopy.

The temperature-sensitivity of the *Cf/Avr*-mediated defense responses can be exploited for cDNA-AFLP analysis to identify new genes involved (Takken, unpublished data). To this aim, *Cf4/Avr4* plants and MM-Cf0 controls were germinated at 23°C and subsequently rescued at 33°C. After two weeks at 33°C, all plants were transferred to 23°C and a differential cDNA-AFLP analysis was performed on RNA isolated from the plants harvested at different time-points after transfer. The isolated fragments were sequenced and computer analysis revealed that about 65% of the fragments showed homology to known sequences whereas approximately 35% had no significant homology to any sequence present in the databases. It was shown previously, that between compatible an incompatible interactions of *C. fulvum* and tomato differences in kinetics of the induction of PR gene expression exist (Danhash et al., 1993; De Wit and Van der Meer, 1986; Joosten and De Wit, 1989; Van Kan et al., 1992). In the future, the fragments identified in the cDNA-AFLP analysis may be used in micro-arrays to test which of these genes are differentially expressed in a compatible and incompatible interaction between *C. fulvum* and tomato. Micro-array analysis can also be employed to identify differences and similarities in gene expression between an (in)compatible *C. fulvum*/tomato interaction on the one hand, and an interaction of tomato with other pathogens, such as the necrotrophic pathogen *Botrytis cinerea*. It has been shown that in tomato, in an incompatible interaction with *C. fulvum* and in a compatible interaction with *B. cinerea*, the same PR genes are induced prior to cell death, albeit with different kinetics (Benito et al., 1998).

Currently, the cDNA-AFLP fragments are cloned into a VIGS vector, aimed at creating knockdowns of the corresponding genes in tomato. Some promising results have already been obtained using a tobacco rattle virus-based VIGS vector (MacFarlane and Popovitch, 2000; Ratcliff et al., 2001), harboring a fragment of the tomato phytoene desaturase (*PDS*) gene. PDS protects the plant against radical formation and silencing of the encoding gene results in a white phenotype as a result of photo bleaching (Baulcombe, 1999) (Gabriëls, Joosten, De Jong, unpublished data). Silencing of the cDNA fragments in resistant tomato plants and subsequent injection of the matching elicitor, may identify genes that are essential for induction of HR. By inoculation of the silenced tomato plants with an avirulent strain of *C. fulvum* it can be assessed whether the corresponding gene is also essential for resistance against the fungus.

Evidence is accumulating that necrotrophic pathogens take advantage of the plants' HR to kill host tissues and by doing so turn the plant HR from defense to defeat (Dickman et al., 2001; Govrin and Levine, 2000; Govrin and Levine, 2002). Comparative micro-array analysis of the genes identified in an incompatible interaction between *C. fulvum* and tomato and compatible interaction between *B. cinerea* and tomato, may provide more insight in how differences of gene expression may turn the HR into a compatible interaction for a necrotrophic pathogen. Furthermore, pathogenicity tests of *B. cinerea* on silenced tomato plants compromised in HR may reveal whether these plants are less or equally susceptible to necrotrophic pathogens.

One of the differentials identified with the cDNA-AFLP analysis revealed high homology with a human PLC gene. Cloning and subsequent silencing of this gene allows studying its role in HR and resistance. Alternatively, the PLC genes cloned from *Nicotiana rustica* and potato (Kopka et al., 1998; Pical et al., 1997) may be used directly for VIGS to assess PLC function. The tobacco genes may be useful to determine which gene is differentially regulated upon AVR4 treatment of *Cf-4* tobacco cells. Similar to *NtCDPK2*, the tobacco PLC genes may be directly used for VIGS in *N. benthamiana* to make an assessment for their requirement in *Cf-4/Avr4* and *Cf-9/Avr9*-mediated HR.

The cloned PLC gene may be tagged and used for localization studies to determine whether the PLC protein is translocated from the cytosol to the plasma membrane upon elicitation. Subsequently, its regulation can be studied and additionally, dominant positive and negative mutations can be made. Agroinfiltrations in tobacco will reveal whether overexpression of wild-type PLC or a constantly active mutant will induce a HR and/or other defense responses.

Proteomic approaches as presently performed in yeast (Gavin and *al.*, 2002; Yuen and *al.*, 2002) hold great promises to analyze plant proteins and their interacting partners. When human orthologs of yeast proteins were transformed to this organism the encoding proteins were retrieved from protein complexes made up of yeast orthologs. Strikingly, these complexes consisted of yeast orthologs of human proteins that were known to be part of a specific complex in humans (Gavin and *al.*, 2002). This indicates that in yeast and humans similar protein complexes occur. With the completion of sequencing the Arabidopsis genome, tagged proteins of other plant species may be transformed to Arabidopsis, the formed complexes may be retrieved, analyzed by mass spectroscopy and matched against the Arabidopsis proteome to determine which Arabidopsis orthologs form complexes with the protein of interest. This may allow researchers working on other model systems than Arabidopsis to take advantage of the detailed information on the Arabidopsis proteome.

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Summary

The outcome of a plant-pathogen interaction is determined by both the presence of resistance (*R*) genes in the plant and matching avirulence (*Avr*) genes in the pathogen. According to the gene-for-gene concept, for a dominant *R* gene in the host plant resistant to a specific strain of a pathogen, a corresponding dominant *Avr* gene exists in that strain of the pathogen. *R* gene-mediated recognition of an *Avr* gene product triggers a signal transduction cascade, eventually resulting in a hypersensitive response (HR). This HR consists of a collapse of plant cells at the primary site of infection, resulting in an arrest of growth of the pathogen. As it is impractical to study defense signaling responses in whole plants, often cell suspensions are used for this purpose. Directly after treatment of cell suspensions with elicitors, activation of signal transduction processes, such as ion fluxes over the plasma membrane (detectable as alkalization of the extra cellular medium), phospholipid signaling and protein phosphorylation occur. Also reactive oxygen species, which are thought to play a role both in defense signaling and in direct defense against the pathogen, are produced after AVR perception.

The interaction between *Cladosporium fulvum* and tomato is a well-studied plant-pathogen interaction that obeys the gene-for-gene concept. From this pathosystem several resistance (*Cf*) and *Avr* genes have been cloned, from which the matching gene pairs *Cf-4/Avr4* and *Cf-9/Avr9* are the best studied. Although many efforts were undertaken to study defense signaling in cell suspensions derived from *Cf*-carrying tomato plants, they were not responsive to the matching AVRs. Therefore, *Cf4*$^+$-and *Cf-9*$^+$-tobacco cell suspensions, which are responsive to the matching AVR protein, were used to study *Cf/Avr*-mediated defense signaling.

In **Chapter 2,** defense responses in *Cf-9*$^+$-tobacco leaves and *Cf-9*$^+$-tobacco cell suspensions induced by both wild-type (WT-AVR9) and mutant AVR9 analogues were studied. Upon injection into leaves of both tomato MM-Cf9 and *Cf-9*$^+$-tobacco leaves, the mutant AVR9 peptides R08K, F10A and F21A showed higher, lower and no necrosis-inducing activity, respectively, as compared to WT-AVR9. Similar relative activities were found for these 4 peptides when assayed in *Cf-9*$^+$-tobacco cell suspensions. R08K showed a stronger, whereas F10A and F21A showed a lower oxidative burst-inducing activity as compared to WT-AVR9. In a medium alkalization assay equal activities were observed for R08K and WT-AVR9, whereas F10A showed a lower activity and no medium alkalization activity at all was observed for F21A. Surprisingly, the oxidative burst was induced at peptide concentrations that were 100 times lower as compared to those inducing medium alkalization. Concentrations inducing a full medium alkalization response are similar to peptide concentrations that induce necrosis in leaves of *Cf-9*-carrying tomato or tobacco plants. Treatment of *Cf-9*$^+$-tobacco cell suspensions with WT-AVR9 resulted in the activation of a MAP kinase, whereas F21A activated the MAP kinase only to a small extent. WT-AVR9 also induced massive cell death at 18 hr after addition to *Cf-9*$^+$-tobacco

cell suspensions, whereas tobacco cells not expressing *Cf-9* remained viable, illustrating the specificity of this response.

In **Chapter 3** we have shown that, upon AVR4 treatment of *Cf-4*$^+$-tobacco cells, levels of the second messenger phosphatidic acid (PA) increased dramatically. This response occurred within 2 min after addition of AVR4 and was highly specific. The PA conversion product diacylglycerol pyrophosphate (DGPP) accumulated between 4-8 minutes after addition of AVR4. Whether DGPP is a second messenger in its own right or serves as a negative regulator for PA signaling is still unclear. A differential labeling strategy showed that AVR4-induced PA accumulation resulted predominantly from the conversion of diacylglycerol (DAG) into PA by diacylglycerol kinase (DGK). DAG can be generated during signaling events by the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by phospholipase C (PLC). Pretreatment of *Cf-4*$^+$-tobacco cells with the PLC inhibitors neomycin and U73122 blocked AVR4-induced PA accumulation, indicating that PA is indeed generated via PLC activity. The AVR4-induced oxidative burst was blocked by the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), whereas it did not block AVR4-induced PA accumulation. Conversely, the PLC inhibitor U73122 blocked both AVR4-induced PA accumulation and oxidative burst in a dose-dependent way. Treatment with a synthetic, water-soluble PA derivative induced a small and transient oxidative burst in *Cf-4*$^+$-tobacco cells. These data demonstrate the importance of phospholipid signaling in the AVR4-induced oxidative burst. Additional studies showed that AVR4-induced medium alkalization and MAP kinase activation were also blocked by PLC inhibitors, suggesting that these responses are also PLC-dependent.

During the experiments described in **Chapter 2** it became clear that AVR9-induced defense responses are temperature sensitive. The temperature-sensitivity of *Cf/Avr*-mediated defense responses is studied in **Chapter 4.** Injection of AVR4 or AVR9 in leaves of tobacco and tomato plants carrying *Cf-4* or *Cf-9,* respectively, resulted in necrosis in the injected area at 20°C, whereas at 33°C this response was suppressed. At 20°C, tomato seedlings expressing both a *Cf* gene and matching *Avr* gene germinate but develop systemic HR after unfolding of the cotyledons and subsequently die. These seedlings could be rescued at 33°C but rapidly died after transfer to 20°C. Gel blot analysis of RNA isolated at different time points after transfer of the rescued *Cf/Avr* seedlings to 20°C, revealed a controlled induction of expression of various typical defense-related genes. This synchronized onset of HR provides an excellent basis for the identification of novel, HR-related genes by cDNA-AFLP analysis.

In cell suspensions we found that both the AVR4- and AVR9-induced medium alkalization response is slowly suppressed at 33°C, but quickly recovers upon transfer to 15°C. For AVR4- and AVR9-induced medium alkalization, differences in the kinetics of the suppression of this response at an elevated temperature were demonstrated. The high affinity binding site for AVR9 is thought to be the AVR9 receptor, which is involved in the initiation of *Cf-9/Avr9*-mediated defense responses. It was shown that binding of AVR9 to microsomal fractions isolated from cell suspensions incubated at 33°C was decreased by

80%, as compared to microsomal fractions isolated from cell suspensions incubated at 20°C. The decrease of AVR9 binding was caused by a decrease in amount of binding sites rather than by a decrease in the affinity of the binding site for AVR9, providing a molecular basis for the temperature sensitivity of *Cf-9/Avr9*-mediated defense.

In **Chapter 5** the results described in this thesis are discussed and some additional unpublished data are included. A model for AVR-induced signaling in the *C. fulvum*/tomato interaction is presented. In addition, new techniques, future experiments and perspectives to further unravel *Cf/Avr*-mediated signaling are discussed.

Samenvatting

De uitkomst van een plant-pathogeen interactie wordt bepaald door zowel de aanwezigheid van resistentie (*R*) genen in de plant en corresponderende avirulentie (*Avr*) genen in het pathogeen. Volgens het gen-om-gen concept bestaat er voor elk dominant *R* gen aanwezig in de waardplant populatie, een corresponderend dominant *Avr* gen in de populatie van het pathogeen. *R* gen-afhankelijke herkenning van een *Avr* gen product (AVR eiwit), activeert een signaal transductie keten, culminerend in een overgevoeligheidsreactie, ofwel hypersensitieve respons (HR). De HR bestaat uit het afsterven (necrotiseren) van een beperkt aantal cellen rond de primaire infectiehaard, waardoor kolonisatie van het plantenweefsel door het pathogeen wordt voorkomen.

Cel suspensies worden vaak gebruikt om vroege afweerreacties van de plant, gerelateerd aan de HR, te bestuderen, omdat dit in intacte planten om praktische redenen vaak niet mogelijk is. Direct na toediening van een AVR eiwit aan celsuspensies die het corresponderende *R* gen tot expressie brengen, vinden er signaal transductie processen plaats, zoals accumulatie van specifieke fosfolipiden, eiwitfosforylatie en ion fluxen over de plasmamembraan (detecteerbaar als een stijging van de pH van het extracellulaire medium). Ook de productie van zuurstofradicalen wordt door AVR eiwitten geïnduceerd. Van deze zuurstofradicalen wordt gedacht dat ze een rol spelen bij de activering van de afweerreactie en betrokken zijn bij directe afweer tegen het pathogeen.

De interactie tussen de plant-pathogene schimmel *Cladosporium fulvum* en tomaat volgt het gen-om-gen concept. Van dit pathosysteem zijn verscheidene resistentie (*Cf*) genen en *Avr* genen gekloneerd. Van deze genen zijn de corresponderende genenparen *Cf-4/Avr4* en *Cf-9/Avr9* het best bestudeerd. Er zijn veel pogingen gedaan om signaal transductie processen die betrokken zijn bij de afweerreactie te bestuderen in celsuspensies afkomstig van Cf tomatenplanten. Deze reageerden echter niet, of gaven een aspecifieke respons, na toediening van de corresponderende AVR eiwitten. Om toch processen in de *Cf/Avr*-afhankelijke signaal transductie te kunnen bestuderen is de toevlucht genomen tot celsuspensies afkomstig van transgene tabaksplanten getransformeerd met het *Cf-4* of *Cf-9* gen. In tegenstelling tot celsuspensies van tomaat, reageren deze celsuspensies van tabak specifiek op behandeling met het corresponderende AVR eiwit.

In **Hoofdstuk 2** zijn de afweerreacties welke worden geïnduceerd door zowel wild-type AVR9 (WT-AVR9) en gemuteerde AVR9 eiwitten in *Cf-9*$^+$-tabaksbladeren en *Cf-9*$^+$-tabakscelsuspensies bestudeerd. Injectie van de gemuteerde AVR9 eiwitten R08K, F10A en F21A in bladeren van zowel de tomaten cultivar MoneyMaker (MM)-Cf9 als *Cf-9*$^+$-tabak liet zien dat deze eiwitten in vergelijking tot WT-AVR9, respectievelijk een hogere, lagere en helemaal geen necrose-inducerende activiteit bezitten. In *Cf-9*$^+$-tabakscelsuspensies werden deze verschillen ook gevonden. In vergelijking tot WT-AVR9 heeft R08K een hogere activiteit wat betreft het induceren van de productie van zuurstofradicalen, terwijl F10A en F21A een lagere activiteit bezitten. WT-AVR9 en R08K verhogen de pH van het

medium op vergelijkbare wijze, terwijl behandeling met F10A de pH minder, en F21A de pH helemaal niet verhoogt. Het is opmerkelijk dat de door de AVR9 eiwitten geïnduceerde productie van zuurstofradicalen al plaats vindt bij concentraties die 100 maal lager zijn in vergelijking tot de concentraties die nodig zijn om de pH van het extracellulaire medium te verhogen. AVR9 eiwit concentraties die een maximale pH verhoging geven zijn vergelijkbaar met concentraties die necrose induceren na injectie in zowel bladeren van MM-Cf9 als *Cf-9*$^+$-tabaksplanten. Behandeling van *Cf-9*$^+$-tabakscelsuspensies met WT-AVR9 resulteerde in de activering van een MAP kinase, terwijl F21A slechts een geringe activering van dit kinase gaf. WT-AVR9 induceerde ook massale celdood 18 uur na toediening aan *Cf-9*$^+$-tabakscelsuspensies. Tabakscellen die geen *Cf-9* tot expressie brengen bleven leven na toediening van dezelfde concentratie AVR9, hetgeen de specificiteit van de inductie van deze afweerreactie weergeeft.

In **Hoofdstuk 3** wordt beschreven dat na behandeling van *Cf-4*$^+$-tabakscellen met AVR4 het fosfolipide "phosphatidic acid" (PA) zich ophoopt. Twee minuten na toediening van AVR4 begon PA reeds te accumuleren. Het omzettingsproduct van PA, diacylglycerol pyrofosfaat (DGPP) hoopte zich op tussen 4 en 8 minuten na toediening van AVR4. Het is nog niet duidelijk of DGPP zelf een signaalstof is, of dat het dient als een negatieve regulator van PA signalering. Een differentiële labelingsstrategie toonde aan dat PA voor het overgrote deel gegenereerd wordt via de omzetting van diacylglycerol (DAG) in PA door het enzym diacylglycerol kinase (DGK). In signaal transductie processen kan DAG zelf weer gegenereerd worden via de hydrolyse van fosfatidylinositol 4,5 bisfosfaat (PIP_2) door het enzym fosfolipase C (PLC). Voorbehandeling van *Cf-4*$^+$-tabakscellen met de PLC remmers neomycine of U73122 blokkeerde de door AVR4-geïnduceerde ophoping van PA. Dit gaf aan dat PA inderdaad gegenereerd wordt via PLC activiteit. De AVR4-geïnduceerde productie van zuurstofradicalen werd onderdrukt door de NADPH oxidase remmer diphenyleeniodonium chloride (DPI), terwijl deze remmer niet de door AVR4-geïnduceerde ophoping van PA remde. Echter, beide AVR4-geïnduceerde responsen werden op een dosis-afhankelijke wijze onderdrukt door de PLC remmer U73122. Hieruit kan worden geconcludeerd dat PLC activatie en PA accumulatie plaatsvinden vóórdat zuurstofradicalen worden geproduceerd. Behandeling van de *Cf-4*$^+$-tabakscellen met een wateroplosbaar, synthetisch PA analoog resulteerde in een acute, kleine, niet-aanhoudende productie van zuurstofradicalen. Deze gegevens demonstreren het belang van fosfolipide signalering in de AVR4-geïnduceerde productie van zuurstofradicalen. Additionele studies toonden aan dat ook AVR4-geïnduceerde verhoging van de pH van het extracellulaire medium en MAP kinase activering door PLC remmers onderdrukt worden. Dit suggereert dat ook deze processen PLC-afhankelijk zijn.

Bij het uitvoeren van de experimenten beschreven in **Hoofdstuk 2** werd duidelijk dat de door AVR9 geïnduceerde afweerreacties bij hoge temperaturen onderdrukt worden. De temperatuur-gevoeligheid van de *Cf/Avr*-afhankelijke afweerreacties is beschreven in **Hoofdstuk 4.** Injectie van AVR4 of AVR9 in bladeren van tomaat of tabak, die respectievelijk *Cf-4* of *Cf-9* tot expressie brengen, resulteerde in necrose bij 20°C, terwijl

bij 33°C deze reactie volledig onderdrukt werd. Bij 20°C necrotiseerden tomatenzaailingen die zowel het *Cf* gen, als het corresponderende *Avr* gen tot expressie brengen spoedig na kieming als gevolg van een systemische HR. Deze zaailingen konden gered worden door ze na kieming te incuberen bij 33°C, maar ze stierven snel af als ze teruggezet werden naar 20°C. Expressie-analyse van verschillende genen betrokken bij de afweerreactie liet zien dat bij 33°C de expressie onderdrukt was, terwijl de expressie van deze genen weer snel en gesynchroniseerd op gang kwam na terugzetten van de zaailingen naar 20°C. Deze gesynchroniseerde inductie van de HR vormt een uitstekende basis voor de identificatie van nieuwe genen betrokken bij de afweerreactie door middel van een differentiële cDNA-AFLP analyse in de tijd nadat de gesynchroniseerde HR in gang gezet is.

In celsuspensies ebde zowel de AVR4- als de AVR9-geïnduceerde pH verhoging van het extracellulaire medium langzaam weg na incubatie bij hoge temperatuur, terwijl deze respons weer snel terugkeerde na terugzetten van de cellen naar 15°C. Ook werden er verschillen in kinetiek tussen het verlies van AVR4- en AVR9-geïnduceerde pH verhoging waargenomen bij hoge temperaturen.

Er is op het plasmamembraan van zowel tomaten- als tabakscellen een bindingsplaats voor AVR9 aanwezig waarvan wordt gedacht dat deze de AVR9 receptor is, die betrokken is bij de inductie van *Cf-9/Avr9*-afhankelijke afweerreacties. De hoeveelheid AVR9 die bindt aan microsomale fracties geïsoleerd uit tabakscellen die geïncubeerd waren bij 33°C, was 80% lager in vergelijking tot microsomale fracties geïsoleerd uit tabakscellen geïncubeerd bij 20°C. Deze afname van AVR9 binding bleek te worden veroorzaakt door een reductie van de hoeveelheid AVR9 bindingsplaatsen en niet door een afname van de affiniteit van de bindingsplaats voor AVR9. Dit gegeven vormt een moleculaire basis voor de temperatuur-gevoeligheid van de *Cf-9/Avr9*-afhankelijke afweerreactie.

In **Hoofdstuk 5** worden de resultaten beschreven in de voorgaande hoofdstukken bediscussieerd, evenals nog niet gepubliceerde resultaten. Een model voor de AVR-geïnduceerde signaal transductie in de *C. fulvum*-tomaat interactie wordt gepresenteerd. Ook worden nieuwe technieken, mogelijke toekomstige experimenten en perspectieven om *Cf/Avr*-afhankelijke signaal transductie processen verder te ontrafelen besproken.

Nawoord

Vanuit deze plaats kijk ik uit over de akker die ik nu zo'n zes jaar bewerkt heb. Ik heb geploegd, geëgd, gezaaid en gewied. Zonneschijn en regen, zomerbries en najaarsstorm volgden elkaar op. Soms was het koud en guur op het land, de hemel van een onbestendig grijs en de zuigende klei leek zich dan oneindig voor de ploeg uit te strekken. Maar nu is de tijd van oogsten aangebroken! Ik wil dan ook graag al die mensen bedanken die me hebben geholpen om het land te bewerken.

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Pierre, je hebt de basis gelegd van het inmiddels wereldbekende *Cladosporium* onderzoek. Omdat je naast mijn promotor ook nog een hele hoop meer bent, heb je het altijd verschrikkelijk druk. Maar als je er was, was de drempel om bij je binnen te stappen erg laag. Daar maakte ik dan ook dankbaar gebruik van! Ook wist je altijd oude referenties aan te halen die niet in de databases stonden. Je liep dan naar je kast en trok het eruit. Ook van de recente literatuur was je uitstekend op de hoogte wat wel eens vervelend kon zijn als ik meer geëxperimenteerd dan gelezen had! Tijdens werkbesprekingen weet je veel van de gebruikte technieken. Hoewel je zelf niet meer op de labvloer staat, kon je vaak de oorzaak van experimentele problemen achterhalen, terwijl wij er onze tanden al op stuk gebeten hadden! Ik ben je ook zeer erkentelijk voor de mogelijkheid die je me bood om het onderzoek voor een half jaar als pre-post doc te kunnen voortzetten, waarbij ik naast het verder uitbreiden van de gene silencing systemen ook nog wat losse eindjes kon afmaken.

Beste Clado's! Dit bestek is te kort om jullie allemaal persoonlijk te bedanken. Ik heb in de afgelopen jaren met veel plezier met jullie samengewerkt. Renier, jij was mijn collega van het eerste uur! De eerste maand samen in de trein weer terug naar Leiden waar je me, uiteraard met behulp van pen en papier, geduldig uitlegde wat bijvoorbeeld een transposon was en dat dit geen wezen uit Startrek was zoals ik eerst veronderstelde. Franck (Salue gamin!) et Richard, on a bien ricolé, bien bu, bien mangé et j'ai bien fumé vos clops! Merci encore! Nienk! Zo vaak een kameleon van kleur veranderd, verander jij van kapsel! Hoewel

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Rianne, we hebben samen gedurende onze promotie tijd heel wat lief en leed gedeeld. "Schaken" hebben we tot een ware kunstvorm weten te verheffen! Je was altijd paraat als ik ergens hulp bij nodig had. We zijn nu geen collega's meer maar onze vriendschap blijft. We gaan snel weer een keer zeilen! Heel erg bedankt voor al je steun!

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Mijn ouders en mijn broer! Ik wil jullie graag bedanken voor jullie onvoorwaardelijke steun en toewijding ook al hadden jullie het zelf niet altijd even makkelijk. Pa, het ziet er nu toch naar uit dat ik net als jij doctor voor mijn naam mag gaan plaatsen. Met mijn natuurwetenschappelijke achtergrond gooi ik het op erfelijke belasting terwijl jij dit gezien je antropologische en sociologische achtergrond waarschijnlijk verklaard door invloed van factoren uit de omgeving maar dat vechten we nog wel een keer uit!

Jan, we zijn samen in Leiden met het eerste jaar scheikunde begonnen en op dezelfde datum in Wageningen allebei als OIO begonnen. Naast het hebben van goede wetenschappelijke discussies over ons beider experimenten kon ik samen met jou onder het genot van een vaasje (of meer) weer eens lekker nostalgisch de Leienaar uithangen. Kortom: Gewóón leuk, man!

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Gaea en Rob, het was altijd bijzonder prettig om 's avonds thuis te komen en samen te kijken wat ieder nog aan levensmiddelen in huis had en daaruit een goede maaltijd te bereiden en onderwijl de dingen van de dag te bespreken. Rob, jij woont inmiddels in Utrecht en Gaea en ik zijn nog steeds buren en zetten de traditie voort, alhoewel niet meer op de Marijkeweg. Gaea, ik vond de lange wandelingen samen met Loekie en Beertje altijd heel erg fijn en ze waren een uitstekende remedie tegen proefschrift stress. Ik wens je veel succes met de laatste loodjes aan de Kunstacademie!

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Curriculum Vitae

Camiel Frido de Jong werd geboren op 27 september 1970 te Amsterdam. Van 1980 tot 1982 woonde hij in Indonesië, te Bogor. In juni 1990 behaalde hij het Athenaeum B diploma aan "Het Rotterdamsch Lyceum". Na het behalen van de propedeuse scheikunde aan de Rijks Universiteit Leiden begon hij aan de bovenbouw studie Bio-Farmaceutische Wetenschappen. In september 1996 rondde hij deze studie af na een éénjarige stage bij de vakgroep farmacognosie (Prof. R. Verpoorte), waar hij onderzoek deed naar de biosynthese van het anti-kanker medicijn paclitaxel in *Taxus* soorten. Op 1 oktober 1996 startte Camiel als onderzoeker in opleiding aan het Laboratorium voor Fytopathologie van Wageningen Universiteit (Prof. P.J.G.M. de Wit), alwaar hij onderzoek deed naar de vroege plant afweer responsen geïnduceerd door avirulentie eiwitten van het tomatenpathogeen *Cladosporium fulvum*. Dit onderzoek werd in eerste instantie begeleid door Dr. G. Honée en later door Dr. M.H.A.J. Joosten en werd gefinancierd door de stichting ALW. De resultaten van dit onderzoek zijn gepresenteerd in dit proefschrift ter verkrijging van de graad van doctor. Na zijn OIO schap was Camiel nog een half jaar werkzaam als pre-post doc aan het Laboratorium voor Fytopatholgie onder begeleiding van Dr. M.H.A.J. Joosten waar hij het werk voortzette aan virus-induced gene silencing assays ten behoeve van de identificatie van genen betrokken bij *Cf/Avr*-afhankelijke signaaltransductie.

List of Publications

Early defence responses induced by AVR9 and mutant analogues in tobacco cell suspensions expressing the *Cf-9* resistance gene
Camiel F. De Jong, Guy Honée, Matthieu H. A. J. Joosten and Pierre J.G.M. De Wit.
Physiologicaly and Molecular Plant Pathology (2000) 56, 169-177

Attenuation of *Cf*-mediated defense responses at elevated temperatures correlates with a decrease in concentration of elicitor-binding sites
Camiel F. De Jong, Frank L.W. Takken, Xinzhong Cai, Pierre J.G.M. De Wit and Matthieu H.A.J. Joosten.
(submitted)

Cf-4/Avr4-dependent defense signaling is mediated by the second messenger PA
Camiel F. De Jong, Ana M. Laxalt, Wilco Ligterink, Matthieu H.A.J. Joosten and Teun Munnik
(submitted)

Fungal (A)virulence and host resistance in the *Cladosporium fulvum*-tomato interaction
Matthieu H.A.J. Joosten, Xinzhong Cai, Renier van der Hoorn, Camiel F. De Jong, Maarten de Kock, Richard Laugé, Rianne Luderer, Ronelle Roth, Frank Takken, Paul Vossen, Rob Weide, Nienke Westerink, Pierre J.G.M. De Wit. Biology of Plant-Microbe Interactions (2000), volume 2 (eds. P.J.G.M. de Wit, T. Bisseling, W.J. Stiekema). St. Paul, Minnesota, USA : International Society for Molecular Plant-Microbe Interactions, 2000. pp. 29-34 .

Geranylgeranyl diphosphate synthase activity and taxane production in *Taxus baccata* cells
Gregory Laskaris, Camiel F. De Jong, Mondher Jaziri, Robert van der Heijden, Georgios Theodoridis and Rob Verpoorte
Phytochemistry (1999), 50 (6) 939-946

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Georgios Theodoridis, Gregory Laskaris, Camiel F. De Jong, André J.P. Hofte and Rob Verpoorte
Journal of Chromatography A (1998), 802, 297-305

Patent Application

"Nucleotide sequences involved in disease resistance" (Cladosporium), submitted by Keygene N.V., Wageningen.

The research presented in this thesis was carried out at the Laboratory of Phytopathology of Wageningen University and was financially supported by a grant from the Dutch foundation for Earth and Life Sciences (ALW), project SLW 805.45.001.

Cf-dependent early defense responses induced by avirulence proteins of the tomato pathogen *Cladosporium fulvum*
De Jong, Camiel Frido

Thesis Wageningen University, The Netherlands
With references – With summary in Dutch

On the cover:
Front: *Cf-9*$^{+}$-tobacco cells treated with water (left flask) and AVR9 (right flask). Photograph by Berry Geerligs, Beeldgroep WUR
Back: Microscopic photograph of the cells depicted on the front cover stained with propidium iodide (red fluorescence, dead cells) and fluorescein diacetate (green fluorescence, living cells) to check for vitality. Photograph by Camiel F. De Jong

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