Functional analysis of tomato genes expressed

during the Cf-4/Avr4-induced

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response

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CHAPTER 1

General Introduction and Outline

GENERAL INTRODUCTION

Plant diseases can cause severe losses to field and glasshouse crops and have threatened global food and feed security several times in the history of mankind. Since pathogen populations are extremely variable in time, space and genetic composition, they remain difficult to control. It is only since the last century that several control measures have been applied successfully to protect crops against multiple types of pathogens. However, public opposition to the extensive use of pesticides has stimulated research for alternative strategies to combat diseases. Minimization of disease is often indeed achievable by making optimal use of our understanding of the biology of plants and their pathogens. In the last few decades, research on molecular plant-microbe interactions has intensified and has led to a better understanding of the molecular communication between plants and their microbes. Thus, in addition to strategies such as plant quarantine, intercropping, crop rotation, biological control and post-harvest protection, approaches based on molecular resistance breeding and biotechnology have become available to develop resistant plants (Strange and Scott, 2005).

The goal of this study is to use the interaction between tomato and the fungus *Cladosporium fulvum* (Joosten and De Wit, 1999; Rivas and Thomas, 2005) to reveal which plant genes are involved in defense signaling pathways. Eventually this knowledge can be exploited to develop durable resistant plants, either by marker-assisted breeding or by transgenesis. In this general introduction, I describe the plant defense barriers that pathogens encounter, the tomato-*C. fulvum* interaction and the approaches taken to unravel part of the "black box" which is present downstream of successful recognition of *C. fulvum* by a resistant tomato plant.

Defense Barriers: Non-Host Resistance

Although plants are constantly exposed to micro-organisms, most plant species are resistant to microbial invaders. This phenomenon is caused by "non-host" resistance (Thordal-Christensen, 2003; Nürnberger et al., 2004), and may result from pathogen species not sufficiently adapted to the basic physiology of the plant species. This form of resistance may also be the consequence of preformed defense factors like physical barriers such as the cell wall, a waxy cuticle, or the presence of antimicrobial compounds. The next "non-host" defense barriers pathogens have to overcome in order to be infectious are the active defense responses. These active defense responses are induced upon recognition of Pathogen-Associated Molecular Patterns (PAMPs). These patterns are functionally important for the pathogen and are shared among several members of a pathogen

group. They include for example chitin, chitosan, glucan, glycoproteins, lipopolysacharides, flagellin and elongation factor Tu (Kunze et al., 2004; Nürnberger et al., 2004). Plant receptors, such as the *Arabidopsis thaliana* receptor-like kinase FLS2 recognize flagellin, a general elicitor protein derived from bacterial flagella (Gómez-Gómez and Boller, 2002; Zipfel et al., 2004; Chinchilla et al., 2005). Upon recognition of these PAMPs, defense responses like cell wall reinforcement, lignification, accumulation of phenolic compounds, production of phytoalexins, papillae formation and expression of Pathogenesis Related (*PR*) genes are induced and pathogen penetration and multiplication will be arrested (Mysore and Ryu, 2004).

In contrast to PAMPs, specific pathogen elicitors like Inf1 from *Phytophthora infestans* induce a Hypersensitive Response (HR) in non-host plants like *Nicotiana benthamiana* (Kamoun et al., 1998). The HR is a form of Programmed Cell Death (PCD) induced in plant cells at the site of penetration by a pathogen (Lam, 2004). In this way, a few cells are sacrificed to prevent further spread of (hemi-) biotrophic pathogens. Mysore et al. (2004) referred to this type of non-host resistance, which is associated with an HR, as type II non-host resistance. Type-II non-host resistance is similar to host-specific resistance which follows the gene-for-gene model (see below).

Defense Barriers: Host-Specific Resistance

It is assumed that certain races or strains of a given pathogen can overcome the above described non-host resistance due to activity of their virulence proteins, which suppresses defense responses and increases virulence by modification of a particular host target. This action of virulence proteins is required to cause disease on susceptible host plants. According to the "guard hypothesis", resistant plants contain resistance (R) proteins which detect the modification of the host target (guardee) eventually leading to an HR and host-specific resistance (Van der Biezen and Jones, 1998).

Indirect recognition of a pathogen may be an advantage since virulence proteins derived from different pathogens may modify the same guardee, so one R protein can potentially activate defense responses against these different pathogens. Thus, detection of modified guardees instead of avirulence proteins might limit the number of R proteins required for resistance to several pathogens. A second advantage for R proteins that function as guards is that recognition of the modified virulence target cannot be circumvented without an effect on its virulence function, while upon direct recognition the Avr protein structure can be altered without a reduced virulence function (Van der Hoorn et al., 2002).

Resistance Proteins

(In)direct recognition of pathogen virulence proteins, which can then be regarded as avirulence factors (Avrs), is mediated by receptors either located in the plasma-membrane or in the cytosol. Depending on their localization and structure, these resistance proteins belong to different classes. The most abundant cytoplasmically localized class consists of NB-LRR proteins, with Nucleotide Binding (NB) sites and Leucine Rich Repeats (LRR) of which the latter are presumably involved in protein-ligand interactions. NB-LRR proteins confer resistance to various pathogens ranging from viruses and bacteria to fungi and nematodes. These R proteins can be subdivided based on their N-terminus in TIR-NB-LRR proteins, with a domain similar to the Drosophila Toll and mammalian Interleukin Receptors (TIR), and CC-NB-LRR proteins, containing a Coiled-Coil (CC) domain. Proteins belonging to other classes of cytoplasmic R proteins are different into structure. Plasmamembrane localized R proteins contain extracellular LRRs and can be divided in Receptor Like Kinases (RLKs) and Receptor Like Proteins (RLPs). The RLK *Xa21* from rice for example, encodes a transmembrane extracellular receptor with an additional intracellular kinase domain. Examples of RLPs are the Cf-, Ve- and RPP27- resistance proteins which confer resistance to *Cladosporium fulvum, Verticillium dahliae* and *Hyaloperonospora parasitica,* respectively (Dangl and Jones, 2001).

In the genomes of plants, *R* genes are usually grouped in clusters, which allows intra- or intergenic recombination resulting in the generation of new *R* genes with potentially new specificities (Hulbert et al., 2001). However, pathogens can evolve and circumvent recognition by their host plant, by mutations in their Avr proteins (Westerink et al., 2004). Due to this evolutionary process, resistance breeding based on the introduction of a single dominant *R* gene is often not durable. However, durability of resistance can be achieved by pyramiding, allowing resistance against multiple isolates of a pathogenic species. This is achievable as long as *R* genes are not allelic but reside at different loci. In addition, genes involved in downstream plant defense signaling pathways, or genes encoding proteins forming a complex with the R protein, are presumably less subjected to evolutionary adaptations. Such genes might even be conserved among resistance pathways present in different plant species and effective against various different pathogens.

The Tomato-*Cladosporium fulvum* interaction

C. fulvum is a foliar, biotrophic fungal pathogen with an epiphytic growth on the abaxial side of the leaf, and finally a parasitic growth in the leaf (Thomma et al., 2005). Runner hyphae that emerge from conidia present on the epidermis enter the intercellular space through stomata. In a

compatible interaction fungal growth progresses towards the vascular tissue possibly triggered by the sucrose gradient around the phloem. Eventually, after sufficient biomass has been produced, conidiophores emerge from the stomata carrying enormous amounts of conidia that are dispersed by wind and water splash, causing secondary infections. Finally, stomata of diseased plants become occupied by numerous conidiophores that prevent normal functioning of the stomata. *C. fulvum* causes leaf mold disease due to this stomatal clogging which affects plant respiration resulting in curling of the leaves, chlorotic spots on the adaxial side of leaves, wilting, abscission and, in case of a severe infection, even death of the plant (Thomma et al., 2005).

In an incompatible interaction, conidia of avirulent strains germinate, produce runner hyphae and penetrate open stomata in a similar way as virulent strains. However, within one to two days post penetration a microscopic HR can be observed preventing further fungal growth (Lazarovits and Higgins, 1976; De Wit, 1977; Joosten and De Wit, 1999; Rivas and Thomas, 2005). In addition to the HR, rapid accumulation of PR proteins such as chitinases and 1-3-β-glucanases, and other host defense responses occur (Thomma et al., 2005).

Tomato is the only host of *C. fulvum* and recognition of specific avirulence factors encoded by *Avr* genes is mediated by matching resistance proteins encoded by *Cf* genes, following the gene-for-gene model. *C. fulvum* Avr proteins are small, secreted, cysteine-rich proteins, and the nomenclature of the races of the various strains of *C. fulvum* is according to their virulence: *C. fulvum* race 5 contains all *Avr* genes except *Avr5,* thus this race can cause disease on tomato Cf0 (not containing any *Cf*-genes) and on tomato containing *Cf-5*. The availability of near-isogenic tomato lines carrying different *Cf* genes, and various strains of *C. fulvum* carrying different *Avr* genes has made the tomato-*C. fulvum* interaction an excellent model system to study the gene-for-gene interaction. In addition, due to the strict extracellular growth of *C. fulvum,* the interaction in planta can be studied by isolation of apoplastic fluid containing both non-specific (PAMPs) and race-specific elicitors (Avrs).

In the 1970s attempts were made to isolate non-specific elicitors from the fungus, recently entitled PAMPs. PAMPs of *C. fulvum* are glycoproteins containing glucose, galactose and mannose (De Wit and Kodde, 1981). Receptors recognizing PAMPs of *C. fulvum* have not been identified, but could well be receptor-like kinases of the plant, similar to FLS2. A speculative model describing PAMP-induced resistance responses by inoculation of *C. fulvum* onto, for example, the non-host *N. benthamiana* is depicted in Figure 1A. PAMPs from *C. fulvum* may be recognized by receptor-like kinases, resulting in activation of PAMP-induced defense responses and finally non-host resistance. Possibly, in tomato plants, *C. fulvum* Avr proteins or additional secreted proteins

modify virulence targets of tomato, leading to inhibition of PAMP-induced responses and disease on tomato plants lacking the Cf protein that matches the Avr (Figure 1B).

Cf proteins from resistant tomato plants are thought to monitor or guard the modification of the virulence target (guardee). Perception of this modification induces resistance responses leading to an HR and resistance (Figure 1C). Indeed, avirulence factor Avr2 of *C. fulvum* acts as an inhibitor of the extracellular cysteine protease Rcr3 (<u>R</u>equired for <u>*Cf-2*</u> <u>R</u>esistance). It appeared that inhibition of Rcr3 by Avr2 is recognized by the Cf-2 protein thereby making Rcr3 the guardee of Cf-2 (Rooney et al., 2005). Since tomato is the only host for *C. fulvum,* the Avrs from this fungus are probably not capable of modifying virulence targets in non-host plants such as those in *N. benthamiana*, and as a consequence the fungus may not be able to inhibit PAMP-induced non-host resistance responses.

To date five *Cf* genes and four of the corresponding *Avr* genes have been cloned. No direct interaction between *C. fulvum* Avr and tomato Cf proteins has been reported, indicating that also for the other Avrs indirect recognition according to the guard hypothesis takes place. Possible functions of virulence proteins might be the induction of nutrient leakage, the suppression of defense responses or the protection against host defenses (Joosten and De Wit, 1999; Rivas and Thomas, 2005; Thomma et al., 2005). The function of Avr2 has been described and its recognition perfectly fits the guard hypothesis (see above). Avr4 contains an invertebrate chitin-binding domain and binds to chitin. It is speculated that Avr4 protects the cell wall of *C. fulvum* against plant chitinases (Van den Burg et al., 2003 and 2004). *C. fulvum* races 2 and 4 contain mutations in *Avr2* and *Avr4,* respectively (Joosten et al., 1994; Luderer et al., 2002; Westerink et al., 2004). Since strains of these races seem not to be affected in virulence, pathogenicity could well rely on a set of virulence proteins rather than on one protein only.
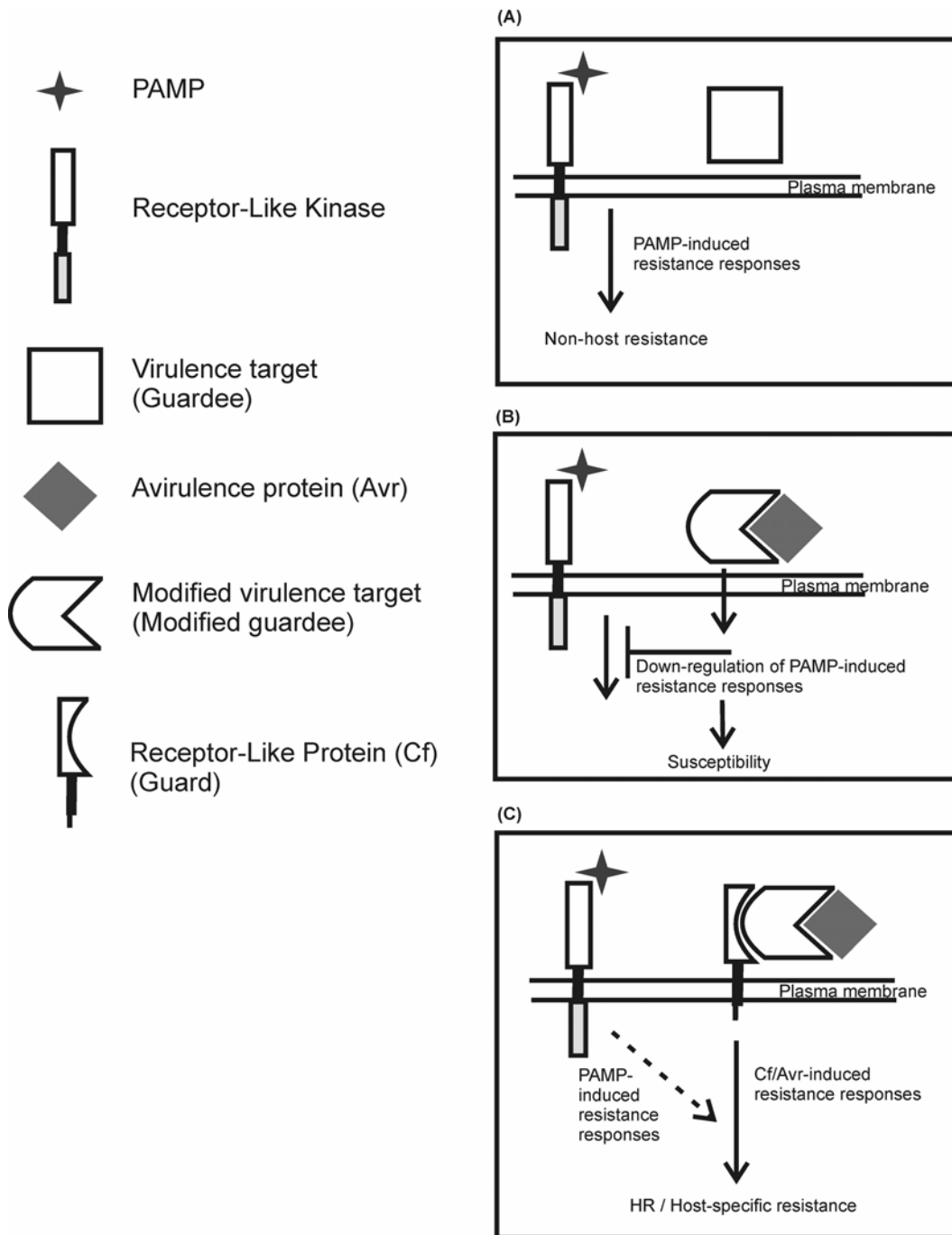
Figure 1. Speculative Model for Signaling Pathways Either Leading to Non-Host Resistance, Susceptibility, or Host-Specific Resistance to *C. fulvum*.

(A) In a non-host plant like *N. benthamiana,* receptor-like kinases might recognize PAMPs from *C. fulvum* and activate PAMP-induced resistance responses leading to non-host resistance.

(B) In the host tomato, *C. fulvum* produces Avr proteins that are able to modify a virulence target, thereby down-regulating the PAMP-induced resistance responses. For example, the virulence target is a regulator of defense which becomes inactivated, subsequently leading to susceptibility.

(C) Resistant tomato plants containing the corresponding Cf receptor-like protein (the guard) detect the modification of the virulence target (the guardee), thereby activating resistance responses, eventually causing an HR and host-specific resistance. The dashed arrow indicates the PAMP-induced resistance responses of which the contribution to tomato-*C. fulvum* resistance is unknown.

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Genes Involved in Downstream Defense Signaling of Tomato

Upon R protein-mediated recognition of the invading fungus through the action of its Avr proteins, several defense responses are induced. Cell cultures were used to analyze rapid changes due to Cf/Avr elicitation. In the first five minutes after treatment of *Cf*-containing tobacco cell cultures with the matching Avr, Reactive Oxygen Species (ROS) accumulate. Further, a calcium-influx occurs, Phospholipase C (PLC) is activated resulting in the accumulation of Phosphatidic Acid (PA) and protein kinases are activated (De Jong et al., (2004); Rivas and Thomas, 2005). ROS function as antimicrobial compounds or induce cell wall cross-linking. PLCs are activated upon treatment of *Cf-4*-containing tobacco cell cultures with Avr4, while PLC inhibitors block *Cf-4/Avr4*-induced responses, indicating the importance of PLC signaling (De Jong et al., 2004).

Protein kinases are signaling components that are activated upon a stimulus, like a pathogen elicitor. For example in *Cf*-transgenic *Nicotiana tabacum* cell cultures WIPK (Wound Induced Protein Kinase) and SIPK (Salicylic acid Induced Protein Kinase) (Romeis et al., 2000b) are activated after elicitation with the corresponding *C. fulvum* Avrs. In addition, a Calcium Dependent Protein Kinase (CDPK) (Romeis et al., 2000a) was identified to be independent, or upstream, of the ROS production pathway. Recently, functional analysis using Virus-Induced Gene Silencing (VIGS) has become a tool to determine which genes are involved in defense pathways. Indeed, VIGS of *NtCDPK* compromised the *Cf-9/Avr9*-dependent HR. In addition, MAPKs (Mitogen-Activated Protein Kinases) are known to be required in several plant defense pathways (Zhang and Klessig, 2001; Pedley and Martin, 2005). Eventually, protein kinase pathways activate their substrates (for example transcription factors) leading to induction of gene expression, such as *PR* genes, and finally leading to an HR.

As discussed above, some players of plant defense pathways are known. However, the major part of plant defense signaling pathways still remains a black box. To identify genes involved in defense signaling, often gene expression levels in "healthy" plants, and in plants undergoing defense responses (including the HR), are compared. However, comparing the transcriptome of susceptible tomato plants inoculated with a virulent strain of *C. fulvum,* with that of resistant plants inoculated with an avirulent strain of *C. fulvum* is difficult, as such infections not only reveal differences in gene expression of the host, but also of the pathogen. To circumvent these limitations we use a system that we refer to as the "dying seedling system", as this allows the synchronous induction of defense responses without the presence of the fungus itself. Near-isogenic tomato lines carrying a *Cf* gene were crossed to transgenic tomato plants that lack functional *Cf* genes but express the matching *Avr* gene. Seeds of the *Cf/Avr*-containing progeny germinate in a similar way

to seeds of the parental lines. However, a few days after emergence of the hypocotyls the seedlings start to express genes involved in HR induction and the plant eventually dies (Cai et al., 2001). The HR is suppressed when the *Cf/Avr*-containing plants are grown at 33ºC, whereas subsequent shift to room temperature results in a synchronized HR (De Jong et al., 2002). This system allows us to compare differences between for example the transcriptome and proteome of *Cf/Avr* plants mounting the HR, and their non-responsive parents. We have chosen to compare transcriptomes enabling direct verification of a role in plant defense by knock-down of the genes corresponding to the differentially expressed transcripts. Comparing these transcriptomes, it was expected that the expression of genes related to the temperature shift will be similar in the parental and the *Cf/Avr*-transgenic plants, whereas genes related to plant defense should be expressed differentially. Thus, this dying seedling system should enable us to identify genes putatively involved in plant defense.

Outline of This Thesis

To determine which genes are involved in *Cf/Avr*-induced defense pathways, cDNA-AFLP analysis was performed on RNA isolated from *Cf-4* controls- and *Cf-4/Avr4* seedlings, at 0, 30, 60 and 90 minutes after a temperature shift from 33ºC to room temperature. This revealed 442 differentially expressed cDNA-AFLP fragments corresponding to genes putatively involved in plant defense, that were designated <u>A</u>vr4-<u>R</u>esponsive <u>T</u>omato (*ART*) genes. To analyze the function of this large set of *ART* genes, high-throughput functional analysis is essential. For this we used VIGS, a technique that allows to make fast transient individual "knock-downs" for relatively large sets of genes (Baulcombe, 1999). Since VIGS as a functional genomics tool was already established in *N. benthamiana* (Ratcliff et al., 2001)*,* first a set of 192 selected *ART* fragments was used to generate knock-downs in transgenic *N. benthamiana* containing the resistance gene *Cf-4* (Gabriëls et al., 2006). Silencing of 20 *ART* fragments revealed a decreased *Cf-4/Avr4-* and *Inf1*-induced HR, indicating that the silenced gene(s) are required for HR-induction. VIGS of five *ART* fragments most clearly affected both *Cf-4/Avr4-* and *Inf1*-induced HR (Gabriëls et al., 2006). Among these five fragments is *ART* transcript number 222, of which the protein encoded by a corresponding full-length cDNA revealed homology to CC-NB-LRR- encoding resistance genes. The protein is designated NRC1 (<u>N</u>B-LRR <u>R</u>equired for HR-associated <u>C</u>ell death 1) (**chapter 2**).

Recent papers describe VIGS as a tool for functional analysis not only in *N. benthamiana* but also in other Solanaceous species (Liu et al., 2002; Brigneti et al., 2004). We optimized VIGS in tomato under our conditions and initiated a high-throughput functional assay to score for compromised resistance to *C. fulvum.* First we showed "proof of principle" by performing VIGS of

Cf-4 and *Rcr3*, in tomato containing *Cf-4* and *Cf-2* respectively. The most efficient method to perform VIGS and to score for compromised resistance was chosen for functional analysis of the genes corresponding to the 192 *ART* fragments that had already been tested for decreased HR in *N. benthamiana*. VIGS using four *ART* fragments resulted in loss of full resistance to *C. fulvum*, among which was *NRC1*, indicating that *NRC1* is required for *Cf-4*-mediated defense (**chapter 3**).

We decided to focus on the NB-LRR gene *NRC1,* required for *Cf-4*-mediated HR and resistance. Until now, NB-LRRs were generally found to be proteins that are involved in the upstream recognition process of avirulent pathogens. However, it was anticipated that NRC1 could play a role more downstream. We investigated whether *NRC1* is involved in multiple HR or resistance pathways by performing VIGS studies in both *N. benthamiana* and tomato. In addition, overexpression studies of NRC1 were performed and a constitutively active mutant of the protein was generated to perform epistasis experiments. Combining the data of several experiments allowed the positioning of *NRC1* in a defense signaling pathway relative to genes known to be required for plant defense (**chapter 4**).

In the general discussion (**chapter 5**) genes involved in innate defense pathways in both plants and mammals are described. Furthermore, the possible roles of NRC1 as a novel component in plant defense signaling pathways are discussed.

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CHAPTER 2

cDNA-AFLP$^{®}$, Combined with Functional Analysis, Reveals Novel Genes Involved in the Hypersensitive Response

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ABSTRACT

To identify genes required for the hypersensitive response (HR), we performed expression profiling of tomato plants mounting a synchronized HR, followed by functional analysis of differentially expressed genes. By cDNA-AFLP® analysis, the expression profile of tomato plants containing both the *Cf-4* resistance gene against *Cladosporium fulvum* and the matching *Avr4* avirulence gene of this fungus, was compared to that of control plants. About 1% of the transcript-derived fragments (442 out of 50,000) were derived from a differentially expressed gene. Based on their sequence and expression, 192 fragments, referred to as Avr4-Responsive Tomato (*ART*) fragments, were selected for Virus-Induced Gene Silencing (VIGS) in *Cf-4*-transgenic *Nicotiana benthamiana*. Inoculated plants were analyzed for compromised HR by agroinfiltration of either the *C. fulvum Avr4* gene, or the *Inf1* gene of *Phytophthora infestans* that invokes an HR in wild-type *N. benthamiana*. VIGS using 15 of the *ART* fragments resulted in a compromised HR, whereas VIGS with fragments of *ART* genes encoding HSP90, a nuclear GTPase, an L19 ribosomal protein and, most interestingly, an NB-LRR type protein, severely suppressed the HR induced both by Avr4 and Inf1. Requirement of an NB-LRR protein (designated NRC1, for NB-LRR protein Required for HR-associated Cell death 1) for Cf resistance protein function as well as Inf1-mediated HR suggests a convergence of signaling pathways and supports the recent observation that NB-LRR proteins play a role in signal transduction cascades downstream of resistance proteins.

INTRODUCTION

The Hypersensitive Response (HR) is a swift reaction of plants to resist pathogen invasion (Dangl and Jones, 2001; De Jong et al., 2004). Although a number of key players involved in the HR have been identified, many steps in the signal transduction cascade that is activated upon recognition of a pathogen by a resistant host remain obscure. To identify novel components of the HR signaling pathway, the interaction between tomato (*Lycopersicon esculentum*) and the biotrophic fungal pathogen *Cladosporium fulvum* is employed (Thomma et al., 2005). Resistance to the fungus depends on the presence of plant resistance (*Cf*) genes matching to pathogen *Avr* genes that encode race-specific elicitors. Five *Cf* genes (Takken et al., 1998; Thomas et al., 1998) and four of the matching *Avr* genes (Joosten et al., 1994; Luderer et al., 2002; Van den Ackerveken et al., 1992; Westerink et al., 2004) have been cloned. In this typical gene-for-gene interaction, a strict

correlation exists between the triggering of an HR and resistance, as the various Avrs induce a specific HR in tomato genotypes carrying the matching *Cf* resistance gene. In susceptible tomato plants that lack the matching *Cf* gene such an HR does not occur. Furthermore, loss of an Avr by the fungus results in evasion of Cf-specific resistance (Joosten et al., 1994; Joosten and De Wit 1999; Van den Ackerveken et al., 1992).

To study HR signaling at the whole plant level and to identify genes involved in this defense response, we have crossed near-isogenic tomato lines carrying a *Cf* gene with transgenic tomato plants that lack functional *Cf* genes and express the matching *Avr* gene. The resulting *Cf*/*Avr* seeds germinate similar to seeds of the parental lines. However, within two to four days after emergence of the hypocotyl, the seedlings mount a systemic HR and express typical defense-related genes (Cai et al., 2001). Interestingly, these defense responses are suppressed by incubating the seedlings at elevated temperature (33ºC) immediately after germination. A subsequent shift to permissive temperature (20–25ºC) results in a synchronized HR that becomes visible within 36 hours (De Jong et al., 2002). However, already within one to four hours after the temperature shift, expression of the HR-marker gene *Lehsr203* (Pontier et al., 1998) and various other defense-related genes such as <u>P</u>athogenesis-<u>R</u>elated (*PR*) genes and *NPR1*, is induced (De Jong et al., 2002). Therefore, this synchronized activation of Cf-mediated signaling allows us to study early transcriptional changes in plants that mount an HR. In absence of the pathogen itself, all differentially expressed genes, except for the genes present on the T-DNA carrying the *Avr* and selection markers, originate from the plant. Furthermore, the constitutive production of the Avr protein continuously triggers HR-signaling throughout the plant, facilitating the detection of the genes involved.

To identify <u>A</u>vr4-<u>R</u>esponsive <u>T</u>omato (*ART*) genes, *Cf-4*/*Avr4* tomato plants undergoing synchronized HR were used for RNA fingerprinting using cDNA-AFLP® (Vos et al., 1995). By comparing the cDNA-AFLP patterns of *Cf-4*/*Avr4* and *Cf-4* control plants, 442 differential *ART* <u>T</u>ranscript-<u>D</u>erived <u>F</u>ragments (TDFs) were identified. A subset of these fragments was used for <u>V</u>irus-<u>I</u>nduced <u>G</u>ene <u>S</u>ilencing (VIGS) (Ratcliff et al., 2001) in *Cf-4*-transgenic *Nicotiana benthamiana*, and the plants were challenged either with Avr4 of *C. fulvum* or the Inf1 elicitor of *Phytophthora infestans* (Kamoun et al., 1997 and 1998). This resulted in the identification of 20 *ART* TDFs for which VIGS compromises the HR. Inoculation with TRV recombinants carrying fragments of the *ART* genes encoding an HSP90, a nuclear GTPase, an L19 ribosomal protein and, most interestingly, an NB-LRR type protein, had the most pronounced effect on the HR.

RESULTS

Analysis of the Transcriptome of *Cf-4/Avr4* Seedlings Mounting an HR

To identify the optimal parameters for the cDNA-AFLP analysis, we first compared the transcriptome of *Cf-4*/*Avr4* and control *Cf-4* plants at different time points after the shift to a permissive temperature. RNA was isolated from the above-ground parts and used to generate *Taq*I/*Mse*I templates which were amplified using a *Taq*I forward primer and a small set of *Mse*I reverse primers with an extension of either two or three selective nucleotides (4 and 16 primer combinations, respectively. See materials and methods). This resulted in about 50 Transcript-Derived Fragments (TDFs) per lane for the more selective primer combinations and about 100 bands per lane for the less selective combinations. For this limited set of AFLP primers no changes in gene expression were observed over the first three time points (0, 10 and 20 min). However, after 30 min TDFs corresponding to both up- and down-regulated genes were detected. For most of these genes changes in expression were observed in both the *Cf-4*/*Avr4* and the control plants, indicating that these differentials are the result of the temperature shift itself, rather than the onset of the HR. Avr4-Responsive Tomato (*ART*) genes can easily be discriminated from Temperature-Responsive Tomato (*TRT*) genes, as *ART* TDFs differ in the *Cf-4*/*Avr4* plants when compared to the controls that were similarly subjected to the temperature shift (Figure 1).

The small scale cDNA-AFLP screen at the various time points eventually resulted in the identification of a total of seven *ART* TDFs using the 16 primer combinations, whereas only one of these TDFs was found using the four primer combinations. Therefore, to identify a representative set of *ART* TDFs, it was decided to carry out the cDNA-AFLP analysis using all 1024 AFLP primer combinations with an extension of 2 and 3 selective nucleotides (see materials and methods). Furthermore, as we were interested in early transcriptional reprogramming and no substantial additional differences became apparent when time points later than 90 min were compared (results not shown), we focused on the analysis of changes in the transcriptome at 0, 30, 60 and 90 min after the temperature shift.
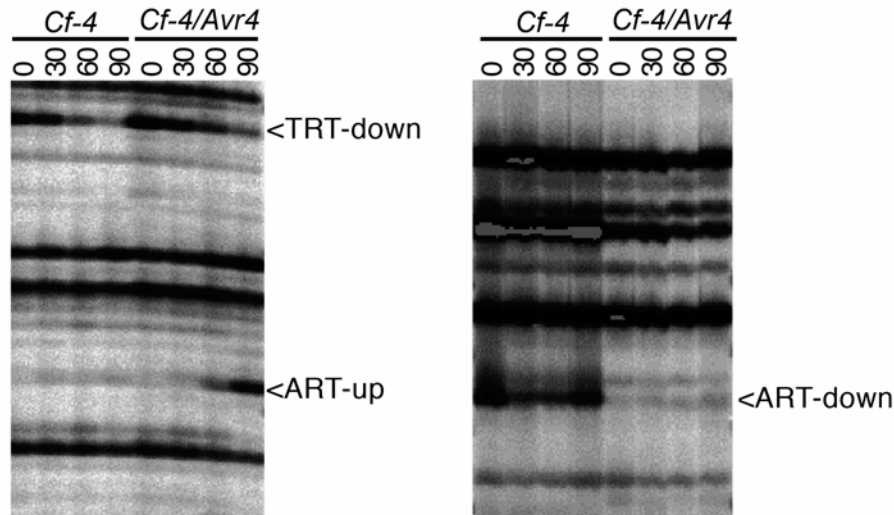
Figure 1. Typical examples of cDNA-AFLP profiles of *Cf-4* and *Cf-4/Avr4* seedlings. The expression profiles at four time points (0, 30, 60 and 90 min) after the temperature shift were analyzed. Two types of TDFs were found; TDFs corresponding to Temperature-Responsive Tomato (*TRT*) genes and TDFs corresponding to Avr4-Responsive Tomato (*ART*) genes. An example of a TRT-down TDF (down-regulated in *Cf-4* as well as in *Cf-4/Avr4* plants) and an ART-up TDF (specifically up-regulated in the *Cf-4/Avr4* plants) is shown in the left panel. An example of an ART-down TDF (in this case constitutively down-regulated in the *Cf-4/Avr4* plants) is shown in the right panel.

Identification of *ART* Fragments, Sequence Analysis and Selection of *ART* TDFs for Functional Studies

To identify *ART* genes, for the four time points and 1024 AFLP primer combinations the presence and intensity of approximately 50,000 bands was compared between the *Cf-4/Avr4* and *Cf-4* samples. TDFs that by eye appeared to be differentially expressed (approximately 5,000) were selected and the band intensities were quantified. These data were used to calculate the differences in expression levels between the *Cf-4/Avr4* and the control plants. Based on these data, 442 *ART* TDFs were selected that showed at least a three-fold difference in expression level at two time points, or at least a two-fold difference at three time points. Remarkably, about 70% of the *ART* genes were already differentially expressed at the time point of the temperature shift. In total 328 *ART* TDFs (74%) corresponded to up-regulated genes, whereas 114 (26%) corresponded to down-regulated genes (supplementary material Table S1).

Out of 442 *ART* TDFs, 405 fragments were successfully cloned and sequenced and analyzed using the Protein Extraction Description and Analysis Tool (PEDANT), to perform BLAST (Basic Local Alignment Search Tool) searches in public databases and determine sequence homology at the protein and DNA level (Frishman et al., 2003). Furthermore, the program determines homology

based on the predicted 3D-protein structure and on domains present in the encoded proteins. Of all *ART* TDFs, we found 85% to be represented in EST libraries (E-value <0.001). To select *ART* TDFs for functional analysis and to exclude TDFs that are derived from the same gene, BLAST searches on the tomato TIGR (The Institute for Genomic Research) database (http://tigrblast.tigr.org/tgi/) were performed. Of the 405 *ART* TDFs, 314 fragments appeared to be unique, whereas the remainder corresponded to 34 different Tentative Contig sequences (TCs), reducing the maximum number of different *ART* genes to 348 (Figure 2). In addition, 5 TDFs were identified that originated from the T-DNA present in the transgenic *Cf-4/Avr4* plants and from homologues of *Cladosporium* resistance gene *Cf-9* (*Hcr9*s) present in the Cf0 *Milky Way* and *Northern Lights* clusters (Parniske et al., 1999), leaving 343 *ART* TDFs for further analysis (Figure 2 and supplementary table). Based on their putative identity and/or expression pattern 192 TDFs were selected for functional analysis (see supplementary table). This resulted in a selection of 147 TDFs corresponding to up-regulated genes (of which 65 showed homology to known genes (E-value <0.001)) and 45 TDFs corresponding to down-regulated genes (of which 21 were homologous to known genes (E-value<0.001)).
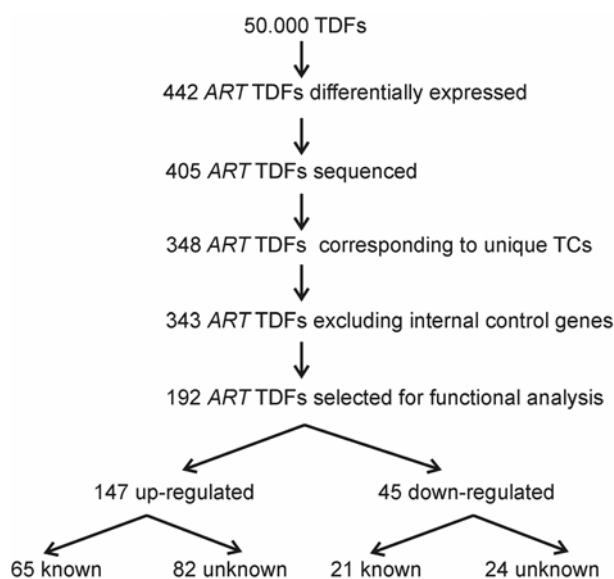

Figure 2. Flow chart showing the selection of *ART* (Avr4-Responsive Tomato) fragments for functional analysis. Eventually, based on their putative identity and/or expression pattern, 192 transcript-derived fragments (TDFs) were selected for functional analysis by VIGS in *Nicotiana benthamiana*. TCs; tentative contig sequences of the tomato TIGR database. See text for details.

Functional Analysis of Selected *ART* Genes by Virus-Induced Gene Silencing

For functional analysis of the selected *ART* genes, virus-induced gene silencing (VIGS) was performed using the binary TRV vectors described by Ratcliff et al. (2001). To identify genes involved in the HR, TRV:ART-inoculated *Cf-4*-transgenic *N. benthamiana* plants (*N. benthamiana:Cf-4*) were analyzed for compromised HR. This HR was induced by agroinfiltration of *C. fulvum Avr4*. Furthermore, we included the *Inf1* gene of the potato late blight pathogen *Phytophthora infestans*, as Inf1 is an elicitor of the HR in wild-type *N. benthamiana* (Kamoun et al., 1997 and 1998). Agroinfiltration of *Avr4* or *Inf1* in non-inoculated or TRV:00-inoculated *N. benthamiana:Cf-4* plants resulted in an HR within three to five days (Figures 3B and 3C; panels 1 and 2), indicating that both elicitors induce a clear HR despite of the TRV-infection. To test whether this response is compromised by silencing of genes known to be involved in either the Avr4 and/or the Inf1 response, TRV:Cf-4 containing a fragment of *Cf-4*, and TRV:SGT1 (Peart et al., 2002) were used for VIGS. TRV:Cf-4-inoculated *N. benthamiana:Cf-4* plants showed a decreased HR upon agroinfiltration of *Avr4*, whereas the HR induced upon agroinfiltration of *Inf1* was not compromised (Figures 3B and 3C; panel 3). The TRV:SGT1-inoculated plants displayed stunted growth (Figure 3A, panel 4) and did not mount an HR upon agroinfiltration of either *Avr4* or *Inf1* (Figures 3B and 3C; panel 4), confirming that the downstream component SGT1 is required for both Avr4- and Inf1-induced HR.

To test the involvement of the selected *ART* genes in the HR, the 192 fragments, varying in length between 50 and 500 bp, were cloned into the TRV vector and inoculated onto *N. benthamiana:Cf-4* plants. In general, TRV-inoculated plants were smaller than non-inoculated plants (Figure 3A, compare panel 1 with panels 2-3 and 12-13). In addition, inoculation with a number of TRV:ART constructs resulted in chlorosis, leaf deformations or stunted growth (Figure 3A; panels 5-11 and 14). The plants were subsequently challenged with Avr4 and Inf1 at three weeks post TRV:ART inoculation. Initially, VIGS using 78 TDFs appeared to compromise the induction of an HR. Re-testing eventually resulted in 20 TDFs for which VIGS reproducibly compromised the Avr4- and/or Inf1-induced HR (marked by an asterisk in the supplementary data). VIGS using five of these fragments; 470, 424a, 614, 653 and 222, resulted in a severely compromised HR (Figures 3B and 3C; panels 5-9), indicating that the orthologous genes in *N. benthamiana* are essential for the HR induced by both elicitors. Fragments 614 and 653 are nearly identical in sequence, indicating that they correspond to the same gene.
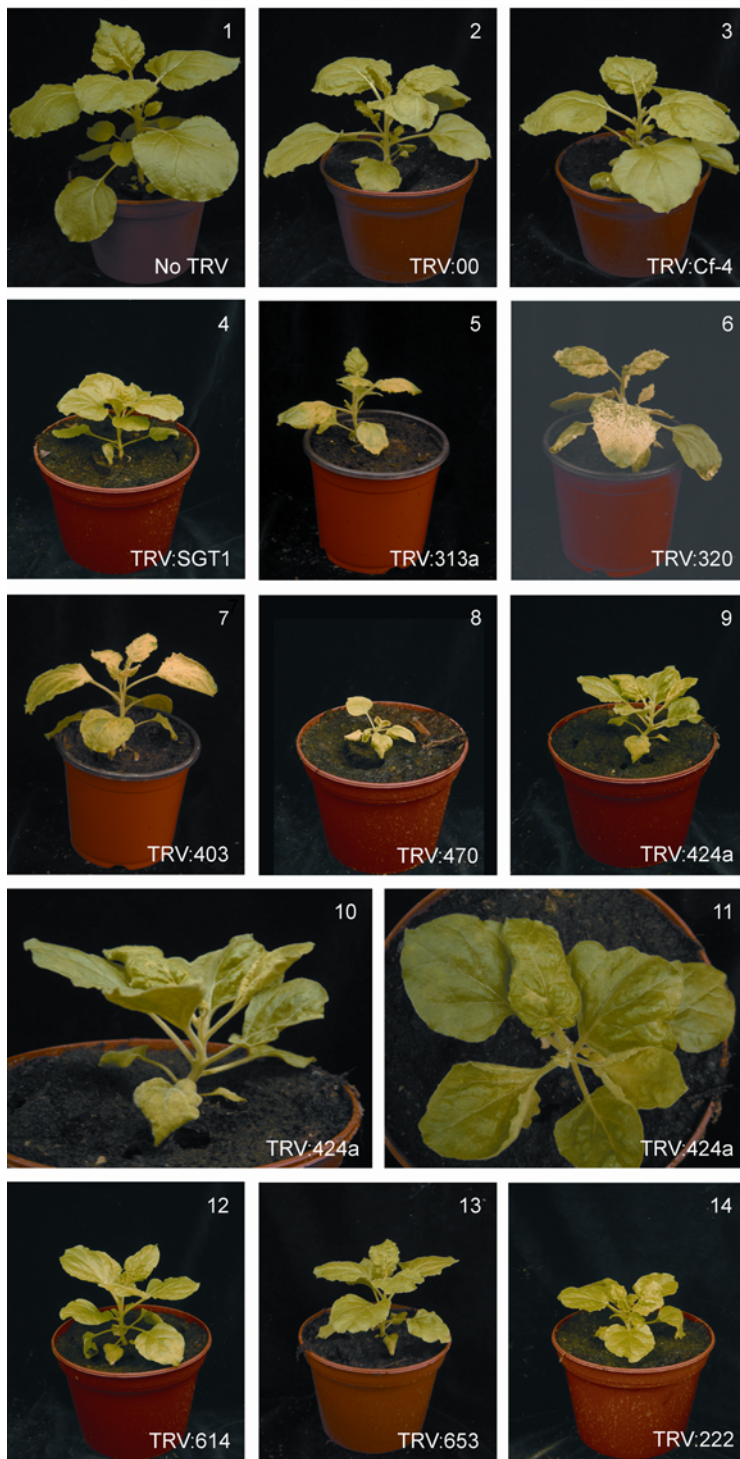
Figure 3. Symptoms of *N. benthamiana* plants expressing *Cf-4*, resulting from inoculation with various TRV:ART recombinants and the effect of the inoculation on the Avr4- and Inf1-induced HR. *Avr4* and *Inf1* were agroinfiltrated three weeks post virus inoculation on the leaf half marked by a white dot and pictures were taken at four days post agroinfiltration.

(A) Phenotype of non-inoculated plants (panel 1) and plants inoculated with TRV:00 (empty vector; panel 2), TRV:Cf-4 (panel 3), TRV:SGT1 (panel 4) and various TRV:ART recombinants (panels 5-14), at 24 days post inoculation. Note that most of the shown TRV:ART-inoculated plants appear chlorotic or stunted, whereas the TRV:424a-inoculated plants also has curled leaves (panels 9-11). Inoculation with TRV:614 and TRV:653 did not result in changes in morphology (panels 12 and 13).
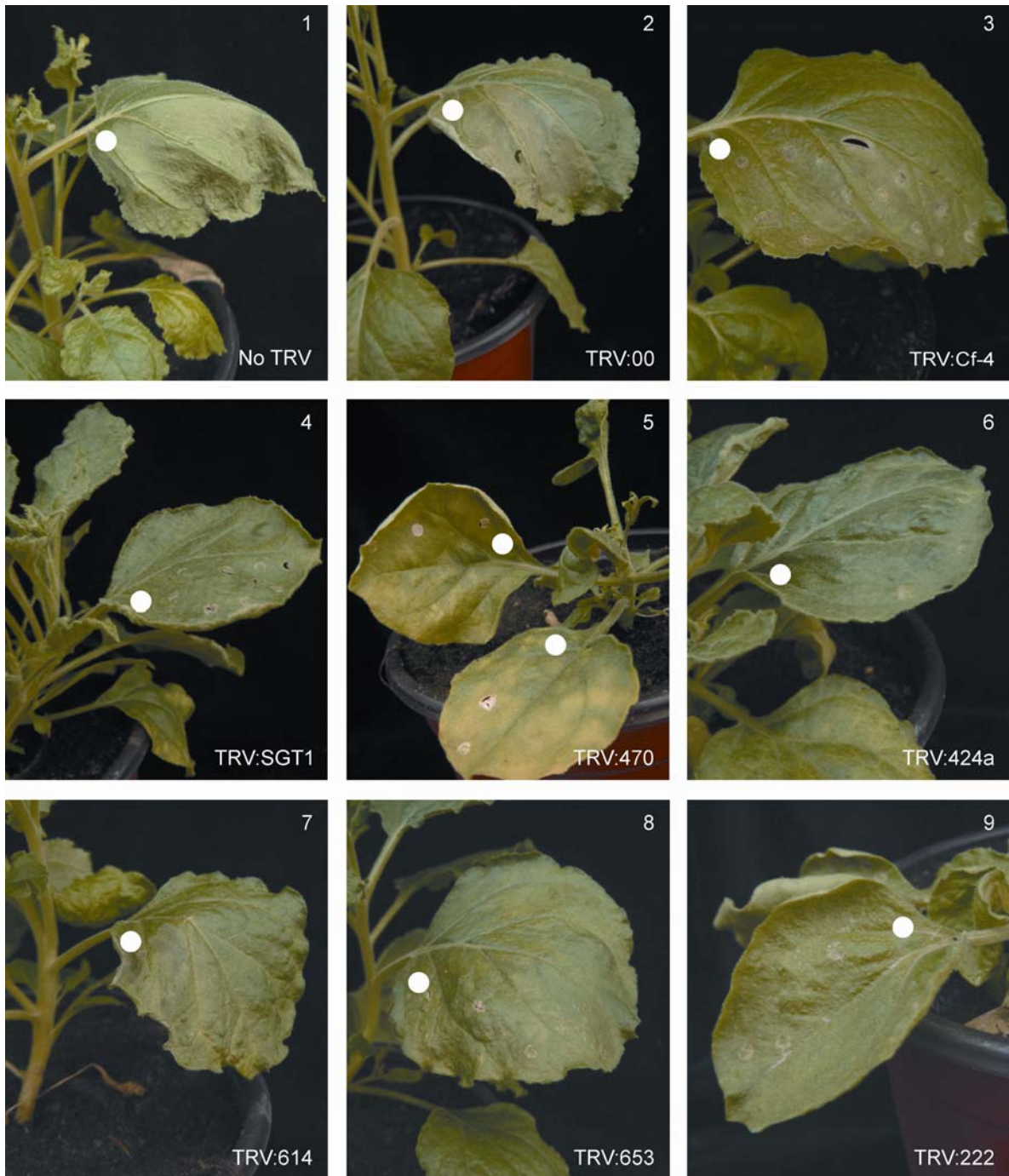
(B) HR induced by agroinfiltration of *Avr4* in TRV:ART-inoculated plants. HR was not compromised in non-inoculated and TRV:00-inoculated plants (panels 1-2), whereas VIGS using TRV:Cf-4, TRV:SGT1, TRV:470, TRV:424a, TRV:614, TRV:653 and TRV:222 severely suppressed the *Avr4*-induced HR (panels 3-9).

For these five fragments we have not been able to confirm 'knock-down' of the corresponding *ART* orthologues in *N. Benthamiana* when using primers designed on the original tomato cDNA sequences for the RT-PCRs. However, isolation of a full-length cDNA for *ART* 424a allowed the identification of a sequence that is highly conserved in tomato, potato and tobacco. Primers designed in the most conserved region allowed to show by RT-PCR that *ART* 424a transcript levels are significantly lower in the TRV:424a-inoculated plants (supplementary material Figure S1).

Characterization of the Four *ART* Genes Required for Avr4- and Infl-Induced HR

VIGS using the five tomato *ART* TDFs 470, 424a, 614, 653 and 222 resulted in a pronounced suppression of both the Avr4- and Infl-induced HR. To allow a more reliable BLAST search, the full-length tomato cDNAs corresponding to the various TDFs were isolated and analyzed (Table 1). Furthermore, the cDNA-AFLP analysis for these particular TDFs was repeated once more, now also including Cf0 plants and the transgenic Cf0 parental line expressing *Avr4* (Cf0/*Avr4*) as controls.

According to the cDNA-AFLP analysis, the gene corresponding to *ART* TDF 470 is up-regulated in the *Cf-4/Avr4* tomato plants when compared to the various control plants (result not shown). *ART* TDF 470 matches the 3'-end of the Open Reading Frame (ORF) of the tomato gene encoding Heat Shock Protein (LeHSP) 90-1 (Liu et al., 2004) (Table 1). This gene was recently shown to be required for plant development, *R* gene-mediated resistance and non-host resistance to *Pseudomonas cichorii* (Kanzaki et al., 2003; Lu et al., 2003; de la Fuente van Bentem et al., 2005).

Our cDNA-AFLP analysis revealed constitutive expression of *ART* gene 424a in *Cf-4/Avr4* plants at all time points, including sampling before the temperature shift, whereas the gene was not expressed in the control plants (result not shown). TDF 424a has a length of 373 bp and the full-length cDNA was isolated from a tomato cDNA library using primers specific for the 424a sequence (Table 1). BLAST analysis of the complete ORF revealed that the *ART* gene matching TDF 424a encodes a nuclear GTPase with an N-terminal nuclear localization signal. In *N. benthamiana*, inoculation with TRV:424a results in stunted plants that also have curled leaves (Figure 3A, panels 9-11).

TDF 614 has a length of 197 bp and the 3' 179 bp are identical to TDF 653, except for four single nucleotide polymorphisms (SNPs), one of which is present in the *Mse*I restriction site used for cDNA-AFLP analysis. TDF 614 is only present in the Cf0 background, whereas TDF 653 is specific for *Cf-4* (result not shown). There are no differences in the expression of the corresponding genes at the various time points, indicating that TDF 614 and 653 do not originate from an *ART* gene but rather that the SNP at the *Mse*I restriction site has resulted in the generation of a

differential TDF. We were not able to clone a cDNA exactly matching either of the two *ART* TDFs. However, one full-length cDNA clone was identified that differed in two SNPs from TDF 614 and also in two SNPs from TDF 653 (Table 1). The encoded protein is an L19 ribosomal protein which is a subunit of ribosomes.

According to the cDNA-AFLP profile, the *ART* gene corresponding to TDF 222 is slightly up-regulated in *Cf-4/Avr4* when compared to the control plants (result not shown). Interestingly, the full-length cDNA matching *ART* TDF 222 encodes a protein of the CC-NB-LRR class of resistance proteins (Table 1). As VIGS using TDF 222 results in suppression of both the Avr4- and Inf1-induced HR, we designated the corresponding protein NRC1, for <u>N</u>B-LRR protein <u>R</u>equired for HR-associated <u>C</u>ell death 1.

Table 1. Characteristics of the tomato *ART* TDFs of which inoculation onto transgenic *N. benthamiana:Cf-4* as a TRV:*ART* recombinant, severely compromises both the Avr4- and Inf1-induced HR.

ART TDF nr.	Size (bp)	Size of ORF of cDNA (bp)	Location of TDF in ORF	Accession nr. of cDNA	Encoded protein
470	373	2100	1637-2009	AY368906	LeHSP90-1
424a	373	1827	1379-1751	DQ304482	Nuclear GTPase
614	197	750	583-750[*]	DQ304483	L19 ribosomal protein
653	179	750	601-750[*]	DQ304483	L19 ribosomal protein
222	252	2664	1793-2044	DQ304484	CC-NB-LRR protein

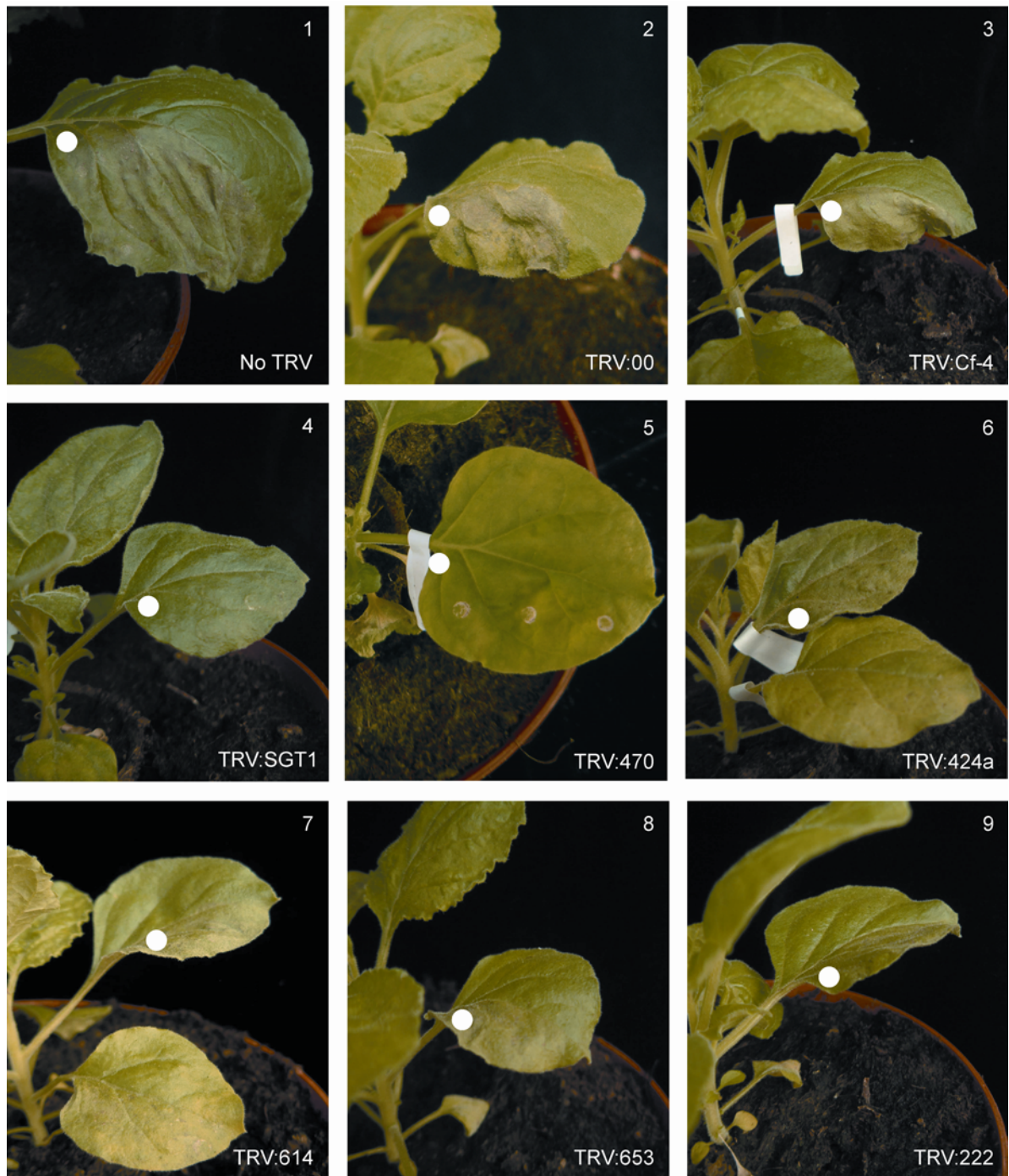[*]: The remainder of the TDF corresponds to the 3'-untranslated region.

Figure 3C. HR induced by *Inf1* agroinfiltration in TRV:ART-inoculated plants. HR was not compromised in non-inoculated, TRV:00- and TRV:Cf-4-inoculated plants (panels 1-3), whereas inoculation with the other TRV recombinants severely suppressed the *Inf1*-induced HR (panels 4-9).

DISCUSSION

Expression Profiling of Cf-4/Avr4 Plants by cDNA-AFLP

Analysis by cDNA-AFLP resulted in the generation of 50,000 fragments of which about 1% corresponded to <u>A</u>vr4-<u>R</u>esponsive <u>T</u>omato (*ART*) TDFs. This proportion is similar to the percentage of differential TDFs (260 fragments) identified amongst the 30,000 fragments that were analyzed by Durrant et al. (2000), who used a transgenic *Cf-9* tobacco cell suspension treated with Avr9. Surprisingly, 70% of the *ART* genes is already differentially expressed before the shift to a lower temperature, when on RNA gel blots no HR-related gene expression is apparent (De Jong et al., 2002) (data not shown). However, when the *Cf-4/Avr4* plants are kept at 33°C for more than four weeks, yellowing of the oldest leaves and stunted growth can be observed (data not shown), suggesting that HR-signaling is not completely eliminated at this temperature. This observation is in agreement with data of De Jong et al. (2002), who used a *Cf-9*-transgenic tobacco cell suspension that had lost Avr9-responsiveness at 33°C but did not show a complete loss of the Avr9-binding capacity at this temperature, suggesting that residual elicitor perception occurs at 33°C. Thus, suppression of HR-signaling at an elevated temperature is not complete and some *Cf*-signaling still occurs. However, this seems not to be sufficient to trigger actual execution of the HR, thereby allowing rescue of the plants. Recently it was shown that an elevated temperature in combination with high humidity (95%) results in complete blocking of the HR (Wang et al., 2005). Shifting the *Cf-4/Avr4* and control *Cf-4* seedlings from 33°C and 95% humidity to a permissive temperature and humidity might enable us to identify additional *ART* genes.

In addition to *ART* TDFs corresponding to genes differentially expressed due to the specific *Cf-4*/*Avr4*-mediated response, various TDFs were identified that originate from known differences between the *Cf-4* control plants and *Cf-4*/*Avr4* cross. Retrieval of a TDF corresponding to *Avr4* and *Hcr9*s derived from the Cf0 background (Parniske et al., 1999), together with the observed redundancy of various identified TDFs, indicates that differential expression profiling by cDNA-AFLP is very sensitive and close to saturation.

Functional Analysis of *ART* Genes by Virus-Induced Gene Silencing (VIGS)

To test the involvement of the *ART* genes in the HR, 192 TDFs that were selected based on their putative identity and expression profile were subjected to VIGS in *N. benthamiana:Cf-4*. For 10 % of the fragments (20 out of 192), VIGS resulted in a decreased HR upon agroinfiltration of *Avr4* and/or *Inf1*. Lu and colleagues (2003) identified 79 cDNAs (1.6%) in a VIGS screen for genes

involved in *Pto*/*AvrPto*-induced signaling, using a normalized library of *N. benthamiana* consisting of 4,992 clones. The higher percentage found here suggests that the cDNA-AFLP screen enriches for genes involved in the HR. Still, we identified only 20 possible candidates, indicating that many additional genes that are expected to be involved in the HR apparently are not identified. This could be explained by the fact that expression of these genes does not change during the induction or execution of the HR, or that a lower number of genes is involved in Avr4- or Inf1-induced HR as compared to AvrPto-induced HR. A third possibility is that the homology of the heterologous tomato TDFs used for VIGS is not sufficient to trigger silencing of the endogenous genes in *N. benthamiana* (Thomas et al., 2001). The latter problem may be overcome by performing the VIGS screen in tomato.

For five *ART* genes, severe HR suppression upon agroinfiltration of *Avr4* and *Inf1* in TRV:ART-inoculated plants was obtained, indicating that these genes, or their close homologues, are essential for the HR. These five *ART* genes encode a heat-shock protein 90 (HSP90) (TDF 470), a nuclear GTPase (TDF 424a), an L19 ribosomal protein (TDFs 614 and 653) and a CC-NB-LRR protein (TDF 222) (Table 1).

ART fragment 470 is derived from the gene encoding heat shock protein 90-1, a member of the HSP90 family (Milioni and Hatzopoulos, 1997). HSP90 is a unique molecular chaperone, as most of its known substrates are signaling proteins (Young et al., 2001). Support for the biological relevance of HSP90 in disease resistance comes from the analysis of *N. benthamiana* plants in which the corresponding gene has been silenced. HSP90 proved to be involved in plant development, *N-, Rx-, Pto-* and *I-2*-mediated resistance pathways, Inf1-induced HR and non-host resistance to *Pseudomonas cichorii* (Kanzaki et al., 2003; Lu et al., 2003; de la Fuente van Bentem et al., 2005). HSP90 associates with the resistance proteins N and I-2 (Liu et al., 2004; de la Fuente van Bentem et al., 2005) and in Arabidopsis its binding to RAR1 and SGT1 (Shirasu and Schulze-Lefert 2003; Takahashi et al., 2003) provides further evidence for the involvement of HSP90 in disease resistance signaling. Our results confirm that the protein is required for plant development and Inf1-induced HR (Figure 3A, panel 8) and we also show that HSP90 plays a role in *Cf-4*/*Avr4*-induced HR (Figures 3B and 3C; panel 5). We observed a milder growth phenotype than described by others (Kanzaki et al., 2003; Lu et al., 2003), as in our experiments the plants are stunted but still develop up to four normal leaves (Figure 3A, panel 8). Probably silencing using a heterologous fragment is less efficient and only results in (partial) silencing of the closely-related HSP90-1/-2-encoding genes, and not of the other HSP90 isoforms (Liu et al., 2004).

The gene corresponding to TDF 424a is specifically and constitutively expressed in the *Cf-4*/*Avr4* plants and has high homology to genes encoding nuclear GTPases. In animals, members of

the nuclear GTPase family are named nucleostemins and prevent stem cells from differentiation. Interestingly, over-expression of a human nucleostemin induces p53-mediated apoptosis, a form of cell death comparable to HR-like cell death (Tsai and McKay 2002). In yeast a homologous gene (*NUG1*) is essential for viability and the GTPase it encodes is physically associated with pre-ribosomal particles (Bassler et al., 2001). VIGS using TRV:424a does not only affect the HR but also influences leaf shape and plant size (Figure 3A, panels 9-11), indicating that the gene, or a close homologue, is also involved in developmental processes in addition to the HR.

The sequences of *ART* TDFs 614, originating from Cf0, and 653, from *Cf-4*, are identical except for four single nucleotide polymorphisms (SNPs) and a 5'-extension of 18 bp in *ART* fragment 614. One SNP is located in the *Mse*I site used for cDNA-AFLP analysis, resulting in two TDFs of different size that discriminate between a Cf0 and *Cf-4* background. Both TDFs were independently identified in the VIGS screen for decreased HR. Isolation of the full-length cDNA from tomato cultivar RZ52201 resulted in a clone that has two SNPs compared to TDF 614 and two SNPs compared to TDF 653. The gene corresponding to this cDNA codes for an L19 ribosomal protein. In a VIGS screen of random cDNAs of a normalized library of *N. benthamiana*, Lu and associates (2003) found that 22 of the 79 cDNAs suppressing *Pto/AvrPto*-induced HR have homology to various ribosomal proteins. Although we identified the gene only due to differences in the genomic background, ribosomal proteins are differentially expressed in *Pti4*-expressing plants in which defense responses are activated (Chakravarthy et al., 2003). Probably the massive transcriptional up-regulation of various defense-related genes in the tomato seedlings mounting the HR (Cai et al., 2001; De Jong et al., 2002), requires a similar increase in translational machinery. Furthermore, the results confirm the importance of *de novo* protein synthesis for activation of the HR (Greenberg, 1997).

The protein encoded by the full-length cDNA corresponding to TDF 222 has high homology to CC-NB-LRR resistance proteins and appears to be involved in the HR induced by at least two non-related elicitors (Avr4 and Inf1). The protein, that we have designated NRC1, also appears to be involved in development as VIGS with a fragment of the encoding gene also causes growth defects (Figure 3A, panel 14). The expression of *NRC1* is slightly up-regulated in *Cf-4/Avr4* plants and silencing of the gene results in a decreased HR, indicating that the encoded protein is a positive regulator of the HR. Recently, Peart and associates (2005) identified a CC-NB-LRR protein that is specifically required for functionality of the *N* gene that provides resistance to TMV. They suggested that NB-LRR proteins also function in signaling downstream of resistance proteins and that possibly many resistance proteins require NB-LRR proteins to be functional. The requirement of an NB-LRR protein downstream of the extracellular receptor-like protein Cf-4 and also

downstream of the protein mediating perception of Inf1 in *N. benthamiana* supports this hypothesis. Our observation could also indicate that NRC1 is acting at a point of convergence of various HR signaling pathways. Furthermore, the CC-NB-LRR requirement for Cf protein function provides a possible direct link to SGT1 and HSP90. The latter proteins have both been shown to be associated with CC-NB-LRR resistance proteins (Liu et al., 2004; Lu et al., 2003; Peart et al., 2002; Shirasu and Schulze-Lefert 2003; Takahashi et al., 2003; de la Fuente van Bentem et al., 2005), whereas silencing of SGT1- and HSP90-encoding genes also compromises the *Cf-4*/*Avr4* response (Figure 3B, panels 4, 5). Further studies focused on NRC1 should reveal whether the protein is also required for other defense signaling pathways and should provide information on the stage where the protein acts in the signal transduction cascade leading to HR.

In conclusion, the combination of transcriptional profiling of plants undergoing a synchronized HR and VIGS using the identified differential TDFs is a powerful method for gene discovery. Still, 90% of the selected *ART* TDFs does not appear to affect the HR when used for VIGS in *N. benthamiana*. Either these *ART* genes are indeed not absolutely required for the HR, or the additional genes that play a role in *N. benthamiana* lack sufficient homology to the tomato TDFs. To circumvent the latter problem, the TDFs will be used for VIGS in tomato, as this will allow us not only to study their role in the HR itself, but also in actual resistance against *C. fulvum*.

MATERIALS AND METHODS

Plant Material and Growth Conditions

A near-isogenic line of tomato cultivar MoneyMaker containing the *Cf-4*-introgressed region providing resistance against *C. fulvum* (referred to as *Cf-4*), was crossed to Cf0 plants (lacking functional *Cf* resistance genes) homozygous for the *Avr4* transgene (De Jong et al., 2002). To synchronize germination, the *Cf-4*/*Avr4* seeds that were obtained, and seeds from *Cf-4* control plants were surface-sterilized with 20% Glorix for 20 min and then thoroughly rinsed with water. After germination under standard greenhouse conditions, the seedlings were transferred to an incubator (Elbanton, Kerkdriel, NL) at 33°C under a 16-8 h light/dark regime. After 10 days the plants were transferred to room temperature (20-25°C) and at 0, 10, 20, 30, 60, 90 min and 2, 4, 8 and 24 h after transfer whole plants, except for the roots, were collected. *Nicotiana benthamiana* plants that were used for VIGS studies were grown under standard greenhouse conditions.

RNA Isolation and cDNA-AFLP Analysis

About 100 mg (fresh weight) of plant material obtained at the various time points was homogenized for 2 min at 1800 rpm in a Micronic deep well tube, using a Retsch MM200 bead mill and 3mm stainless steel beads. The lysate was cleared using a Qiashredder homogenizer (Qiagen, Venlo, NL) and subsequently total RNA was isolated using RNA-easy 96 plates (Qiagen, Venlo, NL). Poly A$^+$-RNA was isolated using magnetic oligo(dT) 25-Dynabeads (Dynal, Oslo, Norway) according to the manufacturer's instructions. First- and second-strand cDNA synthesis was carried out according to standard protocols, using Superscript II (Sambrook and Russell, 2001).

The final volume after cDNA synthesis was 50μl, from which 20μl (10-500ng of cDNA) was subjected to the standard AFLP template production protocol (Bachem et al., 1996; Vos et al., 1995). Restriction enzymes used for template preparation were *Mse*I as frequent cutter and *Taq*I as rare cutter. The AFLP adaptor primers 5'-CTCGTAGACTGCGTAC-3' and 5'-CGGTACGCAGTCT-3' (*Taq*I-adaptor primers, 5'-protruding end underlined) and 5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT- 3' (*Mse*I-adaptor primers, 5'-protruding end underlined) were ligated onto the restriction fragments. A non-selective pre-amplification was performed using the non-selective adaptor primers without additional nucleotides. The selective PCR amplification steps were performed using ^{33}P-dATP-labelled AFLP primers with additional nucleotides complementary to the *Taq*I or *Mse*I protruding ends of the cDNA fragments (underlined) and with selective nucleotides (indicated by N, representing an A, C, G or T): 5'-GTAGACTGCGTACCGANN-3 (*Taq*I-primer; a +2 primer) and 5'-GATGAGTCCTGAGTAANNN-3' (*Mse*I-primer; a +3 primer). The initial small scale screen using four and 16 AFLP primer combinations was done using a *Taq*I forward primer with an AA extension in combination with either four +2 *Mse*I reverse primers (extension AA, AC, AG or AT) or 16 +3 *Mse*I reverse primers (extension AAN, ACN, AGN or ATN), respectively. The large scale cDNA-AFLP analysis was essentially carried out as described by Vos et al. (1995) using all possible (4^2 x 4^3=1024) AFLP primer combinations.

All amplification reactions were performed on a PE-9600 thermocycler, using Taq DNA polymerase (PE Biosystems, Foster City, CA, USA), the oligonucleotides were obtained from MWG Biotech AG (Ebersberg, Germany) and *Mse*I and *Taq*I were from NE-Biolabs Inc. (New Brunswick, NE, USA). After the selective PCR, the samples were denatured and separated on 4.5% polyacrylamide sequencing-type gels, which were fixed by placing them in 10% acetic acid for 30 min. After washing for 10 min in water, the gel was dried on a glass plate at 80°C, after which a phosphor-imaging screen was exposed to the gel for 20 h. Labeled DNA fragments were visualized

by scanning of the screens with a Fuji BAS-2000 phosphor-imager analysis system (Fuji Photo Film Company Ltd., Tokyo, Japan).

Isolation, Sequencing and Analysis of *ART* TDFs

Fragments corresponding to genes differentially expressed in *Cf-4*/*Avr4* seedlings when compared to *Cf-4*, were excised from the dried gel and eluted in 200µl Tris-EDTA (pH 8.0), after which 5µl was reamplified using the same primer set as for the initial PCR amplification. The fragments were cloned in the pCR2.1 vector using the HTP TOPO TA cloning Kit (Invitrogen, Carlsbad, CA) and transformed to *E. coli* DH5α. Four independent colonies were picked and the insert sizes were determined on a sequencing gel. One clone containing an insert of the correct size was sequenced using ABI Big-Dye cycle sequencing (Amersham, Roosendaal, NL). The sequences were analyzed for homology to various databases (Altschul et al., 1997), using the program PEDANT (Frishman et al., 2003).

Plasmid Constructs

From the 426 *ART* TDFs, 192 TDFs were re-amplified from the pCR2.1 vector, using M13 primers with *Hind*III and *Asp*718 sites. After purification, the PCR fragments were cloned into *Hind*III/*Asp*718-digested pTV:00, the binary Tobacco Rattle Virus (TRV) RNA2 vector that facilitates virus-induced gene silencing in *N. benthamiana* (Ratcliff et al., 2001), resulting in TRV:ART recombinants. As 15 TDFs contained internal *Hind*III or *Asp*718 sites, the fragments used for VIGS of the corresponding genes were smaller than the original TDF. For VIGS of *Cf-4*, a *Cf-4* fragment was PCR-amplified from vector pCf4 (pRH48; containing 35S:*Cf-4*) (Van der Hoorn et al., 2000) using Expand-Taq polymerase and the primers 5'-GTGAC<u>GGATCC</u>TTCCGAATTCACTCCTAAACC-3' (*Bam*HI site underlined) and 5'-CTCTGGGAATCCTGTACTCTCATC-3'. As the resulting PCR fragment contains an internal *Hind*III site, *Bam*HI/*Hind*III digestion results in a 404 bp fragment (bp 1163-1566; covering the part of the ORF encoding LRRs 15 to 21, (Thomas et al., 1997), which was cloned into the *Bam*HI and *Hind*III sites of pTV:00.

Transformation of Agrobacterium tumefaciens

The various TRV:ART binary constructs were transformed to *Agrobacterium tumefaciens* strain GV3101, containing the helper plasmid pSoup (Hellens et al., 2000), by electroporation (Takken et al., 2000).

Transformation of *N. benthamiana* with the *Cf-4* Resistance Gene

To obtain *N. benthamiana* plants that respond to the Avr4 elicitor of *C. fulvum*, the plants were transformed with plasmid pFT44. This binary vector is derived from pCGN1548 (Calgene, Davis, CA, USA) and contains a 5.8 kb *Pst*I fragment encompassing the *Cf-4* gene. The *Pst*I fragment was isolated from a λ clone identified in a genomic λgem11 library from *Cf-4* tomato plants. In addition to the *Cf-4* coding sequence, it contains 2.9 kb of promoter sequence and 0.8 kb of 3'-sequence (Takken, 1999). Plant transformation was performed following the protocol of Horsch et al. (1985).

VIGS using TRV:ART Recombinants and Analysis for a Decreased HR

Four-week-old *Cf-4*-transgenic *N. benthamiana* plants were inoculated by co-infiltration of Agrobacterium carrying pBintra6 (the binary TRV1 vector (Ratcliff et al., 2001)) and Agrobacterium carrying the various TRV:ART recombinants, in a 1:1 ratio. For each of the TRV:ART clones three or four plants were used. Three weeks post virus inoculation the third, fourth and fifth leaf above the primary inoculated leaves were challenged with Avr4 and Inf1 by agroinfiltration, whereas after another two weeks also the sixth and seventh leaf were challenged with these elicitors. Agroinfiltrations were performed as described by Van der Hoorn et al. (2000), using either Agrobacterium strain GV3101 containing the binary vector pRH87 (35S:*Avr4* construct) (Van der Hoorn et al., 2000), at an OD of 0.03 or strain MOG101 carrying the binary vector pInf1 (35S:Inf1 construct) (Kamoun et al., 2003), at an OD of 1.0. Three to five days post infiltration the leaves were examined for the development of an HR.

RT-PCR

Four leaf discs (approximately 100 mg (fresh weight) of tissue) were collected from the third, fourth and fifth leaf above the primary inoculated leaves of TRV-infected *N. benthamiana* plants, at three weeks after inoculation. Total RNA was extracted using the QIA-Gen RNAeasy extraction method (Qiagen, Venlo, NL) and treated with RNase-Free DNase (Bio-Rad, Veenendaal, NL). First strand cDNA was synthesized from 1μg of total RNA using the Bio-Rad cDNA synthesis kit (Bio-Rad, Veenendaal, NL), according to the manufacturers' instructions. PCR primers for actin were based on the sequence of TC8012 (ActinF: 5'-TATGGAAACATTGTGCTCAGTGG-3'; ActinR: 5'-CCAGATTCGTCATACTCTGCC-3'), generating a fragment of 217bp. For *PDS* (TC9930), primers PDSF (5'-CCTGAGAGACTTTGCATGCC-3') and PDSR (5'-CTTCAGTTTTCTGTCAAACCATATATG-3') were used, generating a fragment of 294bp.

Primers to amplify the *N. benthamiana ART* 424a orthologue were designed on a highly conserved sequence present in tomato *ART* 424a and the *ART* 424a orthologue of potato (TC121093) and tobacco (BP134842) (ART424aF: 5'-AACCAGGAATGAGGGGGAGCATT-3' and ART424aR: 5'-GTTTAGTGTTCAACATGCCTTCCTC-3'), generating a fragment of 360bp. PCRs were performed with for each cycle denaturing at 95°C for 15 sec, annealing at 60°C for 45 sec, and elongation at 72°C for 60 sec.

Isolation of Full-length *ART* cDNAs

For full-length cDNA isolation, 5'- and 3'-flanks of the *ART* TDFs were isolated by PCR using *ART* TDF-specific primers, from a cDNA library derived from *L. esculentum* cultivar RZ52201 infected with potato aphids (*Macrosiphum euphorbiae*). In this library, the cDNA was directionally cloned into the *Eco*RI and *Xho*I sites of Lambda ACT that can be converted into a yeast two-hybrid shuttle plasmid, using Cre-lox excision. Primers were designed matching the ends of the TDFs

(222F: 5'-AACTTTGGAACGTACAAACCCTCATTGT-3';

222R: 5'-AGAGAACATACTCAGTGCAGCTTTCTGGT-3';

614/653F: 5'-AGAGAACATACTCAGTGCAGCTTTCTGGT-3';

614/653R: 5'-GAGCTAGTTTGTTAGTGTTTTTTCAGGGCA-3';

424aF: 5'-TAAAGTCATCACCGTCCATATCAATGGCA-3;

424aR: 5'-CGATGAAGTTTATGGAAGTGAATCCTCCA-3'). Because the orientation of the TDF in the cDNA was unknown, each primer was used in combination with both a vector-specific forward primer (5'-GGGATGTTTAATACCACTACAATGGATGA-3') and reverse primer (5'-AAGTGAACTTGCGGGGTTTTTCAGTATCT-3'). Amplified fragments were inserted into pGEM-T easy and subjected to sequence analysis. 5'- and 3'- flanks were assigned to one cDNA when the overlapping parts were identical.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

(A)

(B)
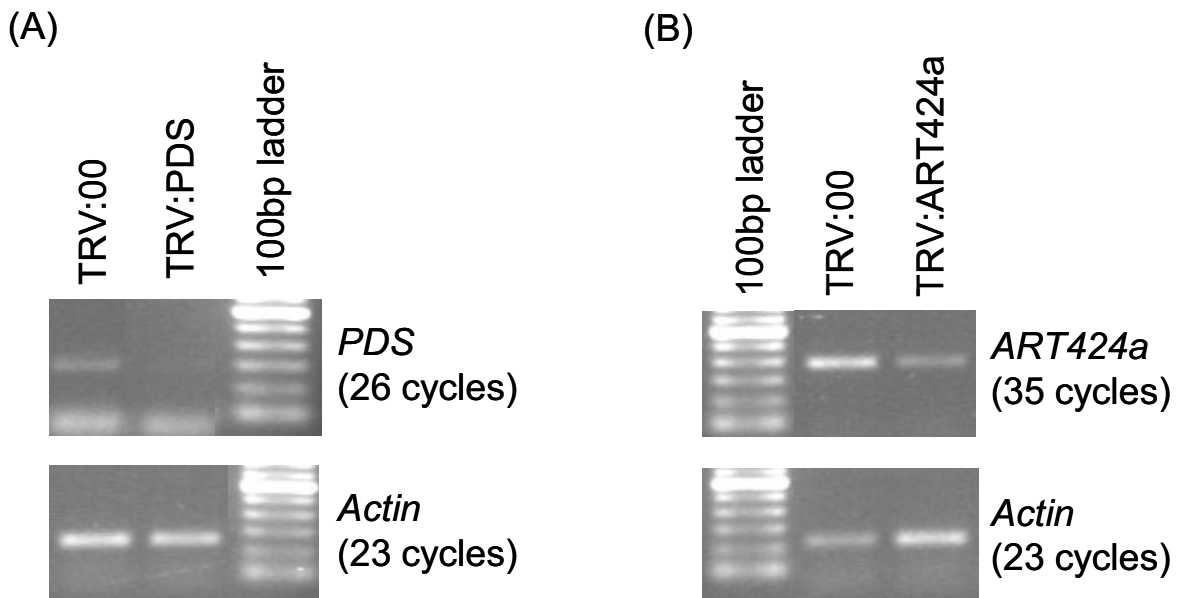
Figure S1. VIGS using TRV induces knock-down of *Nicotiana benthamiana* target genes. *N. benthamiana* plants were inoculated with TRV:PDS, TRV:ART424a or TRV:00 (= empty vector) and total RNA was isolated at three weeks after inoculation. RT-PCR was performed to determine relative expression levels of *PDS* and *ART* 424a genes targeted by VIGS. As a control for cDNA input we used primers targeting the actin gene.

(**A**) Relative expression levels of *PDS*.

(**B**) Relative expression levels of the *ART* 424a orthologue of *N. benthamiana*.

Table S1. Characteristics of the 442 transcript-derived fragments (TDFs) corresponding to <u>A</u>vr4-<u>R</u>esponsive Tomato (*ART*) genes identified by cDNA-AFLP analysis. For all 442 *ART* TDFs the following information is provided; the TDF number, the accession number, whether they are selected for VIGS (ns: not selected; s: selected) and the expression in the *Cf-4/Avr4* seedlings compared to the *Cf-4* controls. Under 'Description of function' the PEDANT blastx results are shown and in case these are not available, blastn results are given. Either the best hit code for the protein or DNA hit is mentioned, followed by the corresponding E-value (E-values higher than 0.001 are excluded). VIGS using *ART* TDFs marked by an asterisk results in a decreased HR in transgenic *Nicotiana benthamiana* expressing the *Cf-4* resistance gene, upon challenge with Avr4 of *Cladosporium fulvum* and/or Inf1 of *Phytophthora infestans*.

TDF nr	accession nr.	Selected for VIGS	Up- or down-regulated	Description of function	Best hit code protein	Best hit code DNA	E-value
1	DV935719	ns	up	no hit			
3	DV935720	s	up	gene: "ROF1"; product: "rof1"; Arabidopsis thaliana FK506 binding protein FKBP62 (ROF1) mRNA, complete cds.	TREMBL:AT49453_1		4.00E-04
4	DV935721	s	up	no hit			
5*	DV935722	s	up	gene: "P0431H09.9"; product: "putative leucine rich repeat containing protein kinase"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0431H09.	TREMBL:AP003248_8		6.00E-26
6	DV935723	s	up	no hit			
7	DV935724	s	up	no hit			
8	DV935725	ns	up	product: "nuclear receptor binding factor-like protein"; Arabidopsis thaliana clone 249370 mRNA, complete sequence.	TREMBL:AY086398_1		1.00E-04
9	DV935726	s	up	omega-6 fatty acid desaturase (EC 1.14.99.-) defense-related - tomato	PIR:T07009		1.00E-40
10	DV935727	ns	down	ribosomal protein L4, chloroplast - common tobacco	PIR:T01739		2.00E-16
11	DV935728	s	up	no hit			
12		ns	up	no sequence data			
13	DV935729	s	up	cytochrome P450 92B1 - garden petunia	PIR:JC7886		3.00E-20
14	DV935730	s	up	no hit			
15		s	down	no proper sequence information			
16a	DV935731	ns	up	gene: "poni1a"; product: "putative membrane protein"; Solanum tuberosum poni1a gene for putative membrane protein, exons 1-3 (allele 1)	TREMBL:STU309301_1		6.00E-50
17	DV935732	ns	up	Cf-4A protein - tomato	PIR:T07015		5.00E-52
18	DV935733	ns	up	gene: "CA"; product: "beta-carbonic anhydrase"; Nicotiana tabacum beta-carbonic anhydrase (CA) mRNA, complete cds; nuclear gene for chloroplast product.	TREMBL:AF454759_1		5.00E-12
19		ns	up	no sequence data			

20	DV935734	s	down	gene: "At2g25910"; product: "expressed protein"; Arabidopsis thaliana chromosome 2 clone F17H15 map B68, complete sequence.	TREMBL:AC005395_6		3.00E-04
21	DV935735	s	down	product: "protein disulphide isomerase"; Elaeis guineensis clone opsc112 protein disulphide isomerase mRNA, partial cds.	TREMBL:AY182168_1		3.00E-16
22	DV935736	s	up	no hit			
23	DV935737	s	up	gene: "PKPI-B10"; product: "Kunitz-type proteinase inhibitor precursor"; Solanum tuberosum Kunitz-type proteinase inhibitor precursor (PKPI-B10) gene, partial cds.	TREMBL:AF536175_1		9.00E-26
24	DV935738	s	down	no hit			
25a	DV935739	s	down	gene: "PKPI-B10"; product: "Kunitz-type proteinase inhibitor precursor"; Solanum tuberosum Kunitz-type proteinase inhibitor precursor (PKPI-B10) gene, partial cds.	TREMBL:AF536175_1		9.00E-26
25b	DV935740	ns	down	gene: "PKPI-B10"; product: "Kunitz-type proteinase inhibitor precursor"; Solanum tuberosum Kunitz-type proteinase inhibitor precursor (PKPI-B10) gene, partial cds.	TREMBL:AF536175_1		9.00E-26
25c	DV935741	ns	down	gene: "ndhA"; product: "NADH dehydrogenase ND 1 subunit"; Nicotiana tomentosiformis ndhA gene for NADH dehydrogenase ND 1 subunit, partial cds, clone:NtndhA-01.	TREMBL:AB098248_1		5.00E-06
26	DV935742	ns	down	ferredoxin 2[4Fe-4S] frxB - common tobacco chloroplast	PIR:FENTB		4.00E-48
27a	DV935743	ns	up	no hit			
28	DV935744	ns	down	nuclear receptor binding factor-like protein - Arabidopsis thaliana	PIR:T47517		9.00E-11
31	DV935745	s	down	no hit			
32	DV935746	ns	up	product: "putative copia-like polyprotein"; Lycopersicon esculentum putative permease gene, partial cds; suppressor-like protein, putative centromere protein, putative auxin growth promotor protein, and putative protein phosphatase genes, complete cds; LT	TREMBL:AF275345_13		2.00E-15
33	DV935747	s	up	peroxidase (EC 1.11.1.7) - common tobacco	PIR:T03686		3.00E-05
34	DV935748	ns	up	no hit			
35	DV935749	ns	up	gene: "CBP4"; product: "cyclic nucleotide-gated calmodulin-binding ion channel"; Nicotiana tabacum cyclic nucleotide-gated calmodulin-binding ion channel (CBP4) mRNA, complete cds.	TREMBL:AF079872_1		2.00E-08
36	DV935750	ns	down	product: "nuclear receptor binding factor-like protein"; Arabidopsis thaliana clone 249370 mRNA, complete sequence.	TREMBL:AY086398_1		2.00E-04

37	DV935751	s	up	receptor protein kinase-like protein - Arabidopsis thaliana	<u>PIR:T45899</u>	4.00E-08
38	DV935752	ns	up	gene: "PDK"; product: "pyruvate dehydrogenase kinase"; Arabidopsis thaliana pyruvate dehydrogenase kinase (PDK) mRNA, nuclear gene encoding mitochondrial protein, complete cds.	<u>TREMBL:AF039406_1</u>	6.00E-33
39a		ns	down	no sequence data		
40	DV935753	s	down	product: "Conserved hypothetical protein"; Vibrio vulnificus CMCP6 chromosome I section 2 of 11 of the complete sequence.	<u>TREMBLNEW:AE016798 _159</u>	9.00E-09
41	DV935754	ns	down	gene: "PKPI-B10"; product: "Kunitz-type proteinase inhibitor precursor"; Solanum tuberosum Kunitz-type proteinase inhibitor precursor (PKPI-B10) gene, partial cds.	<u>TREMBL:AF536175_1</u>	1.00E-21
42	DV935755	ns	up	gene: "OJ1743A09.16"; product: "Putative leucine-rich repeat transmembrane protein kinase"; Oryza sativa (japonica cultivar-group) chromosome 3 clone OJ1743A09, complete sequence.	<u>TREMBL:AC105364_16</u>	1.00E-06
43	DV935756	ns	up	no hit		
45	DV935757	ns	down	hypothetical protein F25G13.100 - Arabidopsis thaliana	<u>PIR:T10203</u>	1.00E-08
46	DV935758	ns	down	product: "unknown"; Davidia involucrata unknown gene.	<u>TREMBL:AF448811_1</u>	1.00E-07
47	DV935759	ns	up	mRNA-binding protein precursor [imported] - tomato (fragment)	<u>PIR:T52071</u>	2.00E-73
48	DV935760	s	up	no hit		
49	DV935761	ns	down	gene: "lap2"; product: "leucine aminopeptidase"; Lycopersicon esculentum leucine aminopeptidase (lap2) mRNA, partial cds.	<u>TREMBL:SL50152_1</u>	1.00E-15
50	DV935762	s	up	no hit		
51	DV935763	ns	down	no hit		
52		ns	up	no sequence data		
53		ns	down	no sequence data		
54	DV935764	ns	up	no hit		
59		s	up	no proper sequence information		
61	DV935765	ns	up	gene: "P0489G09.3"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0489G09.	<u>TREMBL:AP002745_3</u>	9.00E-07
62		ns	up	no sequence data		
64	DV935766	ns	up	gene: "OSJNBa0090L05.14"; product: "hypothetical protein"; Oryza sativa chromosome 3 BAC OSJNBa0090L05 genomic sequence, complete sequence.	<u>TREMBL:AC084765_14</u>	5.00E-50
65		ns	up	no sequence data		
66	DV935767	ns	down	no hit		
67a	DV935768	s	down	no hit		
68	DV935769	s	up	gene: "NH8"; product: "nam-like protein 8"; Petunia x hybrida nam-like protein 8 (NH8) mRNA, complete cds.	<u>TREMBL:AF509871_1</u>	8.00E-12
69		ns	down	no sequence data		

70	DV935770	s	up	product: "DnaJ-like protein"; Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MJJ3.	TREMBL:ATAB5237_16		2.00E-15
71	DV935771	s	up	Alfalfa histone H3 (H3-1.1) gene, complete cds.	TREMBL:MSHISH3A_1		2.00E-05
72	DV935772	ns	down	Mouse DNA sequence from clone RP23-401I8 on chromosome 2		EMBL:AL663062	7.00E-04
73	DV935773	ns	up	no hit			
74	DV935774	s	down	no hit			
75	DV935775	s	up	product: "receptor-like protein kinase"; Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MCK7.	TREMBL:AB019228_17		9.00E-28
76	DV935776	s	up	product: "protein kinase-like protein"; Capsicum annuum protein kinase-like protein mRNA, partial cds.	TREMBL:AF367985_1		3.00E-22
77	DV935777	s	up	no hit			
78	DV935778	s	up	gene: "OSJNBa0090L05.14"; product: "hypothetical protein"; Oryza sativa chromosome 3 BAC OSJNBa0090L05 genomic sequence, complete sequence.	TREMBL:AC084765_14		8.00E-48
79	DV935779	s	down	no hit			
80	DV935780	s	up	no hit			
81	DV935781	s	down	no hit			
82	DV935782	s	up	no hit			
83	DV935783	s	up	gene: "P0665A11.7"; product: "putative receptor-protein kinase"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0665A11.	TREMBL:AP003106_7		5.00E-26
84	DV935784	ns	up	gene: "F9E11.4"; product: "disease resistance protein, putative; 1096-4664"; Arabidopsis thaliana chromosome 1 BAC F9E11 genomic sequence, complete sequence.	TREMBL:AC079678_1		2.00E-10
85	DV935785	s	up	no hit			
86	DV935786	s	up	gene: "lap2"; product: "leucine aminopeptidase"; Lycopersicon esculentum leucine aminopeptidase (lap2) mRNA, partial cds.	TREMBL:SL50152_1		1.00E-15
88	DV935787	ns	up	gene: "beta-geo"; product: "beta-geo"; Cloning vector CA1, complete sequence.	TREMBL:CVU93512_1		1.00E-20
89	DV935788	ns	up	histone H4 - tomato	PIR:S32769		6.00E-18
90		ns	up	no sequence data			
91	DV935789	s	down	no hit			
92a	DV935790	ns	up	gene: "P0001B06.11"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0001B06.	TREMBL:AP002537_11		5.00E-04
93	DV935791	ns	up	no hit			
94	DV935792	ns	up	gene: "At5g57870"; product: "eukaryotic initiation factor 4, eIF4-like protein"; Arabidopsis thaliana eukaryotic initiation factor 4, eIF4-like protein (At5g57870) mRNA, complete cds.	TREMBL:BT000467_1		2.00E-06

95	DV935793	s	down	no hit			
96	DV935794	s	up	no hit			
97	DV935795	ns	up	no hit			
98	DV935796	ns	down	no hit			
99	DV935797	s	up	hypothetical protein F15K9.23 - Arabidopsis thaliana	PIR:H86162		3.00E-12
100	DV935798	ns	down	Homo sapiens BAC clone RP11-288M20 from 2, complete sequence.		EMBL:AC068542	9.00E-04
101	DV935799	ns	down	no hit			
102	DV935800	s	down	no hit			
103	DV935801	s	down	product: "serine/threonine-specific protein kinase-like protein"; Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MRB17.	TREMBL:AB016879_9		4.00E-20
104	DV935802	s	down	no hit			
105		ns	down	no sequence data			
106	DV935803	s	up	product: "hydrolase-like protein"; Arabidopsis thaliana clone 122353 mRNA, complete sequence.	TREMBL:AY084939_1		3.00E-32
107	DV935804	ns	up	product: "hydrolase-like protein"; Arabidopsis thaliana clone 122353 mRNA, complete sequence.	TREMBL:AY084939_1		9.00E-36
108	DV935805	s	up	peroxidase (EC 1.11.1.7) 1 precursor - tomato	PIR:S04763		5.00E-09
109a	DV935806	ns	down	unnamed ORF; Sequence 1 from Patent WO0183788.	TREMBL:AX298155_1		2.00E-07
110a	DV935807	ns	up	no hit			
111	DV935808	ns	up	gene: "poni1a"; product: "putative membrane protein"; Solanum tuberosum poni1a gene for putative membrane protein, exons 1-3 (allele 1)	TREMBL:STU309301_1		1.00E-44
112	DV935809	ns	down	hypothetical protein F25P22.13 [imported] - Arabidopsis thaliana	PIR:C96764		1.00E-29
113	DV935810	s	up	Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MRH10.	TREMBL:AB006703_6		5.00E-04
114	DV935811	s	up	Lycopersicon esculentum mRNA for SPY protein (SPINDLY-related protein)		EMBL:AJ312094	2.00E-20
115	DV935812	s	up	gene: "catsSt"; product: "catalase"; S.tuberosum cat gene encoding catalase (partial)	TREMBL:STCATGP_1		2.00E-13
116	DV935813	s	down	gene: "lap2"; product: "leucine aminopeptidase"; Lycopersicon esculentum leucine aminopeptidase (lap2) mRNA, partial cds.	TREMBL:SL50152_1		2.00E-15
117*	DV935814	s	up	subtilisin-like proteinase (EC 3.4.21.-) precursor P69B, pathogenesis-related - tomato	PIR:T07184		6.00E-25
118		ns	up	no sequence data			
119	DV935815	s	up	no hit			
120	DV935816	s	down	no hit			
121	DV935817	ns	down	no hit			
122		ns	up	no sequence data			
123		ns	down	no sequence data			
124		ns	up	no sequence data			
125		ns	up	no sequence data			
126		ns	up	no sequence data			

201	DV935818	ns	down	no hit		
202	DV935819	s	down	gene: "EEF13"; Solanum melongena EEF13 mRNA, complete cds.	TREMBL:AB032753_1	1.00E-11
203	DV935820	s	up	auxin-regulated protein GH3 homolog At2g46370 - Arabidopsis thaliana	PIR:A84902	8.00E-07
204*	DV935821	s	up	no hit		
205	DV935822	ns	up	disease resistance protein - tomato	PIR:T17460	3.00E-04
206	DV935823	ns	up	no hit		
207	DV935824	ns	up	product: "cellulose synthase catalytic subunit"; Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MYH9.	TREMBL:AB016893_7	6.00E-06
208		ns	up	no sequence data		
209	DV935825	ns	up	gene: "krp2"; product: "p27KIP1-related-protein 2"; Lycopersicon esculentum mRNA for p27KIP1-related-protein 2 (krp2 gene)	TREMBL:LES441250_1	6.00E-17
210	DV935826	s	up	gene: "CalS1"; product: "callose synthase 1 catalytic subunit"; Arabidopsis thaliana callose synthase 1 catalytic subunit (CalS1) mRNA, complete cds.	TREMBL:AF237733_1	2.00E-53
211	DV935827	ns	up	hypothetical protein a - maize transposable element Ac	PIR:T02916	4.00E-15
212	DV935828	ns	down	gene: "NH10"; product: "nam-like protein 10"; Petunia x hybrida nam-like protein 10 (NH10) mRNA, complete cds.	TREMBL:AF509873_1	9.00E-25
213	DV935829	ns	up	no hit		
214	DV935830	ns	up	product: "osmotin"; N.tabacum mRNA for osmotin	TREMBL:NTOSMOTIN_1	4.00E-06
215	DV935831	s	up	no hit		
216	DV935832	ns	up	disease resistance protein - tomato	PIR:T17460	8.00E-73
217		ns	up	no sequence data		
218	DV935833	s	up	no hit		
219	DV935834	ns	down	product: "leucine aminopeptidase"; Solanum lycopersicum clone TPP6 leucine aminopeptidase mRNA, partial cds.	TREMBL:LE20593_1	3.00E-10
220	DV935835	ns	down	product: "leucine aminopeptidase"; Solanum lycopersicum clone TPP6 leucine aminopeptidase mRNA, partial cds.	TREMBL:LE20593_1	3.00E-10
221	DV935836	s	down	no hit		
222*	DV935837	s	up	no hit		
223	DV935838	ns	up	no hit		
224	DV935839	ns	up	gene: "OSJNBb0035A12.2"; Oryza sativa genomic DNA, chromosome 4, BAC clone: OSJNBb0035A12.	TREMBL:OSJN00142_2	3.00E-09
225*	DV935840	s	down	product: "inhibitor of yeast proteinase A"; Solanum lycopersicum partial gene for inhibitor of yeast proteinase A	TREMBL:SLY289776_1	9.00E-44
226	DV935841	ns	up	importin alpha - tomato	PIR:T04329	2.00E-48
227	DV935842	ns	up	pathogenesis-related protein P6 precursor - tomato	PIR:VCTO14	1.00E-35

228	DV935843	ns	down	gene: "AtPPC6;1"; product: "protein phosphatase 2C"; Arabidopsis thaliana AtPPC6;1 mRNA for protein phosphatase 2C, partial cds.	TREMBL:AB079669_1		2.00E-10
229	DV935844	ns	up	gene: "At5g18120"; product: "unknown protein"; Arabidopsis thaliana clone RAFL14-98-K17 (R20305) unknown protein (At5g18120) mRNA, complete cds.	TREMBL:BT002852_1		7.00E-25
230	DV935845	ns	down	no hit			
231	DV935846	ns	down	homeotic protein Athb-7 - Arabidopsis thaliana	PIR:S47137		2.00E-07
232	DV935847	ns	down	naringenin-chalcone synthase (EC 2.3.1.74) B - garden petunia	PIR:SYPJCB		7.00E-19
233	DV935848	ns	down	no hit			
234a	DV935849	ns	up	disease resistance E - tomato	PIR:T17462		5.00E-29
236	DV935850	ns	up	no hit			
237	DV935851	ns	down	product: "leucine aminopeptidase"; Solanum lycopersicum clone TPP6 leucine aminopeptidase mRNA, partial cds.	TREMBL:LE20593_1		1.00E-09
238	DV935852	ns	down	no hit			
239	DV935853	s	down	vacuolar proton-ATPase chain E - potato	PIR:T07110		3.00E-06
240	DV935854	ns	up	no hit			
241	DV935855	s	up	product: "At1g04130/F20D22_10"; Arabidopsis thaliana At1g04130/F20D22_10 mRNA, complete cds.	TREMBL:AY054170_1		7.00E-45
242	DV935856	s	up	CYTOCHROME P450 LXXVIIA1 (EC 1.14.14.1) (P-450EG6) (FRAGMENT).	SWISSPROT:C771_SOLME		5.00E-20
243	DV935857	ns	down	no hit			
244	DV935858	ns	up	product: "chlorophyll a,b binding protein type I"; S.tuberosum gene for chlorophyll a/b binding protein type I	TREMBL:STCHLABP_1		2.00E-05
245	DV935859	s	up	no hit			
246	DV935860	ns	down	no hit			
247	DV935861	s	down	gene: "AATL1"; product: "amino acid transporter-like protein 1"; Arabidopsis thaliana AATL1 gene for amino acid transporter-like protein 1, complete cds.	TREMBL:AB030586_1		9.00E-21
248	DV935862	ns	down	Potato gene for proteinase inhibitor, complete cds.		EMBL:D17331	3.00E-06
249	DV935863	s	up	heat shock protein 18p - common tobacco	PIR:T03958		1.00E-16
250	DV935864	ns	down	Pseudomonas fluorescens ribosomal RNA operon rrnB, complete sequence.		EMBL:AF134704	1.00E-101
251	DV935865	ns	up	phosphopyruvate hydratase (EC 4.2.1.11) - tomato	PIR:JQ1185		3.00E-28
252	DV935866	ns	up	subtilisin-like proteinase (EC 3.4.21.-) p69e - tomato	PIR:T06579		3.00E-13
253	DV935867	s	down	gene: "lap2"; product: "leucine aminopeptidase"; Lycopersicon esculentum leucine aminopeptidase (lap2) mRNA, partial cds.	TREMBL:SL50152_1		4.00E-10

254	DV935868	ns	up	gene: "pgm I"; product: "cytosolic phosphoglucomutase"; Solanum tuberosum mRNA for cytosolic phosphoglucomutase	TREMBL:STU240054_1	3.00E-06
255	DV935869	s	up	Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K3K7.	TREMBL:AB017063_15	2.00E-30
256	DV935870	ns	down	no hit		
257	DV935871	s	up	gene: "AT2G03810_1/F19B11.11"; product: "hypothetical protein"; Arabidopsis thaliana hypothetical protein (AT2G03810_1/F19B11.11) mRNA, complete cds.	TREMBL:AF517846_1	3.00E-23
258	DV935872	s	up	product: "receptor-like protein kinase"; Arabidopsis thaliana genomic DNA, chromosome 5, P1 clone:MXF12.	TREMBL:AB016892_2	1.00E-19
259	DV935873	ns	up	product: "polyprotein-like"; Lycopersicon chilense retrotransposon TLC1.1, complete sequence.	TREMBL:AF279585_1	4.00E-14
260	DV935874	ns	down	gene: "At2g26980"; product: "putative protein kinase"; Arabidopsis thaliana chromosome 2 clone T20P8 map B68, complete sequence.	TREMBL:AC005623_4	4.00E-35
261	DV935875	s	down	gene: "GM5"; product: "env-like protein"; Gossypium barbadense env-like protein (GM5) gene, partial cds.	TREMBL:AY081177_1	3.00E-05
262	DV935876	ns	up	product: "At3g01370"; Arabidopsis thaliana At3g01370 gene, complete cds.	TREMBLNEW:BT010594_1	1.00E-07
263	DV935877	s	up	gene: "ORF"; A.thaliana mRNA for unknown protein (cDNA101)	TREMBL:ATORF101_1	2.00E-26
264	DV935878	ns	up	no hit		
265	DV935879	ns	up	no hit		
266	DV935880	ns	down	product: "Unknown"; Vibrio vulnificus CMCP6 chromosome I section 4 of 11 of the complete sequence.	TREMBLNEW:AE016800_256	5.00E-21
267	DV935881	ns	up	no hit		
268	DV935882	s	up	gene: "At2g20190"; product: "unknown protein"; Arabidopsis thaliana unknown protein (At2g20190) mRNA, complete cds.	TREMBL:AY091025_1	8.00E-29
269*	DV935883	s	up	no hit		
270	DV935884	ns	down	gene: "At2g15790"; product: "expressed protein"; Arabidopsis thaliana chromosome 2 clone F19G14 map mi398, complete sequence.	TREMBL:AC006438_17	2.00E-27
271	DV935885	ns	down	hypothetical protein F16A16.170 - Arabidopsis thaliana	PIR:T04527	2.00E-04
273	DV935886	s	down	product: "leucine aminopeptidase"; Solanum lycopersicum clone TPP6 leucine aminopeptidase mRNA, partial cds.	TREMBL:LE20593_1	3.00E-10

274	DV935887	s	up	gene: "mdr10"; product: "MDR-like ABC transporter"; Oryza sativa (japonica cultivar-group) mdr10 gene for MDR-like ABC transporter, exons 1-11	TREMBL:OSA535062_1	8.00E-05
275*	DV935888	s	up	no hit		
276	DV935889	s	up	no hit		
277	DV935890	ns	up	pathogenesis-related protein P6 precursor - tomato	PIR:VCTO14	1.00E-61
278	DV935891	s	up	serine/threonine protein kinase - Arabidopsis thaliana	PIR:T50501	3.00E-10
279	DV935892	ns	up	Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K3D20.	TREMBL:AP002031_4	2.00E-12
280	DV935893	ns	up	product: "disease resistance gene homolog Mi-copy1"; Lycopersicon esculentum disease resistance gene homolog Mi-copy2 gene, complete cds; resistance gene pseudogene, complete sequence; disease resistance gene homolog Mi-copy1 gene, complete cds; and unkn	TREMBL:U81378_3	1.00E-08
281	DV935894	s	up	O.berteriana mitochondrial gene for protein involved in cytochrome c biogenesis	EMBL:X69555	9.00E-12
282	DV935895	s	up	product: "putative copia-like polyprotein"; Lycopersicon esculentum putative permease gene, partial cds; suppressor-like protein, putative centromere protein, putative auxin growth promotor protein, and putative protein phosphatase genes, complete cds; LT	TREMBL:AF275345_13	8.00E-18
283	DV935896	ns	up	gene: "At1g51110/F23H24_8"; product: "unknown protein"; Arabidopsis thaliana At1g51110 mRNA for unknown protein, complete cds, clone: RAFL19-77-I08.	TREMBL:AK118545_1	2.00E-07
284	DV935897	ns	down	product: "hypothetical protein"; Bacillus megaterium plasmid pBM400, complete sequence.	TREMBLNEW:AF142677_51	4.00E-10
285	DV935898	ns	up	product: "peptidylprolyl isomerase"; Arabidopsis thaliana genomic DNA, chromosome 5, TAC clone:K15N18.	TREMBL:AB015468_3	1.00E-56
286	DV935899	ns	down	gene: "StNR5"; product: "nitrate reductase"; Solanum tuberosum StNR5 mRNA for nitrate reductase, partial cds.	TREMBL:AB062142_1	9.00E-26
287	DV935900	ns	up	no hit		
288	DV935901	ns	down	RIBULOSE BISPHOSPHATE CARBOXYLASE LARGE CHAIN PRECURSOR (EC 4.1.1.39).	SWISSPROT:RBL_SOLTU	4.00E-20
289	DV935902	s	up	product: "receptor protein kinase"; Arabidopsis thaliana genomic DNA, chromosome 3, P1 clone:MMG15.	TREMBL:AB028616_8	1.00E-04

290	DV935903	ns	up	ribulose-bisphosphate carboxylase (EC 4.1.1.39) small chain 3A precursor - tomato	PIR:RKTO3C	3.00E-30
291	DV935904	s	up	no hit		
292	DV935905	ns	up	no hit		
293	DV935906	s	up	gene: "P0409B11.32"; product: "putative PS60; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 7, PAC clone:P0409B11.	TREMBLNEW:AP005185_20	5.00E-09
294	DV935907	ns	up	subtilisin-like proteinase (EC 3.4.21.-) 3 - tomato	PIR:T07169	1.00E-21
295	DV935908	ns	up	no hit		
296	DV935909	s	up	hypothetical protein F26K9.90 - Arabidopsis thaliana	PIR:T48055	7.00E-26
297	DV935910	s	down	no hit		
298a	DV935911	ns	up	no hit		
299	DV935912	s	up	Lycopersicon esculentum BAC clone Clemson_Id 2O7, partial sequence.	EMBLNEW:AF411806	2.00E-79
300	DV935913	s	up	no hit		
301	DV935914	s	up	no hit		
302	DV935915	ns	up	gene: "P0410E03.6"; product: "putative ABC transporter protein"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0410E03.	TREMBL:AP002844_6	3.00E-18
303	DV935916	s	up	gene: "P0681B11.1"; product: "putative protein kinase APK1B"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0681B11.	TREMBL:AP003022_1	4.00E-08
304	DV935917	ns	up	no hit		
305	DV935918	s	up	no hit		
306	DV935919	s	up	gene: "p69c"; product: "P69C protein"; Lycopersicon esculentum p69c gene, complete CDS	TREMBL:LES17277_1	3.00E-08
307	DV935920	ns	up	gene: "PH1"; product: "anthocyanin 5-O-glucosyltransferase"; Petunia x hybrida PH1 mRNA for anthocyanin 5-O-glucosyltransferase, complete cds.	TREMBL:AB027455_1	3.00E-23
308	DV935921	s	up	no hit		
309a	DV935922	s	up	no hit		
310		ns	up	no sequence data		
311	DV935923	s	up	probable helicase [imported] - Arabidopsis thaliana	PIR:H84432	2.00E-19
312	DV935924	ns	up	no hit		
313a	DV935925	s	down	gene: "gln"; product: "glutamine synthetase GS2"; Solanum tuberosum glutamine synthetase GS2 (gln) mRNA, partial cds; nuclear gene for plastid product.	TREMBL:AF302113_1	2.00E-05
314		ns	down	no sequence data		
315	DV935926	ns	down	product: "conserved hypothetical protein"; Corynebacterium efficiens YS-314 DNA, complete genome, section 6/11.	TREMBL:AP005219_73	1.00E-06
316*	DV935927	s	up	no hit		

317	DV935928	s	up	gene: "At1g67890"; product: "putative protein kinase"; Arabidopsis thaliana putative protein kinase (At1g67890) mRNA, complete cds.	TREMBL:AY059769_1		2.00E-08
318	DV935929	s	up	subtilisin-like proteinase (EC 3.4.21.-) p69e - tomato	PIR:T06579		4.00E-21
319	DV935930	ns	up	subtilisin-like proteinase (EC 3.4.21.-) p69e - tomato	PIR:T06579		2.00E-16
320	DV935931	s	up	L.esculentum mRNA for lycopene beta-cyclase		X86452	0
401	DV935932	ns	down	no hit			
402	DV935933	ns	up	no hit			
403a	DV935934	s	up	gene: "F6F9.23"; product: "Unknown protein"; Arabidopsis thaliana Unknown protein (F6F9.23) mRNA, complete cds.	TREMBL:AY062497_1		1.00E-12
404		ns	up	no sequence data			
405	DV935935	ns	up	gene: "dof1"; product: "Dof zinc finger protein"; Solanum tuberosum mRNA for Dof zinc finger protein (dof1 gene)	TREMBL:STU242853_1		1.00E-14
406	DV935936	s	down	no hit			
407	DV935937	s	down	class I patatin - potato	PIR:T07592		3.00E-08
408	DV935938	ns	down	Tomato 25S ribosomal RNA gene		EMBL:X13557	7.00E-14
409	DV935939	ns	up	product: "GUS3-3 protein"; Synthetic construct GUS3-3 protein gene, complete cds.	TREMBL:AY100472_1		7.00E-26
410		ns	up	no sequence data			
411	DV935940	s	up	gene: "WRKY1"; product: "WRKY DNA binding protein"; Solanum tuberosum mRNA for WRKY DNA binding protein (WRKY1 gene)	TREMBL:STU278507_1		5.00E-22
412	DV935941	ns	up	no hit			
413	DV935942	ns	up	product: "unknown"; Arabidopsis thaliana clone 21639 mRNA, complete sequence.	TREMBL:AY086125_1		2.00E-10
414	DV935943	ns	up	probable calcium binding protein - Arabidopsis thaliana	PIR:T45708		3.00E-05
415	DV935944	s	up	no hit			
416	DV935945	ns	up	cytochrome P450 76A2 - eggplant	PIR:S38534		5.00E-19
417	DV935946	ns	up	no hit			
418	DV935947	s	up	no hit			
419	DV935948	s	up	no hit			
420a	DV935949	s	up	no hit			
421*	DV935950	s	up	no hit			
422	DV935951	ns	up	no hit			
423	DV935952	s	up	no hit			
424a*	DV935953	s	up	gene: "F17A9.21"; product: "putative GTPase"; Arabidopsis thaliana chromosome III BAC F17A9 genomic sequence, complete sequence.	TREMBL:AC016827_20		9.00E-24
424b	DV935954	ns	up	probable inositol polyphosphate 5'-phosphatase [imported] - Arabidopsis thaliana	PIR:T00670		9.00E-26
425	DV935955	ns	up	no hit			
426	DV935956	ns	up	no hit			
427	DV935957	s	down	product: "extensin-like protein"; Solanum tuberosum mRNA for extensin-like protein, partial	TREMBL:STAJ3220_1		6.00E-36
428	DV935958	ns	up	gene: "NtEIG-E80"; Nicotiana tabacum NtEIG-E80 mRNA, complete cds.	TREMBLNEW:AB041515_1		9.00E-08

429	DV935959	s	up	no hit		
430	DV935960	s	up	product: "mitochondrial protein-like protein"; Cucumis sativus mRNA for mitochondrial protein-like protein, partial cds.	TREMBL:AB047269_1	4.00E-05
431	DV935961	s	up	hypothetical protein T26I12.30 - Arabidopsis thaliana	PIR:T47654	8.00E-10
432	DV935962	s	up	gene: "pdr7"; product: "PDR-like ABC transporter"; Oryza sativa (japonica cultivar-group) pdr7 gene for PDR-like ABC transporter, exons 1-23	TREMBL:OSA535048_1	2.00E-06
433	DV935963	s	up	no hit		
434	DV935964	ns	up	no hit		
435	DV935965	s	up	no hit		
436	DV935966	ns	up	gene: "poni1b"; product: "putative membrane protein"; Solanum tuberosum poni1b gene for putative membrane protein, exons 1-3 allele 2	TREMBL:STU309302_1	4.00E-24
437	DV935967	ns	up	hypothetical protein F22M8.4 [imported] - Arabidopsis thaliana	PIR:H86150	4.00E-07
438	DV935968	ns	up	callose synthase catalytic subunit-like protein - Arabidopsis thaliana	PIR:T49914	2.00E-10
439	DV935969	ns	down	gene: "psbA"; product: "photosystem II D1 protein"; Amaranthus powellii isolate Elma 4 photosystem II D1 protein (psbA) gene, partial cds; chloroplast gene for chloroplast product.	TREMBL:AF441081_1	7.00E-12
440	DV935970	ns	up	no hit		
441*	DV935971	s	up	gene: "CYP81B1l"; product: "cytochrome P450"; Helianthus tuberosus mRNA for cytochrome P450, CYP81B1l	TREMBL:HTCYP81L_1	7.00E-31
442	DV935972	ns	up	gene: "krp2"; product: "p27KIP1-related-protein 2"; Lycopersicon esculentum mRNA for p27KIP1-related-protein 2 (krp2 gene)	TREMBL:LES441250_1	2.00E-05
443	DV935973	ns	up	no hit		
444	DV935974	s	up	no hit		
445	DV935975	s	up	no hit		
446	DV935976	s	up	product: "receptor protein kinase-like protein"; Capsicum annuum receptor protein kinase-like protein mRNA, complete cds.	TREMBL:AY064216_1	4.00E-43
447	DV935977	s	down	gene: "Td"; product: "threonine deaminase"; L.esculentum threonine deaminase (Td) mRNA, 3' end.	TREMBL:LEILV1A_1	1.00E-50
448	DV935978	ns	down	no hit		
449	DV935979	ns	up	product: "AT3g48690/T8P19_200"; Arabidopsis thaliana AT3g48690/T8P19_200 mRNA, complete cds.	TREMBL:AY064980_1	2.00E-04
450	DV935980	s	down	cysteine-rich extensin-like protein 2 precursor - common tobacco	PIR:B48232	9.00E-19
451	DV935981	s	up	no hit		

452	DV935982	s	up	gene: "At1g50600"; product: "putative scarecrow protein"; Arabidopsis thaliana clone C105359 putative scarecrow protein (At1g50600) mRNA, complete cds.	TREMBL:BT000883_1		3.00E-19
453	DV935983	s	up	no hit			
454	DV935984	s	up	gene: "atpB"; product: "H(+)-transporting ATP synthase"; Helicteres baruensis chloroplast atpB gene, partial	TREMBL:HBAJ3078_1		3.00E-04
455	DV935985	s	up	hypothetical protein T26I12.30 - Arabidopsis thaliana	PIR:T47654		1.00E-08
456	DV935986	s	up	product: "cytochrome P450"; Pyrus communis cytochrome P450 mRNA, complete cds.	TREMBL:AF386512_1		1.00E-17
457	DV935987	ns	down	Petunia axillaris 26S ribosomal RNA gene, complete sequence.		EMBL:AF479174	1.00E-82
458	DV935988	s	up	Solanum demissum late blight resistance protein (R1) gene, complete cds.		EMBLNEW:AF447489	2.00E-19
459a	DV935989	s	up	hypothetical protein T5N23.70 - Arabidopsis thaliana	PIR:T47630		2.00E-09
461	DV935990	ns	up	no hit			
462	DV935991	ns	up	no hit			
463	DV935992	ns	up	no hit			
464	DV935993	ns	up	gene: "NtPDR1"; product: "pleiotropic drug resistance like protein"; Nicotiana tabacum NtPDR1 mRNA for pleiotropic drug resistance like protein, complete cds.	TREMBL:AB075550_1		5.00E-44
465	DV935994	ns	up	product: "glutamate decarboxylase isozyme 3"; Nicotiana tabacum glutamate decarboxylase isozyme 3 mRNA, complete cds.	TREMBLNEW:AF353615_1		3.00E-35
466	DV935995	ns	down	hypothetical protein F21B7.8 - Arabidopsis thaliana	PIR:T00894		3.00E-06
467	DV935996	s	up	product: "glucose-6-phosphate/phosphate translocator 2"; Solanum tuberosum glucose-6-phosphate/phosphate translocator 2 gene, promoter region and complete cds; nuclear gene for plastid product.	TREMBL:AY163867_1		6.00E-48
468	DV935997	s	up	cytochrome P450 92B1 - garden petunia	PIR:JC7886		2.00E-18
469	DV935998	ns	down	no hit			
470*	DV935999	s	up	product: "molecular chaperone Hsp90-1"; Lycopersicon esculentum molecular chaperone Hsp90-1 mRNA, complete cds.	TREMBLNEW:AY368906_1		7.00E-59
471	DV936000	ns	down	hypothetical protein F20D10.300 - Arabidopsis thaliana	PIR:T05645		1.00E-07
472	DV936001	ns	up	product: "AT3g25150/MJL12_9"; Arabidopsis thaliana AT3g25150/MJL12_9 mRNA, complete cds.	TREMBL:AY070733_1		2.00E-17
473	DV936002	ns	down	proteinase inhibitor I precursor - tomato	PIR:A24048		5.00E-41
474	DV936003	ns	down	no hit			

475	DV936004	s	up	no hit			
476	DV936005	ns	up	product: "polymerase"; Rice ragged stunt virus polymerase mRNA, complete cds.	TREMBL:AF015682_1		2.00E-04
477*	DV936006	s	up	no hit			
478	DV936007	ns	down	no hit			
479	DV936008	ns	down	gene: "ssa-13"; product: "putative senescence-associated protein"; Pisum sativum ssa-13 mRNA for putative senescence-associated protein, partial cds.	TREMBL:AB049723_1		7.00E-07
480	DV936009	s	up	STH-2 protein - potato	PIR:S35161		4.00E-33
481	DV936010	ns	up	gene: "H25N7.13"; product: "hygromycin-b kinase"; Trypanosoma brucei 221 VSG expression site BAC	TREMBL:TBH25N7_13		2.00E-37
482	DV936011	ns	up	no hit			
483	DV936012	s	up	hypothetical protein At2g15780 [imported] - Arabidopsis thaliana	PIR:C84533		3.00E-09
484	DV936013	ns	up	hypothetical protein F25G13.100 - Arabidopsis thaliana	PIR:T10203		4.00E-20
485	DV936014	s	up	product: "callose synthase-like protein"; Oryza sativa (japonica cultivar-group) callose synthase-like protein mRNA, partial cds.	TREMBL:AY324384_1		1.00E-10
486	DV936015	ns	up	no hit			
487*	DV936016	s	up	gene: "At1g27200"; product: "unknown protein"; Arabidopsis thaliana unknown protein (At1g27200) mRNA, complete cds.	TREMBL:AF370145_1		1.00E-14
488	DV936017	s	up	gene: "P0496D04.2"; product: "putative casein kinase 1, delta isoform 1"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 7, PAC clone:P0496D04.	TREMBLNEW:AP004670_1		6.00E-10
489	DV936018	ns	up	Sequence 35 from Patent WO02057464.		EMBLALERT:AX481974	5.00E-76
490	DV936019	s	up	gene: "poni1b"; product: "putative membrane protein"; Solanum tuberosum poni1b gene for putative membrane protein, exons 1-3 allele 2	TREMBL:STU309302_1		2.00E-25
491	DV936020	ns	up	Sequence 1 from patent US 5824862.		EMBL:AR050092	6.00E-20
492	DV936021	ns	up	no hit			
493	DV936022	s	up	gene: "At3g50940/F18B3_220"; product: "putative BCS1 protein"; Arabidopsis thaliana At3g50940 mRNA for putative BCS1 protein, complete cds, clone: RAFL16-84-L10.	TREMBL:AK117288_1		2.00E-10
494	DV936023	ns	up	Caenorhabditis elegans cosmid C01F4, complete sequence.		EMBL:U97192	5.00E-04
495	DV936024	ns	up	product: "receptor protein kinase-like protein"; Capsicum annuum receptor protein kinase-like protein mRNA, complete cds.	TREMBL:AY064216_1		5.00E-42
496	DV936025	ns	up	no hit			
497a	DV936026	s	down	leucyl aminopeptidase (EC 3.4.11.1) DR57 - tomato	PIR:A48788		2.00E-10

498	DV936027	s	up	Lycopersicon esculentum beta-1,3-glucanase mRNA, complete cds.		EMBL:M80604	2.00E-11
499	DV936028	s	up	gene: "OSJNBa0006L06.21"; product: "putative protein kinase"; Oryza sativa chromosome 10 BAC OSJNBa0006L06 genomic sequence, complete sequence.	TREMBL:AC022457_26		2.00E-04
500	DV936029	s	up	product: "AT3g48690/T8P19_200"; Arabidopsis thaliana AT3g48690/T8P19_200 mRNA, complete cds.	TREMBL:AY064980_1		2.00E-04
501	DV936030	s	up	no hit			
502	DV936031	ns	up	gene: "ei2"; product: "Erwinia induced protein 2"; Solanum tuberosum Erwinia induced protein 2 (ei2) mRNA, complete cds.	TREMBL:AY187626_1		1.00E-10
503a	DV936032	ns	down	no hit			
504	DV936033	s	up	no hit			
505*	DV936034	s	up	no hit			
506		ns	up	no sequence data			
507	DV936035	ns	up	no hit			
508		ns	down	no sequence data			
509	DV936036	s	up	protein F15H18.11 [imported] - Arabidopsis thaliana	PIR:A86318		8.00E-07
560	DV936037	ns	up	no hit			
603		ns	up	no sequence data			
604		ns	up	no sequence data			
605	DV936038	ns	up	no hit			
606	DV936039	s	up	phosphoinositide-specific phospholipase C (EC 3.1.4.-) plc2 - potato	PIR:T07424		2.00E-10
607	DV936040	s	up	Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, clone:P0702F03.	TREMBL:AP002481_22		1.00E-29
608		ns	up	no sequence data			
609	DV936041	ns	up	hypothetical protein F19F18.160 - Arabidopsis thaliana	PIR:T04724		1.00E-07
610	DV936042	s	down	no hit			
611	DV936043	ns	up	probable ABC transporter [imported] - Arabidopsis thaliana	PIR:F84487		8.00E-12
612	DV936044	ns	up	gene: "P0504E02.19"; product: "photosystem II protein W-like protein"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 1, PAC clone:P0504E02.	TREMBL:AP003269_17		5.00E-30
613	DV936045	s	up	shaggy protein kinase 4 (EC 2.7.1.-) - garden petunia	PIR:S51105		1.00E-05
614*	DV936046	s	up	hypothetical protein T5C23.60 - Arabidopsis thaliana	PIR:T04210		2.00E-19
615	DV936047	s	up	product: "putative ammonium transporter AMT1;1"; Lotus japonicus putative ammonium transporter AMT1;1 mRNA, complete cds.	TREMBL:AF182188_1		1.00E-16
616	DV936048	ns	up	Solanum tuberosum hexokinase-related protein 1 (HxkRP1) mRNA, complete cds.		EMBL:AF118134	2.00E-09
617		ns	up	no sequence data			
618	DV936049	s	up	no hit			

619	DV936050	ns	up	no hit		
620a	DV936051	ns	up	gene: "At2g43950"; product: "expressed protein"; Arabidopsis thaliana chromosome 2 clone F6E13 map CIC10F02, CIC02E07, complete sequence.	TREMBL:AC004005_8	5.00E-04
620b	DV936052	ns	up	gene: "StNR3"; product: "NADH nitrate reductase"; Solanum tuberosum NADH nitrate reductase (StNR3) mRNA, complete cds.	TREMBL:STU95317_1	6.00E-06
621	DV936053	ns	up	product: "GUSA (N358Q); hexaHis tagged"; Binary vector pCAMBIA-1381Xa, complete sequence.	TREMBL:AF234303_1	4.00E-14
622	DV936054	ns	up	product: "GUSA (N358Q); hexaHis tagged"; Binary vector pCAMBIA-1381Xa, complete sequence.	TREMBL:AF234303_1	4.00E-15
623		ns	up	no sequence data		
624	DV936055	s	down	product: "Cell wall-associated hydrolase"; Vibrio vulnificus CMCP6 chromosome I section 2 of 11 of the complete sequence.	TREMBLNEW:AE016798_155	7.00E-20
625	DV936056	ns	down	leucyl aminopeptidase (EC 3.4.11.1) (clone pBlap1) precursor, wound-induced - tomato	PIR:T07849	9.00E-10
626	DV936057	ns	down	no hit		
627a	DV936058	ns	up	no hit		
628		ns	up	no sequence data		
629	DV936059	ns	up	no hit		
630	DV936060	ns	up	no hit		
631*	DV936061	s	up	Rice chloroplast apocytochrome b6 (petB) gene, complete cds.	TREMBL:OSPETB_2	9.00E-17
632	DV936062	s	down	product: "Unknown"; Vibrio vulnificus CMCP6 chromosome I section 4 of 11 of the complete sequence.	TREMBLNEW:AE016800_256	4.00E-18
633	DV936063	s	up	hypothetical protein F25G13.100 - Arabidopsis thaliana	PIR:T10203	2.00E-15
634		ns	up	no sequence data		
635		ns	up	no sequence data		
636		ns	up	no sequence data		
637		ns	up	no sequence data		
638	DV936064	ns	down	product: "mRNA binding protein precursor"; Lycopersicon esculentum mRNA binding protein precursor, mRNA, nuclear gene encoding chloroplast protein, complete cds.	TREMBL:AF106660_1	3.00E-40
639	DV936065	s	up	no hit		
640	DV936066	ns	up	no hit		

641	DV936067	ns	up	AVR4 protein - fungus (Cladosporium fulvum)	PIR:S41047		6.00E-11
642	DV936068	s	up	product: "F3M18.17"; Genomic sequence for Arabidopsis thaliana BAC F3M18 from chromosome I, complete sequence.	TREMBL:AC010155_17		2.00E-04
643	DV936069	s	up	gene: "At5g02850"; product: "unknown protein"; Arabidopsis thaliana clone RAFL21-19-B03 (R51384) unknown protein (At5g02850) mRNA, complete cds.	TREMBL:BT008646_1		2.00E-15
644	DV936070	ns	up	gene: "At5g02850"; product: "unknown protein"; Arabidopsis thaliana clone RAFL21-19-B03 (R51384) unknown protein (At5g02850) mRNA, complete cds.	TREMBL:BT008646_1		2.00E-15
645	DV936071	s	up	gene: "At2g20300"; product: "putative protein kinase"; Arabidopsis thaliana putative protein kinase (At2g20300) mRNA, complete cds.	TREMBL:AY091071_1		3.00E-17
646*	DV936072	s	up	hypothetical protein At2g06990 [imported] - Arabidopsis thaliana	PIR:B84481		1.00E-16
647b	DV936073	ns	up	product: "putative peroxidase"; Arabidopsis thaliana clone 126030 mRNA, complete sequence.	TREMBL:AY085060_1		2.00E-08
648	DV936074	ns	up	product: "glutamine synthetase"; Lycopersicon esculentum glutamine synthetase mRNA, partial cds.	TREMBL:LE14754_1		1.00E-13
649	DV936075	s	down	no hit			
650a	DV936076	s	down	no hit			
650b	DV936077	ns	down	hypothetical protein F13F21.5 [imported] - Arabidopsis thaliana	PIR:H96531		3.00E-04
651	DV936078	ns	up	gene: "F16P17.17"; product: "Unknown protein"; Arabidopsis thaliana Unknown protein (F16P17.17) mRNA, complete cds.	TREMBL:AF387005_1		9.00E-07
652		ns	up	no sequence data			
653*	DV936079	s	down	hypothetical protein T5C23.60 - Arabidopsis thaliana	PIR:T04210		1.00E-17
654	DV936080	s	up	no hit			
655	DV936081	ns	down	gene: "lap2"; product: "leucine aminopeptidase"; Lycopersicon esculentum leucine aminopeptidase (lap2) mRNA, partial cds.	TREMBL:SL50152_1		2.00E-10
656	DV936082	ns	up	hypothetical protein [imported] - Arabidopsis thaliana	PIR:B86234		1.00E-05
657		ns	up	no sequence data			
658	DV936083	ns	up	Nicotiana tabacum enr-T1 gene		EMBL:Y13862	5.00E-15
659	DV936084	ns	up	hypothetical protein F25G13.100 - Arabidopsis thaliana	PIR:T10203		1.00E-08
660	DV936085	ns	up	hypothetical protein F25G13.100 - Arabidopsis thaliana	PIR:T10203		4.00E-08
661	DV936086	ns	up	no hit			
662	DV936087	ns	up	probable RNA-binding protein [imported] - Arabidopsis thaliana	PIR:B84807		6.00E-20
663	DV936088	s	up	no hit			
664	DV936089	s	up	no hit			
665	DV936090	ns	up	no hit			

666	DV936091	ns	down	no hit			
667	DV936092	s	up	product: "conserved hypothetical protein"; Corynebacterium efficiens YS-314 DNA, complete genome, section 6/11.	TREMBL:AP005219_73		6.00E-09
668	DV936093	ns	up	no hit			
669	DV936094	s	up	no hit			
670		ns	up	no sequence data			
671	DV936095	s	up	no hit			
672*	DV936096	s	down	gene: "P0534A03.32"; product: "putative S-receptor kinase"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 7, PAC clone:P0534A03.	TREMBL:AP004401_23		2.00E-05
673	DV936097	s	up	gene: "OJ1457_D07.22"; product: "putative far-red impaired response protein"; Oryza sativa (japonica cultivar-group) genomic DNA, chromosome 7, BAC clone:OJ1457_D07.	TREMBL:AP003956_19		7.00E-04
674	DV936098	ns	up	gene: "MS"; product: "methionine synthase"; Solanum tuberosum methionine synthase (MS) mRNA, complete cds.	TREMBL:AF082893_1		1.00E-30
675	DV936099	ns	up	no hit			
676a	DV936100	s	down	Ralstonia solanacearum GMI1000 megaplasmid, complete sequence; segment 6/11		EMBL:AL646081	2.00E-25
677	DV936101	ns	up	no hit			
678	DV936102	s	up	hypothetical protein [imported] - Arabidopsis thaliana	PIR:B86253		5.00E-12
679	DV936103	ns	up	no hit			
680	DV936104	ns	up	gene: "psbC"; product: "photosystem II CP43 protein"; Spathiphyllum wallisii photosystem II D2 protein (psbD) and photosystem II CP43 protein (psbC) genes, partial cds; chloroplast genes for chloroplast products.	TREMBL:AF239794_2		5.00E-05
681	DV936105	s	down	probable cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) - apple tree	PIR:T16995		3.00E-24
682	DV936106	s	up	no hit			
683	DV936107	ns	up	gene: "Glu-1E1-x"; product: "high-molecular-weight glutenin x-type subunit"; Thinopyrum elongatum high-molecular-weight glutenin x-type subunit (Glu-1E1-x) gene, promoter region and complete cds.	TREMBL:AY280616_1		7.00E-08
684	DV936108	ns	up	Lycopersicon esculentum BAC clone Clemson_Id 2O7, partial sequence.		EMBLNEW:AF411806	1.00E-120
685	DV936109	ns	up	Lycopersicon esculentum BAC clone Clemson_Id 2O7, partial sequence.		EMBLNEW:AF411805	2.00E-27
686	DV936110	s	up	no hit			
687	DV936111	ns	up	catechol oxidase (EC 1.10.3.1) precursor [similarity] - tomato	PIR:S33544		4.00E-29
688	DV936112	s	down	no hit			

689	DV936113	ns	up	no hit			
690	DV936114	ns	up	no hit			
691	DV936115	s	up	no hit			
692a	DV936116	ns	up	no hit			
692b	DV936117	ns	up	no hit			
693	DV936118	ns	up	hypothetical protein F25G13.100 - Arabidopsis thaliana	PIR:T10203		1.00E-08
694	DV936119	s	up	Lycopersicon esculentum BAC clone Clemson_Id 2O7, partial sequence.		EMBLNEW:AF411806	1.00E-36
695	DV936120	s	up	hypothetical protein F25G13.100 - Arabidopsis thaliana	PIR:T10203		1.00E-08
999		ns	up	no proper sequence information			

CHAPTER 3

Functional Analysis of Avr-Responsive Tomato Genes Reveals Their Role in Resistance to Cladosporium fulvum

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ABSTRACT

We use the tomato-*Cladosporium fulvum* interaction as a model to study defense signaling initiated by recognition of race specific elicitors. cDNA-AFLP® analysis revealed 442 Avr4-Responsive Tomato (*ART*) transcripts either up- or down-regulated upon initiation of the *Cf-4/Avr4*-induced hypersensitive response (HR). 192 *ART* fragments were selected for functional analysis by Virus-Induced Gene Silencing (VIGS) in tomato and subsequently these plants were screened for decreased resistance to *C. fulvum*. First, we compared three different methods for their effectiveness to silence the visual marker gene *PDS* (phytoene desaturase) by VIGS in tomato. Next, two different methods for their effectiveness to sensitively detect growth of an avirulent strain of *C. fulvum* upon silencing of *Cf-4* or *Rcr3* (Required for *Cf-2* Resistance) were compared. The most effective method was subsequently employed to silence the genes corresponding to the 192 *ART* fragments in tomato and was combined with the most sensitive method to detect *C. fulvum* growth. Silencing of four *ART* transcripts corresponding to an NB-LRR protein (that we designated NRC1, for NB-LRR Required for HR-associated Cell death 1), a histon H3 protein, a ribosomal protein L1 and an unknown protein, resulted in loss of full *Cf-4*-mediated resistance to a race of *C. fulvum* expressing *Avr4*.

INTRODUCTION

Unraveling the mechanisms by which plants protect themselves against pathogens allows the development of new strategies to engineer disease-resistant plants (Stuiver and Custers, 2001). Transcriptomics and proteomics approaches are useful to identify large sets of genes and gene products, respectively, related to plant defense. Nowadays, high-throughput functional analyses by VIGS enable us to confirm whether the gene, or gene product, is actually required for active resistance (Baulcombe, 1999; Liu et al., 2002a; Brigneti et al., 2004; Gabriëls et al., 2006).

VIGS based on the Tobacco Rattle Virus (TRV) has been described previously for both *Nicotiana benthamiana* (Baulcombe, 1999) and tomato (Liu et al., 2002a). Replication of a recombinant TRV virus containing part of the sequence of an endogenous plant gene to be targeted, results in double-stranded viral RNA, including the RNA corresponding to the gene of interest, thereby triggering post-transcriptional silencing of the gene (Baulcombe, 1999). TRV is a bipartite positive strand RNA virus, consisting of RNA1 and RNA2, with a wide host range including

tobacco, potato and tomato (MacFarlane, 1999). RNA1 codes for the replicase- and movement-proteins, while RNA2 codes for the coat protein and nematode transmission proteins. Two binary vector systems containing cDNA clones of TRV1 and TRV2 are available, in both cases the nematode transmission proteins of TRV2 have been replaced by a multiple cloning site allowing insertion of a fragment of a gene of interest (Ratcliff et al., 2001; Liu et al., 2002b). The main difference between the vectors developed by Ratcliff et al. (2001), and those developed by Liu et al. (2002b), is the presence of a double 35S promoter driving the TRV1 and TRV2 transcripts and a self-cleaving ribozyme sequence at the 3'end, in the latter RNA strand.

VIGS of phytoene desaturase (*PDS*) provides a visual marker for silencing, as successful silencing of *PDS* results in suppression of carotenoid biosynthesis, thereby causing photobleaching which is manifested as white tissue (Kumagai et al., 1995). By using TRV:PDS, both TRV vector systems and three different TRV-inoculation methods were compared for their effectiveness of *PDS* silencing in *Solanum* species (Brigneti et al., 2004). It was shown that silencing by spray-inoculation of Agrobacterium containing the TRV binary vectors occurs one week later than silencing either initiated by agroinfiltrating the Agrobacterium culture, or by inoculation of plant-sap containing the virus-particles. The efficiency of the TRV vector system varied depending on the *Solanum* species used (Brigneti et al., 2004). Other methods to initiate VIGS are vacuum-infiltration and agrodrench of Agrobacterium cultures containing the TRV binary vectors (Ekengren et al., 2003; Ryu et al., 2004).

To identify genes involved in plant-defense we use the tomato-*Cladosporium fulvum* interaction. This pathosystem follows the gene-for-gene model (Joosten and De Wit, 1999; Rivas and Thomas, 2005; Thomma et al., 2005), in which *Cf* genes from tomato mediate *Avr*-triggered resistance to *C. fulvum.* Cf proteins are membrane-anchored Receptor-Like Proteins (RLPs) that mediate recognition of secreted Avrs of the fungus. In transgenic tomato seedlings containing *Cf-4* and expressing the matching avirulence gene *Avr4,* the HR can be synchronously induced by growing these *Cf-4/Avr4* seedlings at 33°C (by which the HR is inhibited) and shifting them to room temperature. cDNA-AFLP analysis of these seedlings at 0, 30, 60 and 90 minutes after temperature shift, revealed 442 Avr4-Responsive Tomato (*ART*) transcripts of which 192 were chosen for functional analysis by VIGS (Gabriëls et al., 2006). VIGS of the 192 selected *ART* fragments in *N. benthamiana* transformed with *Cf-4* (*N. benthamiana:Cf-4),* proved to be an efficient method to identify genes required for Avr4- or Inf1- induced HR (Gabriëls et al., 2006). The latter is an elicitor from *Phytophthora infestans,* which causes avirulence of this oomycete on *N. benthamiana* (Kamoun et al., 1998).

To identify genes involved in resistance to *C. fulvum,* we aimed to optimize VIGS in tomato (which is the only host for this fungus) in combination with a highly sensitive method to detect growth of *C. fulvum in planta*. Since VIGS in tomato is patchy, silencing of genes that are involved in resistance is expected to cause only partial loss of resistance rather than full susceptibility (Rowland et al., 2005). In addition, genes downstream in signaling pathways are likely to be functionally redundant, minimizing the chance to observe a phenotypic effect upon knock-down. Therefore, we first performed VIGS in tomato using genes upstream in defense signaling, like the resistance gene *Cf-4* itself and a gene required for *Cf-2*-mediated resistance, *Rcr3*. The latter is an apoplastic cysteine protease which is targeted by the avirulence gene product Avr2 of *C. fulvum,* thereby inhibiting this protease (Krüger et al., 2002; Rooney et al., 2005).

Based on pilot experiments that we performed using a fragment of *PDS* inserted in the multiple cloning site of the TRV binary vector developed by Ratcliff et al. (2001) and Liu et al. (2002b), it was decided to use the latter vector for VIGS in tomato. Three different TRV-inoculation systems (agroinfiltration, sap-inoculation and vacuum-infiltration), and two different methods to score for decreased resistance upon VIGS using TRV:Cf-4 and TRV:Rcr3 were compared. The most suitable TRV inoculation method and the most reliable *C. fulvum* detection method were eventually chosen for VIGS of all 192 selected *ART* fragments derived from genes that are differentially expressed during *Cf-4/Avr4*-induced HR. VIGS of three different *ART* fragments resulted in chlorosis, similar as the phenotype observed upon VIGS of these fragments in *N. benthamiana* (Gabriëls et al., 2006). VIGS of four *ART* fragments corresponding to the NB-LRR protein NRC1 (<u>N</u>B-LRR <u>R</u>equired for HR-associated <u>C</u>ell death 1), histon H3, a ribosomal protein L1 and an unknown protein resulted in loss of full resistance to *C. fulvum.*

RESULTS

Comparison of Three Different TRV Inoculation Methods for VIGS in Tomato

VIGS in tomato has been described as a method to obtain transient knock-downs of target genes within three weeks post TRV inoculation (Liu et al., 2002a). Here, we aimed to optimize VIGS in tomato in combination with a sensitive method to detect *C. fulvum* growth in silenced plants.

As a marker for efficiency of silencing we used the *PDS* (phytoene desaturase) gene, which results in a phenotype that can be visually scored. PDS is involved in the carotenoid biosynthesis pathway and silencing of its encoding gene results in photobleaching and development of white

tissue (Kumagai et al., 1995). Pilot experiments in which we silenced *PDS* using the TRV vectors described by Ratcliff et al. (2001) and by Liu et al. (2002b), revealed that the number of white tomato leaflets is higher, and the development of white tomato leaflets occurs faster, using the vectors described by the latter. Therefore, it was decided to use these TRV binary vectors for further studies aimed at optimizing VIGS in tomato.

We compared the efficiency of *PDS* silencing in ten-day-old tomato seedlings upon inoculation with TRV:PDS either through agroinfiltration (Liu et al., 2002a), sap-inoculation (Brigneti et al., 2004), or vacuum-infiltration (Ekengren et al., 2003). In all cases approximately three weeks post inoculation most leaflets of the second, third and fourth compound leaves had turned white, indicating silencing occurred within a time-span that plants are still suited for inoculation with *C. fulvum*. Two to three months post inoculation photobleaching extended to flowers (results not shown) and fruits (Figure 1A). In addition, we could confirm down-regulation of *PDS* mRNA by comparing RNA isolated from TRV:PDS- and TRV:00-infected plants with RT-PCR (Figure 1B).

Although usually silencing of *PDS* in sap-inoculated plants occurred a few days earlier (7-9 dpi) compared to silencing in agro- or vacuum-infiltrated plants (10 dpi), it was decided to routinely use the less laborious agroinfiltration method in most of the next experiments.

(A)

(B)

Figure 1. Silencing of *PDS* in Tomato Results in Photobleaching of Leaves and Fruits.

(A) Ten-day-old tomato seedlings were inoculated with TRV:PDS-containing virus-sap. Pictures of leaves and fruits were taken at the indicated days post inoculation (dpi).

(B) Individual leaflets of a TRV:00-infected and a TRV:PDS-infected plant were analyzed for *PDS*-mRNA levels by RT-PCR. Upper panel: *PDS*-mRNA levels. Lower panel: Actin-mRNA levels. Note that actin levels in the TRV:00- and TRV:PDS-inoculated plants are equal, whereas the amount of *PDS* transcript is lower in the TRV:PDS-inoculated plants.

VIGS of *Cf-4* in *Cf-4*-Containing Tomato and *Rcr3* in Cf2 Tomato Results in Loss of Full Resistance

Before we initiated our high-throughput screen, we first tested whether silencing of the *Cf-4* resistance gene in *Cf-4*-containing tomato plants (*Cf-4/*TRV:Cf-4), would result in compromised resistance to *C. fulvum* expressing *Avr4.* As controls either non-TRV inoculated or TRV:00-inoculated (empty vector control) Cf0 tomato plants that are susceptible to all strains of *C. fulvum* we used. The *Cf-4*-containing plants were either non-TRV inoculated, inoculated with TRV:00 or with TRV:Cf-4. Three weeks post TRV inoculation, all tomato plants were inoculated with *C. fulvum* race 5-*pGPD*::*GUS,* which expresses *Avr4* and the β-glucuronidase (*GUS*) gene under control of the constitutive Glyceraldehyde-3-Phosphate Dehydrogenase (*GPD)* promoter (Punt et al., 1988). The latter gene allows visualization of fungal growth after incubation of leaflets in X-gluc. Two weeks post *C. fulvum* inoculation, Cf0 tomato leaflets were completely colonized and covered with mycelium of the fungus. On TRV:Cf-4-infected leaflets of *Cf-4*-containing tomato clear patches of fungal growth were visible, while on the resistant *Cf-4/*non-TRV-, and *Cf-4/*TRV:00-infected plants no fungal growth was visible (Figure 2A, first row, page 70). To further confirm these results, leaflets of all *C. fulvum*-inoculated plants were incubated with X-gluc. Indeed, in susceptible Cf0 and *Cf-4/*TRV:Cf-4-infected leaflets blue staining was visible confirming intercellular fungal growth (Figure 2A, second row). In some cases also on the *Cf-4/*non-TRV- and *Cf-4/*TRV:00-infected leaflets faint blue staining was visible, however, microscopic observation revealed that this was the result of some epiphytic fungal growth on the surface of the leaflets (Figure 2A, third row). As race-5 of *C. fulvum* also secretes Avr9 during growth *in planta*, colonization could further be confirmed by isolating apoplastic fluid (AF) (De Wit and Spikman, 1982), followed by injection into Cf9 tomato. Indeed, only AF isolated from the *C. fulvum*-inoculated Cf0 and *Cf-4/*TRV:Cf-4-infected plants contained sufficient quantities of Avr9 to induce HR in Cf9 tomato (Figure 2B, page 70). These results confirm that VIGS of *Cf-4* in *Cf-4*-containing plants results in compromised resistance, as reflected by the macroscopic- and microscopic-observation of fungal growth and by the production of Avr9.

Subsequently, we investigated whether besides silencing of a *Cf* gene, also silencing of a gene encoding accessory components would compromise resistance of Cf2 tomato to *C. fulvum* expressing *Avr2.* Therefore we silenced *Rcr3* which is known to be required for *Cf-2* mediated resistance (Krüger et al., 2002; Rooney et al., 2005). Cf2 tomato plants were either not inoculated with TRV, inoculated with TRV:00 or with TRV:Rcr3. As controls we used Cf0 (inoculated with TRV:00) and *Cf-2/rcr3-1* (not inoculated with TRV) tomato plants, of which the latter contain a

single nucleotide change in the *Rcr3* gene that partly compromises *Cf-2*-mediated resistance (Krüger et al., 2002). Three weeks post TRV-inoculation all plants were inoculated with *C. fulvum* race 5-*pGPD*::*GUS* which expresses *Avr2,* and two weeks later fungal growth was visible on Cf0 and *Cf-2/rcr3-1* plants. However, on the *Cf-2/*TRV:Rcr3-infected tomato plants, no fungal growth was macroscopically visible (data not shown). Next, leaflets of the *C. fulvum*-inoculated plants were incubated with X-gluc. Cf0 and *Cf-2/rcr3-1* leaflets showed clear blue staining whereas no blue staining occurred in the *Cf-2/*non-TRV- and *Cf-2/*TRV:00-infected leaflets. On *Cf-2/*TRV:Rcr3-infected leaflets local intercellular blue staining was visible, indicating loss of full resistance due to silencing of *Rcr3* (Figure 3A, page 71). AF isolated from all plants was analyzed for the presence of Avr9 by injection into Cf9 tomato. AF from Cf0 and *Cf-2/rcr3-1* tomato plants resulted in a clear HR, whereas AF from the *Cf-2/*non-TRV- and *Cf-2/*TRV:00-infected plants did not. AF isolated from *Cf-2/*TRV:Rcr3-infected plants gave HR and chlorosis in 50% of the cases (Figure 3B, page 71), confirming that silencing of *Rcr3* in Cf2 tomato plants results in loss of full resistance to *C. fulvum* expressing *Avr2.*

VIGS of *Cf-4* in *Cf-4*-Containing Tomato and *Rcr3* in Cf2 Tomato Results in Loss of *Cf-4/Avr4-* and *Cf-2/Avr2*-Induced HR, Respectively

Silencing of *Cf-4* is not only expected to cause compromised resistance to *C. fulvum*, but also to cause a decreased *Cf-4/Avr4*-dependent HR. Therefore, Avr4 was injected into leaflets of *Cf-4*-containing tomato plants that had been inoculated with either TRV:00- or TRV:Cf-4-, or into leaflets of Cf0 tomato plants inoculated with TRV:00 as a control. In the Cf0/TRV:00-infected plants none of the injected sites mounted an HR, while in the *Cf-4/*TRV:00-infected plants nearly all injections resulted in an HR. In *Cf-4/*TRV:Cf-4-inoculated plants approximately half of the injected sites mounted an HR, indicating a decreased HR due to silencing of *Cf-4* (Figure 4, first row, page 74).

To score for decreased HR due to silencing of *Rcr3*, Avr2 was injected into TRV:Rcr3-infected Cf2 tomato plants. As controls, Cf2 plants that had been inoculated with TRV:00, and *Cf-2/rcr3-1* plants, were injected with Avr2. In TRV:00-infected Cf2 plants all injections resulted in an HR, while only two out of ten injections resulted in an HR in the *Cf-2/rcr3-1* plants. In the *Cf-2/*TRV:Rcr3-infected plants the HR was delayed and approximately half of the injected sites mounted an HR (Figure 4, second row). This indicates that the *Cf-2/Avr2*-induced HR is compromised as a result of VIGS of *Rcr3* in Cf2 tomato plants.

(A)

(B)
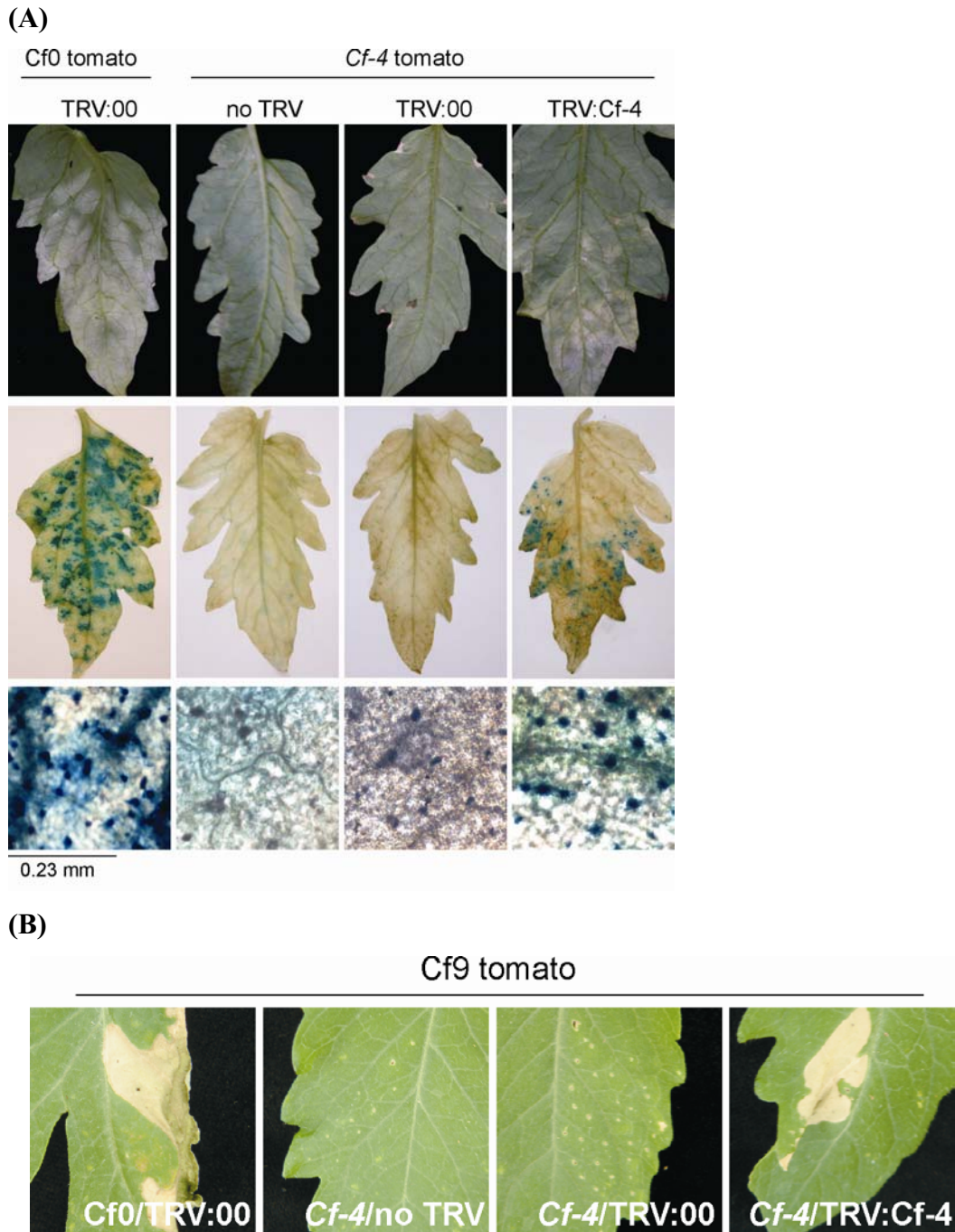
Figure 2. Growth of *C. fulvum* in Leaflets of *Cf-4*-Containing Tomato Plants Silenced for *Cf-4*. Intercellular Colonization by the Fungus was Evaluated Macroscopically, by GUS Staining and by the Presence of Avr9 in AF Isolated from the *C. fulvum*-Inoculated Plants.

(A) Susceptible Cf0 and resistant *Cf-4*-containing tomato plants were inoculated with the indicated TRV constructs. After three weeks, all plants were inoculated with *C. fulvum* race 5-*pGPD::GUS* and two weeks post inoculation fungal growth was scored either macroscopically (first row), or by an X-gluc assay (second and third row).

(B) HR induced in Cf9 tomato plants by Avr9 present in AF isolated from the indicated *C. fulvum*-inoculated plants. Note that AF obtained from *Cf-4*-containing tomato plants infected with TRV:Cf-4 (*Cf-4*/TRV:Cf-4) contains Avr9, indicating successful colonization of these plants by *C. fulvum* after silencing of *Cf-4*.

(A)

(B)
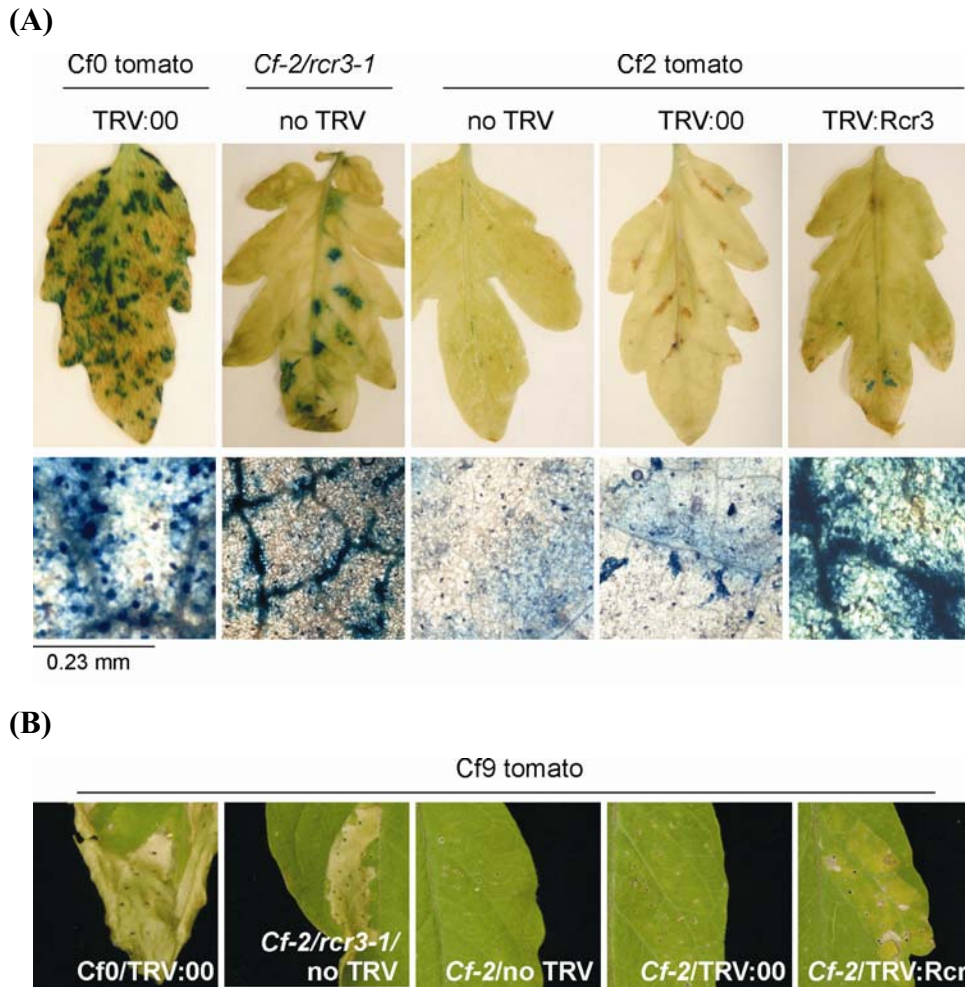
Figure 3. Growth of *C. fulvum* on Leaflets of Cf2 Tomato Plants Silenced for *Rcr3*. Intercellular Colonization by the Fungus was Evaluated by GUS Staining and by the Presence of Avr9 in AF from the *C. fulvum*-Inoculated Plants.

(A) Susceptible Cf0, partially susceptible *Cf-2/rcr3-1,* and resistant Cf2 tomato plants were inoculated with the indicated TRV constructs. After three weeks, all plants were inoculated with *C. fulvum* race 5-*pGPD::GUS* and two weeks post inoculation fungal growth was determined by an X-gluc assay.

(B) HR induced in Cf9 tomato plants by Avr9 present in AF isolated from the indicated *C. fulvum*-inoculated plants. Note that AF obtained from Cf2 tomato plants infected with TRV:Rcr3 (*Cf-2/*TRV:Rcr3) contains Avr9, indicating successful colonization of these plants by *C. fulvum* after silencing of *Rcr3*.

VIGS using *ART* Fragments and Screening for Decreased Resistance to *C. fulvum*

In order to perform VIGS in tomato, we set out to re-clone all 192 tomato *ART* fragments used for VIGS in *N. benthamiana:Cf-4* (Gabriëls et al., 2006), into the TRV2 binary vector appropriate for VIGS in tomato (Liu et al., 2002a; Liu et al., 2002b). From the 192 *ART* fragments 175 were successfully recloned inserts and used for VIGS in *Cf-4*-containing tomato.

Similar to our observations in *N. benthamiana* (Gabriëls et al., 2006), VIGS of *ART* fragments 313a, 320 and 403a also resulted in chlorosis in tomato. VIGS of *ART* 239 and 470 resulted in severe necrosis leading to plant death, and VIGS of *ART* 424a resulted in plants with curled leaves (Figure 5A, page 75). Three weeks post TRV inoculation all TRV-infected tomato plants were inoculated with *C. fulvum* race 5-*pGPD*::*GUS* and two weeks later leaflets were incubated with X-gluc. In each individual experiment in which VIGS of the *ART* genes was performed, *Cf-4*-containing plants silenced for *Cf-4* were included as controls. Besides leaflets of TRV:Cf-4-infected plants, also leaflets of several TRV:ART-infected plants showed small patches of intercellular blue staining, indicating that resistance was compromised (Figure 5B, page 75). Silencing of the *ART* fragments compromising resistance was repeated at least three times using either agroinfiltration or sap-inoculation. The number of times decreased resistance was observed per independent experiment is indicated in Table 1. VIGS of *ART* fragments 11, 71 (picture not shown), 222 and 403a showed patches of blue staining indicating loss of full resistance in at least three independent experiments (Figure 5B and Table 1). The blast hits corresponding to the ART fragments mentioned in this chapter are shown in Table 2.

Table 1. Frequency by which decreased resistance was observed

Decreased resistance / # experiments	*ART* fragments used for VIGS						
1/5 [*1]	4	14	452	424a	470	490	
1/3	299	606					
2/5	75	76	430	431	454	453	653
2/4	406						
3/5	71	222					
4/5	11						
4/4	403a						

[*1]: Note that "1/5" indicates that decreased resistance to *C. fulvum* was observed in one out of five independent experiments.

Table 2. Blast hits corresponding to the *ART* fragments mentioned in the text and in Table 1.

ART nr.	Size (bp)	Accession nr. of *ART* fragment	Blast hit
4	107	DV935721	No hit
11	125	DV935728	No hit
14	103	DV935730	No hit
71	632	DV935771	Histon H3 from maize
75	294	DV935775	Receptor-like protein kinase
76	211	DV935776	Protein kinase-like protein
222	252	DV935837	CC-NB-LRR
239	78	DV935853	26S proteasome regulatory subunit 6B
299	227	DV935912	Lycopersicon esculentum BAC clone
313a	104	DV935925	Glutamine synthetase
320	350	DV935931	Lycopene beta-cyclase
403a	180	DV935934	Ribosomal protein L1
406	142	DV935936	No hit
424a	373	DV935953	Nuclear GTPase
430	88	DV935960	Putative mitochondrial protein
431	166	DV935961	Hypothetical protein
452	235	DV935982	Putative scarecrow protein
453	129	DV935983	No hit
454	94	DV935984	H+ transporting ATPase
470	373	DV935999	Molecular chaperone Hsp90-1
490	164	DV936019	Putative membrane protein
606	150	DV936039	Phosphoinositide-specific phospholipase C
653	179	DV936079	Hypothetical protein
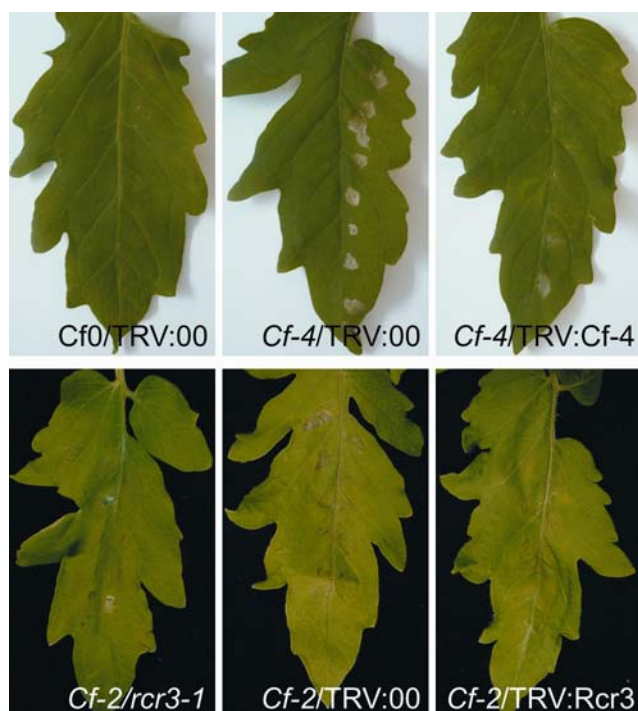
Figure 4. VIGS of *Cf-4* in *Cf-4*-Containing Tomato and *Rcr3* in Cf2 Tomato Results in Compromised *Cf-4/Avr4-* and *Cf-2/Avr2*-Induced HR, Respectively.

First row: HR induced by Avr4 (8 injections per leaflet) in Cf0 tomato and *Cf-4*-containing tomato, infected with the indicated TRV constructs. Note that the number of injected sites mounting an HR is decreased in *Cf-4*-containing tomato plants infected with TRV:Cf-4, as compared to the *Cf-4*-containing tomato plants infected with TRV:00.

Second row: HR induced by Avr2 (10 injections per leaflet) in *Cf-2/rcr3-1* plants and Cf2 tomato plants, infected with the indicated TRV constructs. All pictures were taken 3 dpi.

Note that the number of injections resulting in an HR is decreased in Cf2 tomato plants infected with TRV:Rcr3, as compared to the Cf2 tomato plants infected with TRV:00.

(A)
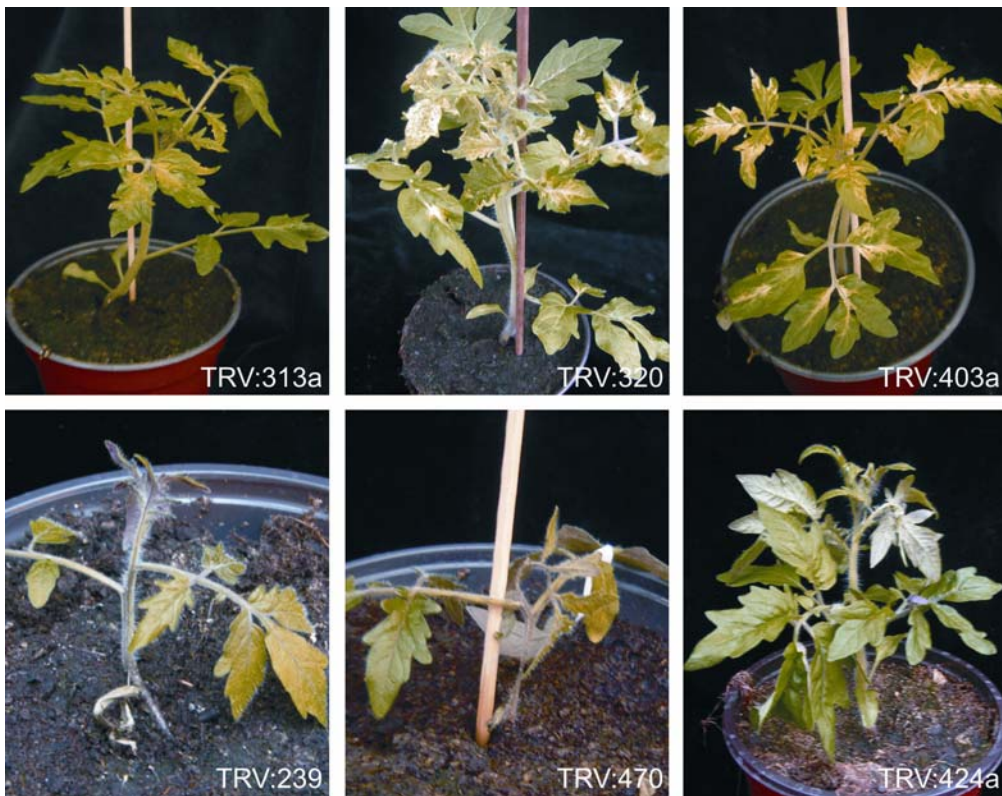
(B)
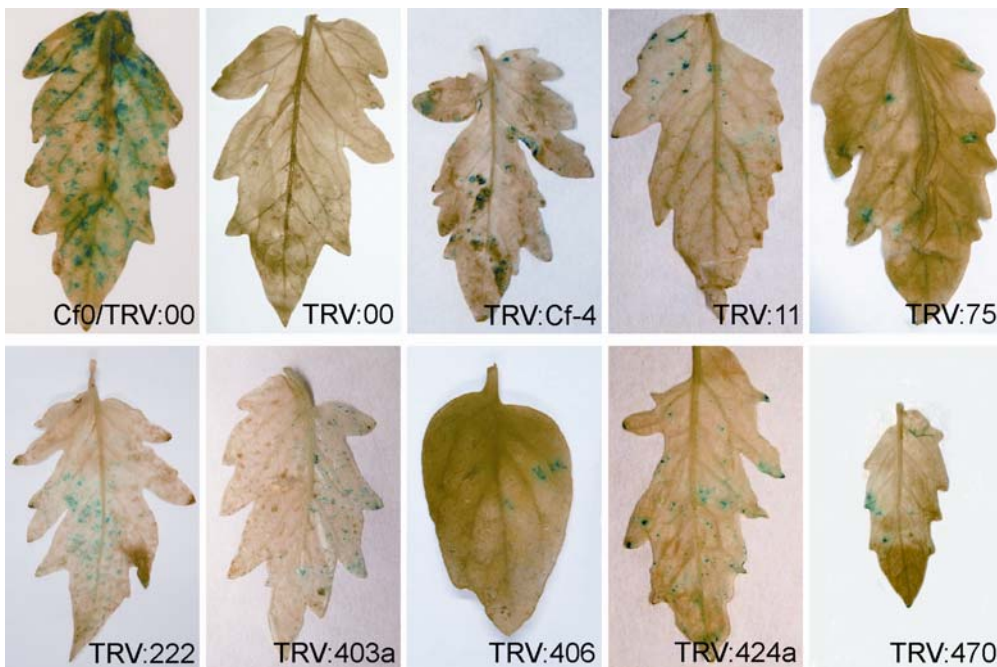
Figure 5. VIGS of *ART* Fragments Affecting Plant Morphology and/or Resistance to *C. fulvum.*

(A) Symptoms on *Cf-4*-containing tomato plants three weeks post inoculation with the indicated TRV constructs (see Table 1 for the blast hits corresponding to the *ART* fragments).

(B) Susceptible Cf0 and resistant *Cf-4*-containing tomato plants were inoculated with the indicated TRV constructs. Subsequently all plants were inoculated with *C. fulvum* race 5-*pGPD::GUS* and two weeks post inoculation fungal growth was scored by the X-gluc assay.

DISCUSSION

Comparing Methods to Perform VIGS in Tomato

For VIGS in plants two TRV binary vector systems have been described (Ratcliff et al., 2001; Liu et al., 2002b). Our experiments indicated that the TRV binary vectors developed by Liu et al. (2002b) are the most effective for VIGS in tomato. This supports the choice for this binary vector system in several other studies involving VIGS in tomato (Liu et al., 2002a; Ekengren et al., 2003; Rowland et al., 2005). Until now, five different methods to inoculate TRV onto plants have been described: agroinfiltration and spray-inoculation (Liu et al., 2002a), vacuum-infiltration (Ekengren et al., 2003), sap-inoculation (Brigneti et al., 2004) and agrodrench (Ryu et al., 2004). Comparisons between these methods using TRV:PDS indicated that on average white tomato leaflets become visible fourteen days post TRV inoculation, except for the spray-inoculation and for the agrodrench method which show a delay of four days and one week, respectively (Brigneti et al., 2004; Ryu et al., 2004). The delay of silencing in leaflets upon TRV inoculation by the agrodrench method is probably due to the time necessary for the virus to move from the roots to the leaves (Ryu et al., 2004). We compared the efficiency of *PDS* silencing in tomato, using agroinfiltration, sap-inoculation or vacuum-infiltration. Although on average the onset of silencing using sap-inoculation is two to three days earlier as compared to the other two methods, it requires some additional steps including agro-infiltration of *N. benthamiana*, followed by harvesting and homogenization of the infected leaves to obtain virus-sap. Vacuum-infiltration is more laborious due to the thorough cleaning of the vacuum dessicator necessary to exclude any contamination by traces of Agrobacterium. Therefore, we decided to use direct agroinfiltration of tomato to perform our high-throughput VIGS screen, whereas for a few interesting candidates VIGS experiments were repeated using both the agroinfiltration and the sap-inoculation method. RT-PCR analysis indicated that expression levels of *PDS* are decreased in TRV:PDS-infected plants as compared to those in the TRV:00-infected control plants.

Detection of *C. fulvum* Growth

Due to patchy silencing in tomato and due to possible functional redundancy of *ART* genes acting downstream of Cf-4, we expected only decreased resistance to *C. fulvum* instead of full susceptibility. Prior to VIGS of all selected *ART* genes, we first assessed whether decreased resistance could be obtained by silencing genes upstream in plant defense signaling, like the resistance gene *Cf-4* itself and the gene required for *Cf-2*-mediated resistance, *Rcr3*. Intercellular

fungal growth was observed on *Cf-4/*TRV:Cf-4- and *Cf-2/*TRV:Rcr3-infected plants, which could be confirmed by an X-gluc assay and in case of the *Cf-4/*TRV:Cf-4-infected plants also by detection of Avr9 elicitor in the isolated AF. Thus, the X-gluc assay is a sensitive method to detect intercellular *C. fulvum* growth in *Cf-4/*TRV:Cf-4- and *Cf-2/*TRV:Rcr3-infected plants. VIGS of *ACIK1* (<u>A</u>vr9-<u>C</u>f9 <u>I</u>nduced <u>K</u>inase) also resulted in loss of full resistance to this fungus, a feature also detectable by an X-gluc assay (Rowland et al., 2005). Furthermore, a decreased HR was observed upon Avr4 and Avr2 injections in *Cf-4/*TRV:Cf-4 and *Cf-2/*TRV:Rcr3-infected tomato plants, respectively. Based on these results, we expect *Cf-4* and *Rcr3*-expression to be significantly down-regulated in both TRV:Cf-4- and TRV:Rcr3-infected plants, respectively.

VIGS of *ART* Genes and Scoring for Genes Involved in Resistance to *C. fulvum*

VIGS of *ART* 239 resulted in plants developing systemic necrosis, which was most severe in the roots and veins. Eventually these plants died within two weeks post TRV:239-inoculation. Blast analysis revealed that the *ART 239* fragment is homologous to a gene coding for the 26S proteasome subunit 6B. The proteasome is involved in the degradation of proteins, and silencing of a gene encoding a subunit of the proteasome might disturb several essential processes in the plant, eventually leading to necrosis. Indeed, also Ryu et al. (2004) reported that silencing of the gene encoding the 20S proteasome in tomato resulted in systemic necrosis.

VIGS of *ART* 313a, 320 and 403a resulted in chlorosis, which is similar to the chlorotic phenotype observed upon VIGS of these *ART* fragments in *N. benthamiana* (Gabriëls et al., 2006). *ART* transcript 313a revealed homology to glutamine synthetase. Glutamine synthetase, together with glutamate synthase, catalyses the conversion of the nitrogen source ammonium, into glutamine and glutamate. Thus, knock-out of glutamine synthetase or glutamate synthase causes accumulation of ammonium, which results in a decrease in the amount of photosynthetic compounds like chlorophyll (Lam et al., 1995; Temple et al., 1998). Indeed, *Arabidopsis thaliana* glutamate synthase photorespiratory mutants are chlorotic (Coschigano et al., 1998), similar to the phenotype of the glutamine synthetase-silenced tomato plants. *ART* transcript 320 reveals homology to lycopene beta-cyclase which is known to control cyclic carotenoid production in Arabidopsis (Cunningham et al., 1996). As already mentioned for *PDS,* knock-down of genes involved in the carotenoid production is known to cause photobleaching, a phenotype which is indeed observed upon VIGS of *ART* 320.

In addition to a chlorotic phenotype, TRV:403a-infected plants also showed loss of full resistance to *C. fulvum.* The 180 bp sequence of *ART* 403a is similar to a gene encoding a

ribosomal protein L1. Interestingly, also VIGS in *N. benthamiana* revealed a ribosomal protein (L19) involved in *Cf-4/Avr4*-induced HR (Gabriëls et al., 2006), whereas several ribosomal proteins involved in *Pto/AvrPto*-induced HR were reported by Lu et al. (2003). The effect of silencing genes encoding ribosomal proteins or proteins required for assembly of a functional proteasome on HR-induction and resistance, indicates that regulation of the proteome (requiring both ribosomal proteins for synthesis, as well as the proteasome for degradation), is essential and its disturbance also affects plant defense.

ART fragment 424a corresponds to a nuclear GTPase. VIGS using TRV:424a resulted in plants with curled leaves, both in tomato and in *N. benthamiana* (Gabriëls et al., 2006). Nuclear GTPases might be involved in developmental processes, similar as mammalian members of the nuclear GTPase family which are known to prevent stem cells from differentiation (Tsai and McKay, 2002). Although VIGS of *ART* 424a affected *Cf-4/Avr4-* and *Inf1-* induced HR in *N. benthamiana,* resistance to *C. fulvum* was not significantly affected upon VIGS using this *ART* fragment in tomato.

ART 470 encodes H̲eat S̲hock P̲rotein 90 (HSP90), which upon silencing resulted in plants with severe necrosis that eventually died. However, in one out of five experiments (Table 1) a few plants survived and showed some intercellular *C. fulvum* growth, indicating loss of full resistance. HSP90 was shown to be required for *Cf-4*-mediated HR in *N. benthamiana* (Gabriëls et al., 2006), and was also reported to be required for *Pto*-mediated HR and *Pto-, Rx-* and *N*-mediated resistance (Lu et al., 2003). HSP90 might function as a chaperone, which is required for stabilization of NB-LRR type-R proteins or R protein complexes (Hubert et al., 2003; Kanzaki et al., 2003; Takahashi et al., 2003; Liu et al., 2004; de la Fuente van Bentem et al., 2005). Our data support that HSP90 is required for multiple disease resistance pathways, including those triggered by RLPs, like the Cf proteins. Since HSP90 is in the cytoplasm and the LRR region of Cf-4 is in the extracellular space, it is hard to envision a role in stabilization of the Cf-4 protein itself. It is therefore tempting to speculate that HSP90 is involved in stabilization of downstream signaling components.

Silencing of *ART* 11, 71, 222 and 403a resulted in patchy intercellular *C. fulvum* growth in three or more independent VIGS experiments (Table 1). Blast analysis of *ART* fragment 11 (125 bp) did not reveal homology to any known gene. The fragment is probably too small and it will be required to isolate a longer cDNA fragment to draw any conclusion about homology to other genes. ART 71 corresponds to histon H3 from maize. In a VIGS-screen using 4992 cDNAs from a normalized *N. benthamiana* library, histon H3- and histon H4 were identified to be required for *Pto/AvrPto*-mediated HR. Moreover, silencing of these genes caused stunting- and mosaic-like symptoms (Lu et al., 2003). A DNA helix winded around a core of eight histone proteins (octomer)

is referred to as a nucleosome, in which the histone proteins play a crucial role in gene regulation (Berger, 2002). Acetylation of histones weakens the histon-octomer complex and increases gene expression, while deacetylation is associated with transcriptional repression. Overexpression of histon deacetylase 19 from *A. thaliana* results in increased resistance to *Alternaria brassicicola* while RNAi of this gene results in decreased resistance to this pathogen (Zhou et al., 2005). Affecting acetylation or deacetylation of histones, or silencing of a histon itself, might result in either up-regulation of negative regulators of defense or down-regulation of genes required for defense, explaining the loss of full resistance to *C. fulvum* after silencing of *ART* 71.

Although VIGS of *ART* 11, 71 and 403a in tomato affects *Cf-4*-mediated resistance to *C. fulvum,* no effect on *Cf-4/Avr4-* or *Inf1*-induced HR was found upon VIGS using these *ART* fragments in *N. benthamiana:Cf-4* (Gabriëls et al., 2006). For *ART* 11 and 71 this discrepancy might be due to the lack of homology of these *ART* fragments to the corresponding endogenous *N. benthamiana* genes, thereby not resulting in sufficient knock-down of the gene (Thomas et al., 2001). For *ART* 403a it could be that the compromised HR could not be assessed due to the chlorosis and fragility of the silenced *N. benthamiana* leaves.

VIGS of *ART* 222 in tomato causes a decreased resistance to *C. fulvum,* and VIGS using this *ART* fragment in *N. benthamiana* has an effect on the *Cf-4/Avr4* and *Inf1*-induced HR (Gabriëls et al., 2006). Isolation of a corresponding full-length cDNA revealed homology to an NB-LRR protein, which was designated NRC1 (NB-LRR Required for HR-associated Cell death 1) (Gabriëls et al., 2006). Most NB-LRR proteins function as intracellular resistance proteins, except for NRG1 (N Requirement Gene 1) (Peart et al., 2005), which is required for *N*-mediated resistance to Tobacco Mosaic Virus (TMV). NRC1 is the first NB-LRR protein involved in *Cf*-mediated defense signaling. These NB-LRRs functioning downstream in defense signaling might be key regulators of multiple defense pathways.

In this chapter we report that both silencing of defense-related genes and scoring for intercellular growth of *C. fulvum* is feasible in tomato. We can not rule out that the genes corresponding to the *ART* fragments of which VIGS did not result in compromised resistance to *C. fulvum* are not involved in defense signaling. Patchy silencing or functional redundancy might mask the effect of knock-down of one specific gene on resistance to *C. fulvum*. Therefore, we have to take into account that this VIGS screen in tomato is aimed to directly identify genes involved in *Cf-4*-mediated resistance of tomato to *C. fulvum.* VIGS in tomato allows a pre-selection of candidate genes involved in *Cf*-mediated resistance. Stable knock-downs using RNAi constructs should provide additional evidence that the silenced genes are indeed required for *Cf*-mediated resistance

and resistance to other pathogens. Future experiments will focus on the role of *NRC1* which upon silencing compromises both *Cf-4*-mediated HR (Gabriëls et al., 2006) and resistance (this chapter).

METHODS

VIGS in Tomato and Detection of *Cladosporium fulvum*

For VIGS in tomato we used the binary tobacco rattle virus constructs pTRV-RNA1 and pTRV-RNA2 (Liu et al., 2002b). For sap-inoculation, first *N. benthamiana* leaves were agroinfiltrated with the binary pTRV-RNA1 and pTRV-RNA2 constructs (combined in a 1:1 ratio), and three to five days post agroinfiltration leaves were harvested and homogenized in phosphate buffer (50 mM, pH 7.2). Subsequently, 6 – 12 μl of virus-sap was inoculated onto cotyledons of ten-day-old tomato seedlings (Brigneti et al., 2004). VIGS in tomato was also performed by agroinfiltration or vacuum-infiltration (Ekengren et al., 2003) of the cotyledons with the binary pTRV-RNA1 and pTRV-RNA2 constructs combined in a 1:1 ratio (Liu et al., 2002a). For each TRV construct either four *Cf-4*-containing tomato plants (Cf0 plants transformed with *Hcr9-4D* (*Cf-4*) (Thomas et al., 1997), resistant to *C. fulvum* expressing *Avr4*, or four Cf2 tomato plants resistant to *C. fulvum* expressing *Avr2* were used (Dixon et al., 1996). As controls we used Cf0 tomato plants (MM-Cf0) fully susceptible to *C. fulvum* and *Cf-2/rcr3-1* tomato plants (Krüger et al., 2002), partially resistant to *C. fulvum* expressing *Avr2*, that were either non-TRV inoculated or TRV:00-inoculated. Three weeks post TRV inoculation, the plants were inoculated with *C. fulvum* race 5-*pGPD*::*GUS* (expressing *Avr2*, *Avr4, Avr9* and the β-glucuronidase (*GUS*) gene, the latter under control of the constitutive *GPD* promoter), for which leaflets were sprayed with a suspension of conidia of the fungus (De Wit, 1977). Fourteen days post *C. fulvum* inoculation, leaflets of the third and fourth compound leaf were harvested and used for incubation in an X-gluc solution (8 leaflets per construct), or used for isolation of apoplastic fluid (AF) (from a mix of four leaflets). AF was injected into leaflets of Cf9 tomato plants (MM-Cf9) with a 100 μl micro-syringe (Ito Corporation, Fuji, Japan).

Detection of Compromised HR upon Injection of the Elicitors of *Cladosporium fulvum*

To determine whether the HR is affected in TRV:Cf-4-infected *Cf-4*-containing tomato or TRV:Rcr3-infected Cf2 tomato, the third compound leaf was injected with Avr4 or Avr2, respectively. The proteins were injected at a concentration of approximately 10 μM, at eight or ten

sites per leaflet, four leaflets per plant, and four plants per TRV construct. All injections were performed with a 100 µl micro-syringe.

RT-PCR

Four leaf discs (approximately 100 mg of tissue (fresh weight)) were collected from the second, third or fourth compound leaf of TRV-infected plants. Total RNA was extracted using the QIA-Gen RNAeasy extraction method (Qiagen, Venlo, NL) and treated with RNase-Free DNase (Bio-Rad, Veenendaal, NL). First strand cDNA was synthesized from 1 µg of total RNA using the Bio-Rad cDNA synthesis kit (Bio-Rad, Veenendaal, NL) and subsequent PCR was performed with denaturing at 95°C for 15 sec, annealing at 60°C for 45 sec, and elongation at 72°C for 60 sec. Samples were taken in the logarithmic amplification phase. The primers that were used do not amplify the fragment which is inserted in the TRV vector; for amplification of cDNA corresponding to *PDS* we used: PDSFnr184; 5'-CCTGAGAGACTTTGCATGCC-3' and PDSRnr190: 5'-CTTCAGTTTTCTGTCAAACCATATATG-3'. Actin primers (ActinFnr182: 5'-TATGGAAACATTGTGCTCAGTGG-3' and ActinRnr183: 5'-CCAGATTCGTCATACTCTGCC-3') were used as a control for the amount of input cDNA.

Plasmid Constructs

The *Cf-4* and *PDS* fragments were PCR-amplified from the TRV RNA2 vector described by Ratcliff et al. (2001), using FR69 and FR70 as forward and reverse vector-specific primers respectively (Ratcliff et al., 2001). The PCR fragment was *Bam*H1/*Asp*718-digested and inserted into the pTRV-RNA2 (pYL156) vector suitable for VIGS in tomato (Liu et al., 2002a; Liu et al., 2002b). From the 192 TRV:ART constructs based on the vector described by Ratcliff et al. (2001) and used for VIGS in *N. benthamiana* (Gabriëls et al., 2006), *ART* fragments were amplified using the primers F1: 5'-TCCTAGAACTAGTGGATCCCC-3' (*Bam*HI site underlined) and Rev1: 5'-CCCAATTCGCCCTATAGTG-3', both annealing to vector-specific sequences. In the pTV00 multiple cloning site the *Asp*718 (isoschizomer of *Acc*651) site is located upstream of primer Rev1. All, except for fourteen (nr 22, 30, 50, 68, 78, 96, 113, 119, 245, 276, 278, 300, 303, and 642 (Gabriëls et al., 2006)), PCR fragments were *Bam*HI/*Acc*651-digested and cloned into the pTRV-RNA2 vector (Liu et al., 2002b), eventually resulting in 178 TRV:ART recombinants. Transformation to *Agrobacterium tumefaciens* strain GV3101 failed for three TRV:ART constructs (TRV:20, TRV:81 and TRV:103 (Gabriëls et al., 2006)) and the remaining 175 TRV:ART recombinants were used for VIGS in tomato. *Rcr3* was amplified from the vectors

pMWBin19:Rcr3^{pim}-His-HA   and pMWBin19:Rcr3^{esc}-His-HA,   containing -HIS-HA-tagged versions of the *Rcr3* genes from *L. pimpinellifolium* and *L. esculentum* respectively, using primers FRCR3Bam300: 5'-GTGAC<u>GGATCC</u>AAATGGTGGAATTTCAAGGG-3' and RevRCR3Asp: 5'-GTGAC<u>GGTACC</u>TCTTATAATTTTCATAAACCC-3' (*Bam*HI and *Asp*718 sites underlined, respectively). These primers amplify regions specific for *Rcr3*^{pim} or *Rcr3*^{esc}. The PCR fragments were *Bam*HI/*Asp*718-digested and inserted into pTRV-RNA2 (Liu et al., 2002b). Silencing using either TRV:Rcr3^{pim} or TRV:Rcr3^{esc} showed similar results, and is referred to as silencing using TRV:Rcr3. All plasmids were transformed to *Agrobacterium tumefaciens* strain GV3101 (Gabriëls et al., 2006).

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CHAPTER 4

An NB-LRR Protein Required for HR Signaling Mediated by Both Extra- and Intracellular Resistance Proteins

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ABSTRACT

Tomato *Cf* resistance genes confer hypersensitive-response (HR)-associated resistance to strains of the pathogenic fungus *Cladosporium fulvum* that express the matching avirulence (*Avr*) gene. Previously, we identified an Avr4-Responsive Tomato (*ART*) gene that is required for *Cf-4/Avr4*-induced HR in *Nicotiana benthamiana* as demonstrated by Virus-Induced Gene Silencing (VIGS). The gene encodes a CC-NB-LRR type resistance (R) protein analogue that we have designated NRC1, for NB-LRR protein Required for HR-associated Cell death 1. Here, we describe that knock-down of *NRC1* in tomato not only affects the *Cf-4/Avr4*-induced HR but also compromises *Cf-4*-mediated resistance to *C. fulvum*. In addition, VIGS using *NRC1* in *N. benthamiana* revealed that this protein is also required for HR signaling induced by other *R*/*Avr* combinations. Agroinfiltration of the gene encoding a constitutively active NRC1 mutant protein (NRC1^{D481V}) results in an elicitor-independent HR which, in combination with VIGS of genes known to be involved in HR signaling, was used to position NRC1 in an HR signaling pathway. Our results indicate that NRC1 requires RAR1 and SGT1 to be functional, whereas it does not require NDR1. NRC1 acts downstream of Cf-4 and EDS1, and upstream of a MAP-kinase pathway. Thus, *Cf*-mediated resistance signaling requires a downstream NB-LRR, which also functions in cell death signaling pathways triggered by other matching *R*/*Avr* combinations.

INTRODUCTION

Resistance (*R*) gene-mediated recognition of an avirulence factor of a pathogen, subsequently triggering active defense of plants, complies with the gene-for-gene model (Dangl and Jones, 2001). To date, several plant *R* genes have been cloned and based on the structure of their products they are divided into several classes (Hammond-Kosack and Jones, 1997). Most *R* genes encode cytoplasmic NB-LRR proteins, containing a nucleotide binding site (NB) and leucine-rich repeats (LRR). This class consists of genes either encoding CC-NB-LRR proteins, containing a coiled-coil domain, or proteins that have a domain similar to mammalian Toll and Interleukin Receptors, the so-called TIR-NB-LRR proteins (Hammond-Kosack and Jones, 1997).

Using highly specific genes in resistance breeding programs has not been very successful since pathogens easily circumvent recognition by their host plant due to mutations in their avirulence factors (Westerink et al., 2004). However, similarity among R proteins suggests the

existence of common resistance pathways (Shirasu and Schulze-Lefert, 2000). Therefore, identification of additional genes required for resistance not only provides information on how such signaling pathways function but also should allow identifying genes that play a more general role in resistance. For example, by silencing studies it was shown that SGT1 (Suppressor of the G2 allele of *SKP1*, a protein first identified in yeast) is involved in *N-*, *Rx-* and *Pto*-mediated HR and resistance, and *Cf-4-* and *Cf-9*-mediated HR (Peart et al., 2002b; Zhang et al., 2004). SGT1 has several functions and is, for example, involved in ubiquitination which is a process that targets proteins for degradation by the proteasome (Schwechheimer and Schwager, 2004). It is hypothesized that perturbation of this process hampers the specific degradation of negative regulators, which is required for defense activation (Azevedo et al., 2002). SGT1 directly interacts with RAR1 (Required for Mla12 Resistance) and in this context SGT1, and also RAR1, could act as a chaperonin-like protein that is involved in the generation and stabilization of R protein recognition complexes (Muskett and Parker, 2003; Shirasu and Schulze-Lefert, 2003).

In several resistance pathways MAPKs (Mitogen-Activated Protein Kinases) are activated (Zhang and Klessig, 2001; Pedley and Martin, 2005). In *Cf-9*-containing tobacco plants and cell cultures challenged with Avr9, NtWIPK (Wound-Induced Protein Kinase) and NtSIPK (Salicylic Acid-Induced Protein Kinase) are activated (Romeis et al., 1999). VIGS of a NtCDPK (belonging to the family of calcium-dependent protein kinases) in *N. benthamiana* inhibits the *Cf-9/Avr9-* and *Cf-4/Avr4*-dependent HR (Romeis et al., 2001). Furthermore, VIGS in tomato of LeACIK1 (Avr/Cf-Induced Kinase 1), which belongs to yet another class of kinases, results in decreased *C. fulvum* resistance (Rowland et al., 2005). The activation of kinases during defense and the decreased resistance upon knock-down of the encoding genes supports their function in defense activation. Ekengren et al. (2003) silenced 21 genes known to be involved in various defense-related pathways and found that two MAPKKs- (LeMEK1 and LeMEK2) and two MAPKs- (LeNTF6 and LeWIPK) are required for *Pto*-mediated resistance of tomato to *Pseudomonas syringae* pv. *tomato*. In another study, over 2400 cDNAs from a normalized cDNA library of *N. benthamiana* were cloned in a potato virus X-based vector and used for VIGS in *N. benthamiana*. About 3% of the cDNAs compromised *Pto*-dependent HR upon silencing, including a MAPKKα gene (Del Pozo et al., 2004).

Lu et al. (2003) performed VIGS in transgenic *Pto*-expressing *N. benthamiana* using 4992 cDNAs from a normalized *N. benthamiana* cDNA library cloned into a PVX vector. 79 cDNAs (1.6%) corresponded to genes involved in *Pto*-mediated HR, of which only six were also required for *Pto*-mediated resistance to *P. syringae* pv. *tabaci* expressing *AvrPto*. VIGS using a cDNA

corresponding to HSP90 abolished not only *Pto*-mediated HR but also *Pto-, Rx-* and *N*-mediated resistance, indicating that HSP90 is required in multiple disease resistance pathways. The same set of cDNAs was also used for VIGS in *N*-transgenic *N. benthamiana,* followed by inoculation with a GFP-tagged TMV strain. Resistance to TMV was most significantly suppressed upon silencing using a cDNA fragment derived from a CC-NB-LRR-encoding gene, referred to as *NRG1* (N Requirement Gene 1) (Peart et al., 2005). *NRG1* was shown to be specifically required for *N* gene function, indicating that CC-NB-LRR proteins do not only act as resistance proteins involved in recognition of avirulence factors, but are also involved in the signaling pathway initiated by the TIR-NB-LRR protein N, which eventually leads to resistance (Peart et al., 2005).

In the interaction between tomato and the pathogenic fungus *Cladosporium fulvum*, Cf resistance proteins mediate recognition of matching avirulence factors (Avrs) that are secreted by the fungus. Recognition results in a typical HR and resistance. We have used cDNA-AFLP analysis, in combination with VIGS in *Cf-4*-transgenic *N. benthamiana* (*N. benthamiana:Cf-4*), to identify genes involved in *Cf-4/Avr4*-dependent HR and resistance (Gabriëls et al., 2006). We identified several genes required for the HR, among which is *ART* 222 (Avr4-Responsive Tomato transcript number 222), which encodes a CC-NB-LRR type resistance protein analogue (Gabriëls et al., 2006). This indicates that similar to N, also Cf-4 requires an NB-LRR protein for downstream signaling and supports the hypothesis that resistance proteins generally require downstream NB-LRRs for cell death signaling (Peart et al., 2005). Interestingly, TIR-NB-LRR proteins such as N are intracellular, whereas Cf resistance proteins are extracellular Receptor-Like Proteins (RLPs) (Kruijt et al., 2005), indicating convergence of signaling pathways triggered by different classes of R proteins. Furthermore, we found that for the induction of an HR by Inf1, which is an elicitor of the oomycete pathogen *Phytophthora infestans*, *ART* 222 is also required and therefore the encoded protein was designated NRC1, for NB-LRR protein Required for HR-associated Cell death 1 (Gabriëls et al., 2006).

Here we describe detailed studies on the involvement of NRC1 in multiple HR and resistance pathways. Knock-down of *NRC1* in tomato confirmed its requirement for *Cf-4/Avr4*-induced HR and revealed that NRC1 is also required for *Cf-4*-mediated resistance to *C. fulvum*. Furthermore, VIGS using *NRC1* suppresses the HR initiated by multiple resistance proteins, whereas for the pathogens tested the actual resistance appeared not to be compromised. To position NRC1 in an HR signaling pathway, agroinfiltration of a constitutively active mutant of the protein (NRC1^{D481V}) was used in combination with VIGS of genes required for HR (Peart et al., 2002b; Ekengren et al., 2003). It appeared that NRC1 operates upstream of a MAP-kinase cascade and downstream of EDS1. We conclude that *Cf*-mediated resistance requires a downstream CC-NB-LRR protein,

which is also involved in cell death signaling pathways triggered by several additional R/Avr protein pairs.

RESULTS

Tomato NRC1; a CC-NB-LRR Protein

VIGS studies have revealed that in *N. benthamiana* a putative orthologue of the *NRC1* gene of tomato is required for *Cf-4/Avr4-* and *Inf1*-induced HR (Gabriëls et al., 2006). The predicted primary structure of tomato NRC1 typically resembles that of CC-NB-LRR resistance proteins (Figure 1A). The protein comprises an amino-terminal coiled-coil (CC) domain, an NB-ARC (Nucleotide Binding adapter shared by Apaf-1, R proteins and CED4) domain (Van der Biezen and Jones, 1998; Aravind et al., 1999) and 13 imperfect Leucine-Rich Repeats (LRRs). As indicated in Figure 1A, comparison with homologous NB-ARC domains revealed the presence of a typical Kinase1A or P-loop motif, four RNBS (Resistance Nucleotide Binding Site) motifs and a GLPL and MHD motif (Meyers et al., 1999; Meyers et al., 2003).

We probed a gel blot of genomic DNA of tomato, digested with various restriction enzymes, with a fragment ranging from nucleotides 1876 to 3168, covering the sequence encoding for LRR four to thirteen and the 3' UTR of *NRC1*. Under conditions of high stringency only one clear band was visible, indicating that *NRC1* is a single-copy gene in tomato (Figure 1B). The 252 bp cDNA-AFLP fragment present in the TRV:NRC1 vector used for VIGS in *N. benthamiana* codes for amino acids 599-681, which cover LRRs four to seven. We probed a DNA gel-blot of genomic DNA of *N. benthamiana* digested with the same set of restriction enzymes with this fragment and found three hybridizing bands at low stringency, whereas these bands disappear at higher stringency (results not shown). This observation suggests that there are three potential *NRC1* orthologues present in the genome of *N. benthamiana* that can be silenced upon inoculation with TRV:NRC1.

(A)

(B)

```
CC-domain

  1    MVDVGVEFLL  ENLKQLVLDN  VELIGGAKDE
 31    IENLRDDLSE  FNAFLKQAAM  VRSENPVLKE
 61    LVRSIRKVVN  RAEDAVDKFV  IEAKVHKDKG
 91    FKGVFDKPGH  YRRVRDAAVE  IKGIRDKMRE
121    IRQNKAHGLQ  ALLQDHDDSI  SRGGEERQPP


NB-ARC domain

151    VVEEDDVVGF  DDEAQTVIDR  LLEGSGDLEV
             Kinase 1A
181    IPVVGMPGLG  KTTLATKIFK  HPKIEYEFFT
             RNBS-A
211    RLWLYVSQSY  KTRELYLNII  SKFTGNTKHC
                                   Kinase 2
242    RDMSEKDLAL  KVQEILEEGG  KYLIVLDDVW
               RNBS-B
271    STDAWDRIKI  AFPKNDKGNR  VLLTTRDHRV
             RNBS-C
301    ARYCNRSPHD  LKFLTDEESW  ILLEKRAFHK
                       GLPL
331    AKCLPELETN  GKSIARKCKG  LPLAIVVIAG
361    ALIGKSKTIK  EWEQVDQSVG  EHFINRDQPN
                               RNBS-D
391    SCDKLVRMSY  DVLPYDWKAC  FLYFGTFPRG
421    YLIPARKLIR  LWIAEGFIQY  RGDLSPECKA
                                     MHD
451    EEYLNELVNR  NLVMVMQRTV  DGQIKTCRVH
481    DMLYEFCWQE  ATTEENLFHE  VKFGGEQS


LRR and C terminus

509    VREVSTH  IRLCIHS  S-VVEFISK
531    KPSGEHV  RSFLCFS  PEKIDTPPTVSANISKAFP
564    LLRVFDT  ESIKINR  FCKEFFQ
585    ---LYHL  RYIAFSF  D-SIKVIPKH
605    VGELWNV  QTLIVNT  Q-QINLDIQAD
629    ILNMPRL  RHLLTNT  SAKLPALANPKTSKTTLVNQSLQTLSTIAPESCTEYV
680    LSRAPNL  KKLGIRG  KIAKLMEPSQSVLLNN
710    VKRLQFL  ENLKLIN  VGQIDQTQLRLPPA
738    SIFPTKL  RKLTLLD  T-WLEWDDMSV
762    LKQLENL  QVLKLKD  NAFKGENWELN
787    DGGFPFL  QVLCIER  ANLVSWNAS
810    GDHFPRL  KHLHISC  D-KLEKIPIG
833    LADICSL  QVMDLRN
847    STKSAAKSAREIQAKKNKLQPAKSQKFELSVFPPDSDVQTAS

       l..l..l  c.l.l..  ...l..lP.
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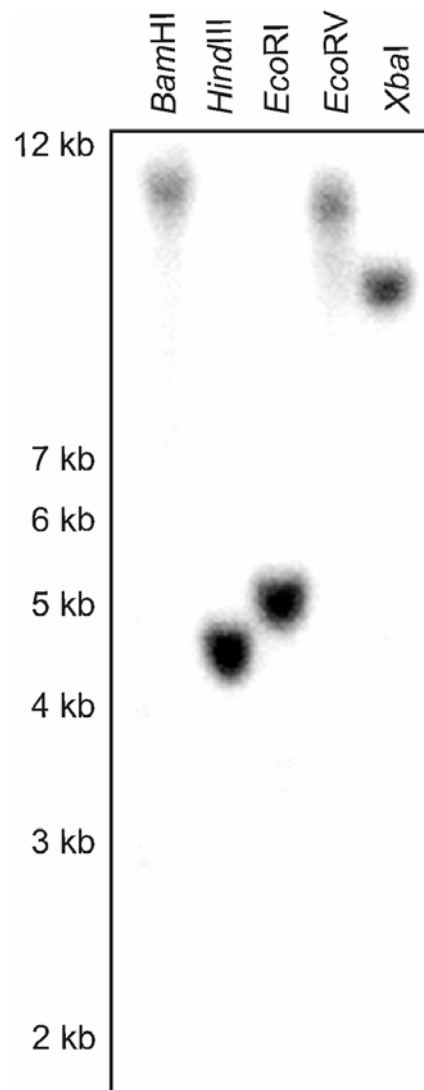
Figure 1. Predicted Sequence of NRC1, Product of a Single-Copy Gene in Tomato.

(A) Predicted sequence of the NRC1 protein. The first 150 amino acid residues represent the coiled-coil (CC) domain and residues that are predicted to form the CC structure are underlined. Residues 151 to 508 comprise the nucleotide-binding (NB-ARC) domain, with the following motifs (underlined and labeled): Kinase1A (P-loop), RNBS-A, Kinase 2, RNBS-B, RNBS-C, GLPL, RNBS-D and MHD. Residues 509 to 847 comprise the 13 imperfect leucine-rich repeats (LRRs); the conserved hydrophobic and proline residues are shown in italic. Below the protein sequence the LRR consensus motif is indicated: 'l' indicates a conserved aliphatic residue, 'c' indicates a conserved charged residue and 'P' indicates a conserved proline residue.

(B) DNA gel-blot analysis of tomato genomic DNA digested with *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV and *Xba*I, and hybridized with a 1293 bp cDNA fragment of *NRC1* (nucleotides 1876 to 3168 of the full-length cDNA sequence). The position of the molecular weight markers is indicated on the left.

NRC1-Silenced Tomato Is Affected in *Cf-4*-Dependent HR and Resistance

To investigate the function of NRC1 in HR-signaling and resistance to *C. fulvum*, we performed VIGS in tomato, the only host plant for this fungus. Ten-day-old tomato seedlings were agroinfiltrated with the TRV:NRC1 recombinant that had also been used for VIGS in *N. benthamiana* and three weeks post infiltration silencing was analyzed by RT-PCR. In TRV:NRC1-infected plants the *NRC1* transcript levels were lower than in the TRV:00-infected plants, indicating that the expression of *NRC1* was knocked-down (Figure 2A, page 95). To confirm that the observed phenotype in tomato is not caused by silencing of additional NB-LRR proteins, tomato was also inoculated with TRV containing a 360 bp fragment targeted to the sequence of *NRC1* encoding LRRs eight to twelve (TRV:NRC1-LRR) and a fragment consisting of 297 bp of the 3'-untranslated region (UTR) of *NRC1* (TRV:NRC1-UTR). Silencing of *NRC1* (using each of the three constructs) results in a mild developmental phenotype as the tomato plants appeared somewhat smaller than TRV:00- or TRV:Cf-4-infected control plants (Figure 2B, page 95). To test whether NRC1 is required for *Cf-4*-mediated HR, Avr4 protein was injected in *Cf-4*-containing tomato plants infected with TRV:NRC1-UTR and compared with the response in TRV:00- and TRV:Cf-4-infected plants. In TRV:Cf-4-infected plants 52% of the total number of Avr4-injected sites responded with an HR (Figure 2C, page 95), indicating a decreased HR due to silencing of *Cf-4*. In TRV:NRC1-UTR-infected plants this percentage was similar (48%, Figure 2C), showing the requirement of NRC1 for *Cf-4*/*Avr4*-induced HR in tomato. Similar results were obtained upon VIGS using TRV:NRC1-LRR in *Cf-4*-containing tomato, whereas also in *Cf-9*-containing tomato inoculation with the various NRC1-VIGS constructs resulted in compromised HR upon injection of Avr9 (results not shown).

Next, we investigated whether NRC1 is also required for full resistance of tomato to *C. fulvum*. Cf0 and *Cf-4*-containing tomato plants were inoculated with TRV:00, TRV:Cf-4 and TRV:NRC1 and after three weeks the plants were inoculated with a strain of *C. fulvum* expressing *Avr4* and the β-glucuronidase (*GUS*) gene, that allowed to visualize fungal growth. Two weeks post *C. fulvum* inoculation leaves were treated with X-gluc. In leaflets of *Cf-4*-containing tomato infected with TRV:00 no growth of *C. fulvum* was detected, whereas in plants infected with TRV:Cf-4 patches of blue staining indicate fungal growth due to compromised *Cf-4*-mediated resistance (Figure 2D, page 95). Also in TRV:NRC1-infected plants small patches of blue staining indicate loss of full resistance against the fungus. Detailed microscopical analysis indeed revealed intercellular fungal growth in TRV:Cf-4- and TRV:NRC1-infected plants, but not in the TRV:00-infected control plants (Figure 2D). Similar results were obtained when a TRV:NRC1-UTR construct was used for VIGS (results not shown). All Cf0 plants displayed extensive colonization

by *C. fulvum*, indicating that neither the TRV infection itself, nor VIGS using TRV:NRC1 affects the susceptibility of these plants to the fungus.

NRC1 Is Required for HR-Induction by Different Matching R/Avr Protein Pairs

In addition to its requirement for *Cf/Avr*-induced HR, also the HR induced in *N. benthamiana* by the Inf1 elicitor of the oomycete pathogen *Phytophthora infestans* was found to be NRC1-dependent (Gabriëls et al., 2006). To further investigate the specificity of NRC1 in defense signaling, we tested its requirement for the HR induced by various different matching R/Avr protein pairs. As controls TRV:00 (empty vector) and TRV:SGT1 were included, since SGT1 is known to be required for the HR induced by several R/Avr pairs (Peart et al., 2002b). As expected, the HR resulting from agroinfiltration of a mix of *Cf-4* and *Avr4* and a mix of *Cf-9* and *Avr9* (Van der Hoorn et al., 2000) was compromised in TRV:NRC1-infected *N. benthamiana* (Figure 3A, page 97). Next, we tested whether the HR induced by the combination of LeEIX2, which is an RLP of tomato, and its ligand, the Ethylene-Inducing Xylanase of *Trichoderma viride* (tvEIX) (Ron and Avni, 2004) is also NRC1-dependent. Indeed, also the LeEix2/tvEIX-induced HR was reduced in TRV:NRC1-infected plants, whereas in the TRV:00-infected plants the HR was not affected (Figure 3A). We also agroinfiltrated *AvrPto* from the bacterial pathogen *Pseudomonas syringae* pv. *tomato* and the gene encoding the coat protein (*CP*) of Potato Virus X (PVX) in *N. benthamiana* expressing the resistance gene *Pto* (Pedley and Martin, 2003) and *Rx* (Bendahmane et al., 1999), respectively. Plants infected with TRV:00 showed an HR, while the HR was severely suppressed upon agroinfiltration of the genes in TRV:NRC1-infected plants (Figure 3A). Thus, in *N. benthamiana* an *NRC1* orthologue is required for HR signaling activated by all R/Avr protein combinations tested. In TRV:SGT1-infected plants in all cases the HR was severely reduced, confirming the observations of Peart et al. (2002b) (Figure 3A). To exclude the possibility that the compromised HR in TRV:NRC1-infected *N. benthamiana* results from a decreased transformation efficiency by Agrobacterium, we infiltrated TRV:00- and TRV:NRC1-infected *N. benthamiana:Cf-4* with Agrobacterium containing the β-glucuronidase (*GUS*) gene (Van der Hoorn et al., 2000). Three days post infiltration the intensity of the blue staining in TRV:00- and TRV:NRC1-infected plants revealed that the transformation efficiency of the plants by Agrobacterium is not affected (Figure 3B, page 97). In addition, the TRV:NRC1-infected plants also showed a reduced HR upon injection with Avr4 protein, while the TRV:00-infected plants showed a normal development of the HR (Figure 3B), confirming the involvement of NRC1 in downstream defense signaling.

(A)

(B)

(D)

(C)
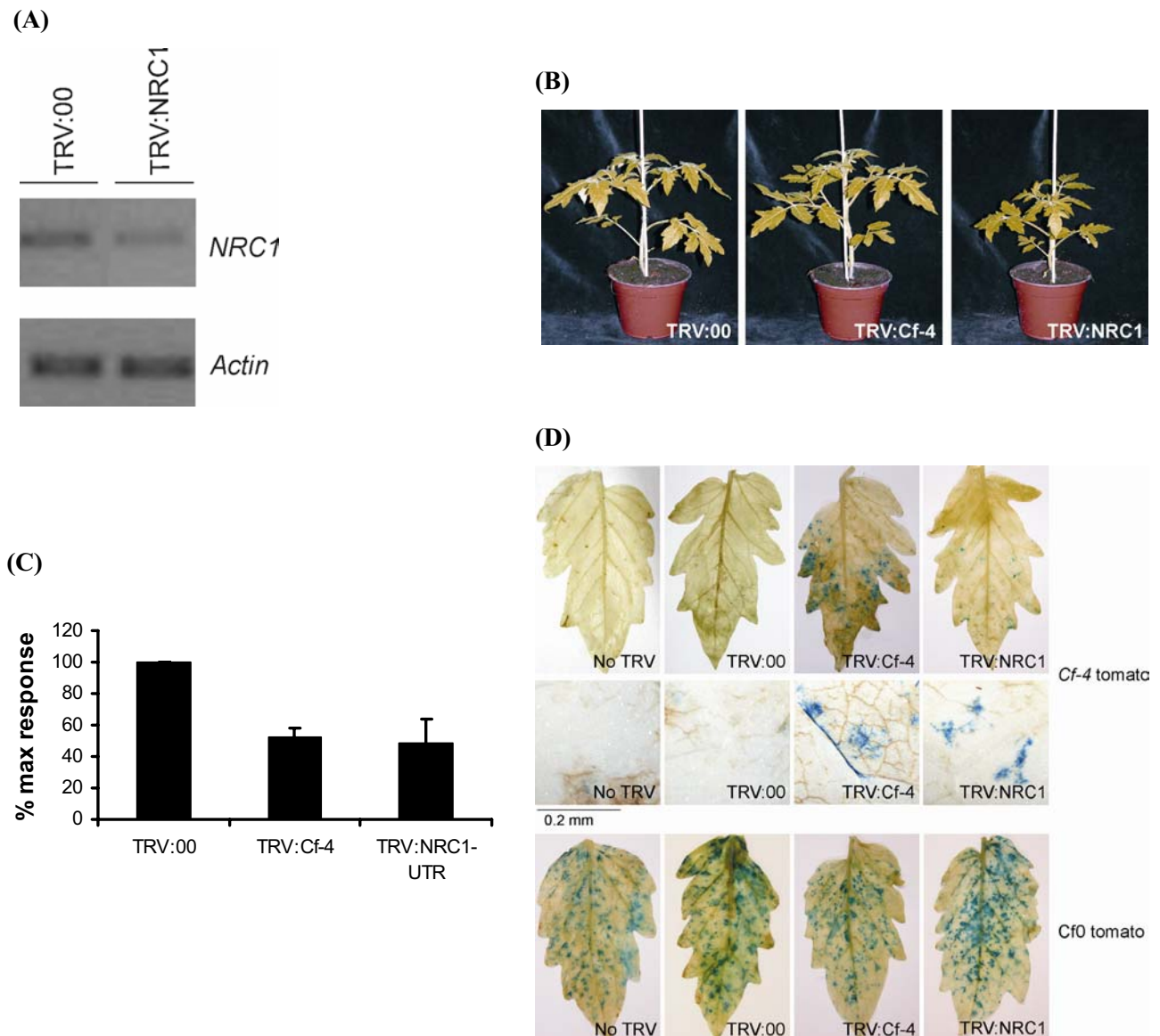
Figure 2. NRC1 Is Required for Full *Cf-4*-Mediated HR and Resistance of Tomato to *Cladosporium fulvum*. Cf0 tomato and *Cf-4*-containing tomato plants were inoculated with the indicated TRV constructs and plants were analyzed three weeks after the onset of VIGS.

(A) RNA was isolated from individual leaflets of a TRV:00-infected plant and a TRV:NRC1-infected plant and analyzed by RT-PCR using *NRC1*-specific primers (upper panel), or actin-specific primers as a control (lower panel).

(B) Phenotype of *Cf-4*-containing tomato plants 3 weeks post inoculation with TRV:00, TRV:Cf-4 or TRV:NRC1.

(C) Leaflets of TRV-infected *Cf-4*-containing tomato plants were injected with Avr4 protein and examined for the development of an HR. The number of injected sites mounting an HR on TRV:00-infected plants was set to 100%. Each error bar represents the standard error from four independent experiments.

(D) Non-TRV-infected- and TRV-infected *Cf-4* or Cf0 plants were inoculated with *C. fulvum-pGPD::GUS* and two weeks post inoculation colonization of the leaflets was studied with an X-gluc assay.

VIGS of *NRC1* Does Not Affect Resistance to *P. syringae* pv. *tomato,* Potato Virus X and Tobacco Mosaic Virus

We found that inoculation of *N. benthamiana* with TRV:NRC1 affects the Pto/AvrPto-induced HR (Figure 3A). To test whether knock-down of *NRC1* expression also compromises resistance to *AvrPto*-expressing bacteria, tomato RG-PtoR (*Pto/Pto, Prf/Prf*), which is resistant to *P. syringae* pv. *tomato* expressing *AvrPto*, was inoculated with TRV:NRC1. As controls, inoculations with TRV:00 and TRV:Prf were included. *Prf* encodes an NB-LRR protein which is required for *Pto*-mediated resistance (Pedley and Martin, 2003). The TRV-infected plants were inoculated with *P. syringae* pv. *tomato* (*AvrPto*) and colonization by the bacteria was assessed at one hour and four days post-inoculation. In TRV:00- and TRV:NRC1-infected plants the number of colony forming units (CFUs) of the bacterium did not increase, indicating that resistance was not compromised (Figure 4A, page 98). In TRV:Prf-infected plants the number of CFUs did increase, indicating loss of full resistance (Figure 4A) (Ekengren et al., 2003).

We also investigated the requirement of NRC1 for Rx- and N-mediated resistance to PVX and TMV, respectively. TRV:00, TRV:SGT1 and TRV:NRC1 was inoculated onto *N. benthamiana*:*Rx* and *N. benthamiana*:*N* and three weeks later the plants were inoculated with PVX:GFP and TMV:GFP, respectively. Subsequently, over a period of two weeks systemic leaves were examined for GFP fluorescence under UV light. In TRV:NRC1-infected plants resistance did not decrease, indicating that knock-down of NRC1 expression is not sufficient to compromise resistance to these viruses (Figure 4B, page 98). Resistance was also not affected in the TRV:00-infected plants, whereas resistance to both viruses was severely compromised in TRV:SGT1-infected plants (Figure 4B).

(A)
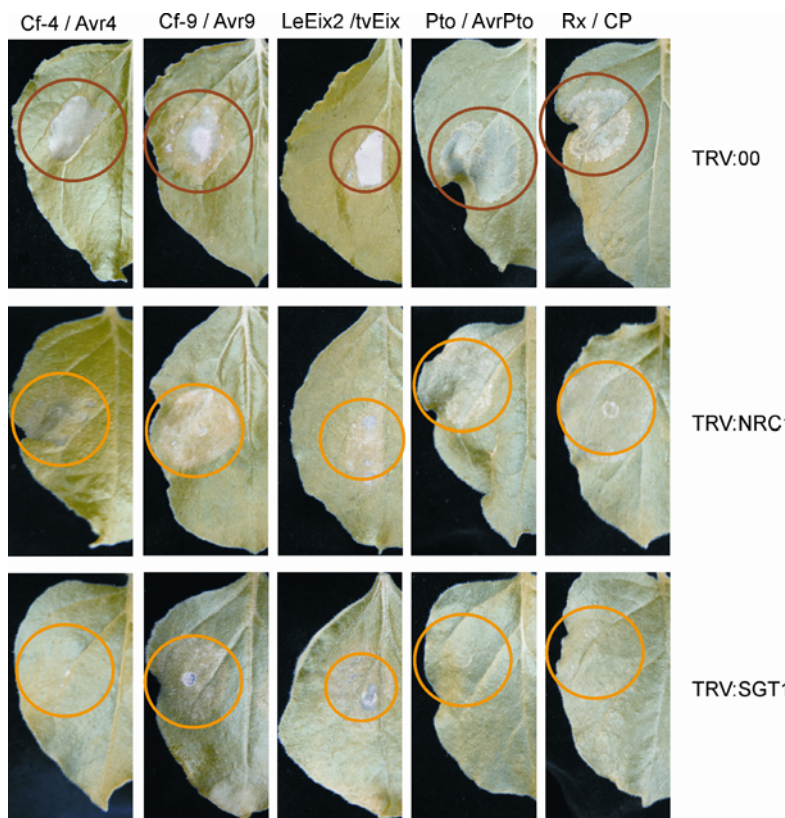
(B)
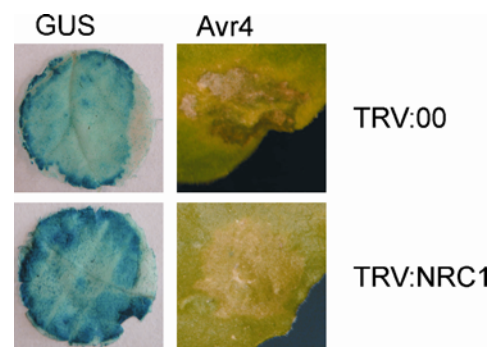
Figure 3. Inoculation of *N. benthamiana* with TRV:NRC1 Affects *Cf/Avr-*, *LeEix2/tvEix-*, *Pto/AvrPto-* and *Rx/CP*-Induced HR.

(A) *N. benthamiana* was inoculated with TRV:00 (empty vector), TRV:NRC1 and TRV:SGT1. Three weeks later leaves were infiltrated with Agrobacteria expressing HR-inducing proteins and pictures were taken at 4 days post infiltration. First, second and third column: leaves of *N. benthamiana* expressing the *Cf-4* resistance gene agroinfiltrated with *Avr4*, or a mix of *Cf-9* and *Avr9*, or a mix of *LeEix2* and *tvEix* (combined in a 1:1 ratio), respectively. Fourth column: leaves of transgenic *N. benthamiana* expressing the *Pto* resistance gene agroinfiltrated with *AvrPto*. Fifth column: leaves of transgenic *N. benthamiana* expressing the *Rx* resistance gene agroinfiltrated with the gene expressing the coat protein of PVX (*CP*). The red circles indicate an HR, orange circles indicate a compromised HR.

(B) Transformation efficiency and response to Avr4 protein of TRV:00- and TRV:NRC1-infected transgenic *N. benthamiana* expressing the *Cf-4* resistance gene. First column: infiltration with Agrobacterium that contains the β-glucuronidase (*GUS*) gene. Leaves were stained for GUS activity at 3 days after infiltration. Second column: leaves were injected with Avr4 protein. Pictures were taken at 2 days after injection.

(A)

(B)
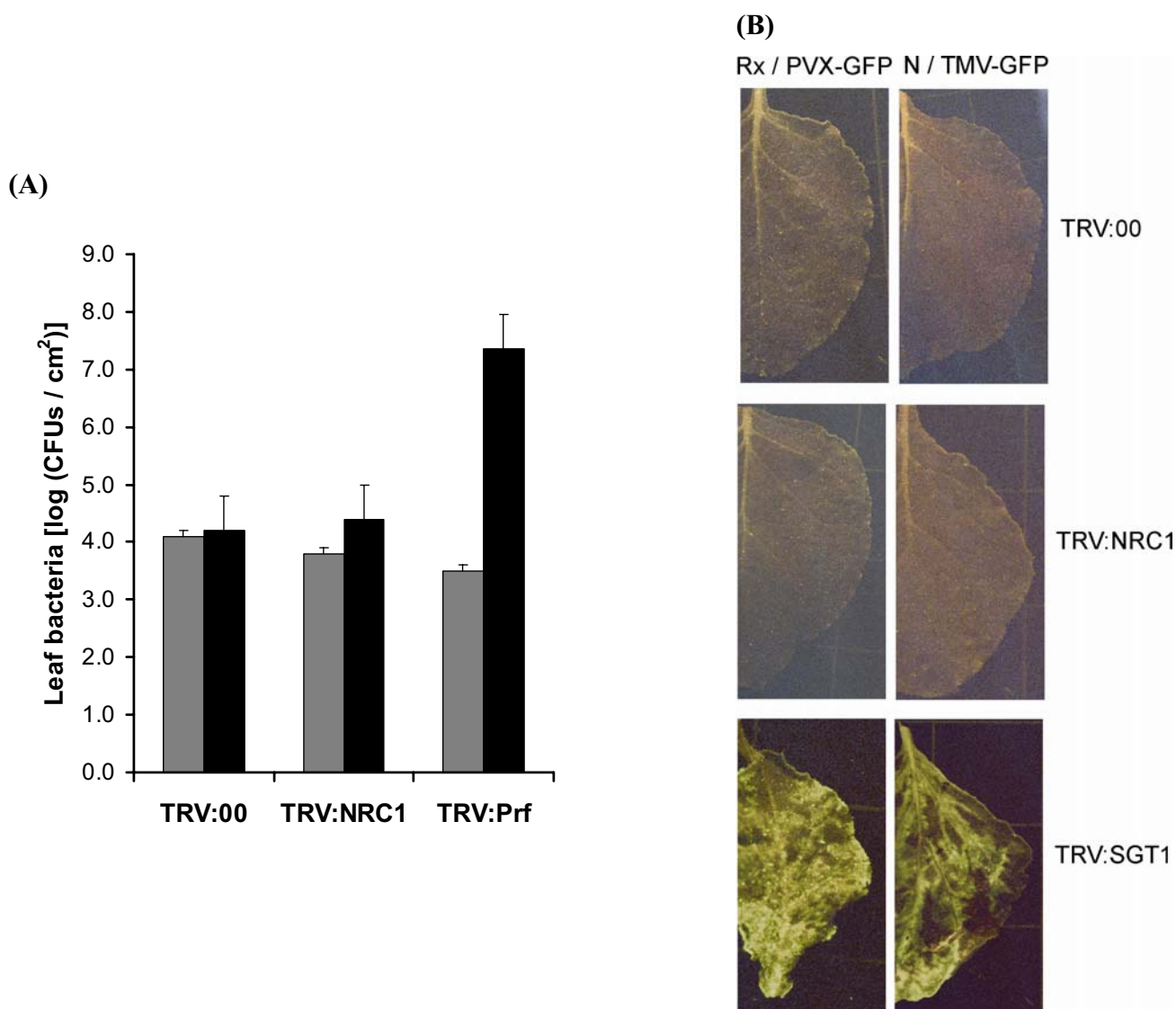
Figure 4. VIGS of *NRC1* Does Not Affect Resistance to *Pseudomonas syringae* pv. *tomato,* Potato Virus X (PVX) or Tobacco Mosaic Virus (TMV).

(A) Tomato RG-PtoR (*Pto/Pto, Prf/Prf*) was inoculated with TRV:00-, TRV:NRC1- or TRV:Prf and three weeks later the plants were inoculated with *Pseudomonas syringae* pv. *tomato* (strain T1 (*AvrPto*)). The number of colony-forming units (CFUs) of the bacterium per square cm of leaf area was determined at one hour (gray bars) and four days post-inoculation (black bars). The error bars represent the standard error of four different TRV-infected plants.

(B) Transgenic *N. benthamiana* was inoculated with the indicated TRV constructs and challenged with either PVX or TMV three weeks later. First column: TRV-infected *N. benthamiana* plants expressing *Rx* were challenged with PVX-GFP. Second column: TRV-infected *N. benthamiana* plants expressing *N* were challenged with TMV-GFP. Pictures were taken two weeks post PVX-GFP or TMV-GFP inoculation.

NRC1 Functions Upstream of a MAPK Cascade in an HR Signaling Pathway

Since NRC1 is required for HR induced by several additional matching *R/Avr* combinations, we assume that this NB-LRR protein is involved in a common HR signaling pathway. A typical host response that precedes the initiation of the HR includes activation of MAPK cascades (Romeis et al., 2001; Del Pozo et al., 2004; Pedley and Martin, 2005). To investigate the requirement of NRC1 for the HR mediated by MAPKs, we performed epistasis experiments in *N. benthamiana*. Plants were inoculated with TRV:00, TRV:SGT1 and TRV:NRC1 and subsequently agroinfiltrated with a gene fragment encoding a constitutively active kinase domain of LeMAPKKKα (*LeMAPKKKα^{KD}*) or constitutively active LeMEK2 (*LeMEK2DD*, which is a MAPKK) (Yang et al., 2001; Del Pozo et al., 2004). Two days post agroinfiltration, expression of the genes was induced by spraying the infiltrated leaves with estradiol. In TRV:00-infected plants an HR was observed, whereas in TRV:SGT1-infected plants the HR was decreased (Figure 5A). In TRV:NRC1-infected plants the HR caused by both constitutively active kinases is not affected (Figure 5A). Agroinfiltration of the corresponding negative controls, *LeMAPKKKα^{KD-}* and wild-type *LeMEK2,* did not result in an HR in any of the TRV-infected plants (data not shown). These results indicate that the MAPKK(K)s that were tested require SGT1 to initiate the HR and that they act downstream, or possibly independent, of NRC1.

To determine which genes are further required for HR signaling by this CC-NB-LRR protein we aimed to perform epistasis experiments by overexpressing *NRC1* in *N. benthamiana*, silenced for various genes required for defense signaling. If NRC1 actively feeds in an HR signaling pathway, overexpression of the encoding gene should result in an HR and suppression of this response would indicate that the silenced gene is required for NRC1 signaling. Therefore the coding sequence of the *NRC1* cDNA was fused to the constitutive 35S promoter and cloned into a binary vector. Agroinfiltration of this construct in *N. benthamiana* did not result in an HR. Only when *NRC1* was agroinfiltrated in combination with the *p19* silencing inhibitor (Voinnet et al., 2003), an elicitor-independent HR was provoked (Figure 5B). However, *p19* can not be used in combination with epistasis experiments in silenced plants. Since mutations in the MHD motif of the NB-LRR resistance proteins Rx (D460V) (Bendahmane et al., 2002; Tameling et al., 2002) and I-2 (D495V) (Bendahmane et al., 2002; Tameling et al., 2002; de la Fuente van Bentem et al., 2005) result in constitutive activity, we generated a similar mutant of *NRC1* (*NRC1^{D481V}*). Indeed, agroinfiltration of *NRC1^{D481V}* resulted in an elicitor-independent HR in leaves of *N. benthamiana* within three days post infiltration, without the requirement of *p19* (Figure 5B).

(A)

(B)

(C)
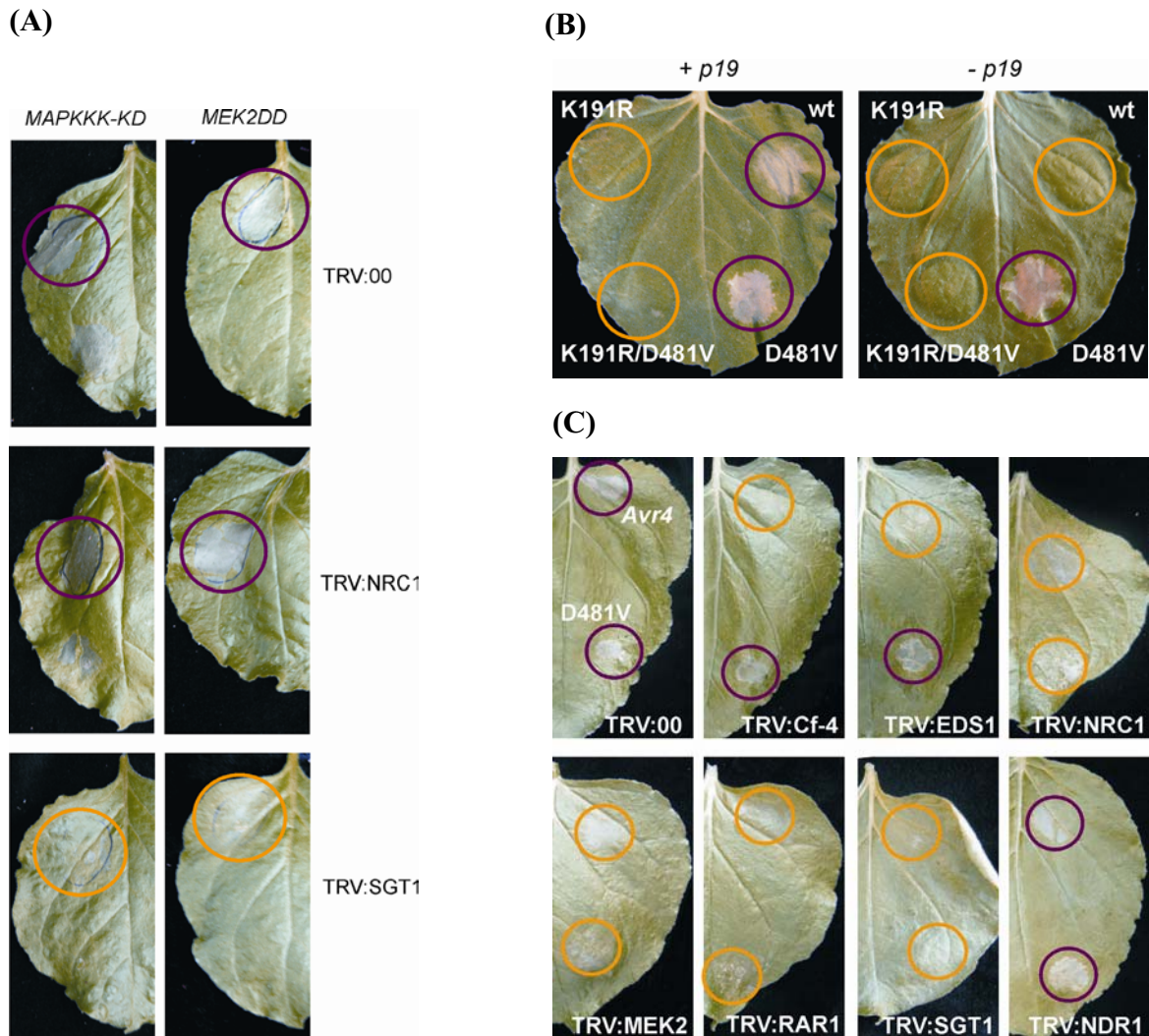
Figure 5. Constitutively Active NRC1 Induces an Elicitor-Independent HR and Allows to Position NRC1 in a Cell Death Signaling Pathway.

N. benthamiana expressing the *Cf-4* resistance gene was agroinfiltrated with the indicated genes. For panels A and C, three weeks prior to agroinfiltration the plants were inoculated with the indicated TRV constructs. Red circles indicate an HR, orange circles indicate a compromised HR.

(A) Agroinfiltration of genes encoding constitutively active MAPKK and MAPK kinases. First column: agroinfiltration of a fragment encoding the constitutively active kinase domain of LeMAPKKKα (*MAPKKK-KD*). Second column: agroinfiltration of the gene encoding a constitutively active mutant of LeMEK2 (*MEK2DD*). Two days post infiltration of *MAPKKK-KD* or *MEK2DD* expression was induced by spraying the leaves with estradiol. Pictures were taken four days post agroinfiltration.

(B) Agroinfiltration of wild-type *NRC1* (wt) and mutated forms of the gene, under control of the 35S-promoter, either mixed in a 1:1 ratio with Agrobacterium containing the gene encoding silencing suppressor p19 (left panel), or alone (right panel). *NRC1^K191R^* (K191R): inactive P-loop mutant of NRC1; *NRC1^D481V^* (D481V): constitutively active NRC1 (mutated in the MHD motif); *NRC1^K191R/D481V^* (K191R/D481V): double mutant of NRC1. Pictures were taken three days post agroinfiltration.

(C) Agroinfiltration of *Avr4* and constitutively active *NRC1^D481V^* (D481V). Pictures were taken three days post agroinfiltration.

Agroinfiltration of a construct encoding a mutant of NRC1 (K191R) in which the P-loop motif has been disrupted, thereby affecting ATP hydrolysis (Tameling et al., 2002), either with or without *p19*, did not result in an HR and no HR was observed either upon agroinfiltration of the double mutant $NRC1^{K191R/D481V}$ (Figure 5B). Furthermore, no HR was induced upon expression of $NRC1^{D481V}$ in *SGT1*-silenced plants (see below). These results indicate that NRC1 functions in a signal transduction cascade leading to HR and that the response induced by $NRC1^{D481V}$, which can be used in epistasis experiments, is specifically due to constitutive activity of the NRC1 protein.

NRC1 Requires RAR1 and SGT1 and Acts Downstream of EDS1 in an HR Pathway

To test whether NRC1 requires *RAR1* and *SGT1*, *N. benthamiana:Cf-4* was silenced for these genes and subsequently agroinfiltrated with *Avr4* and constitutively active $NRC1^{D481V}$. Furthermore, additional genes required for HR signaling including *NDR1* (Non race-specific Disease Resistance) (Century et al., 1995), *EDS1* (Enhanced Disease Susceptibility) (Aarts et al., 1998) and *MEK2* (Ekengren et al., 2003) were tested and VIGS using TRV:00, TRV:Cf-4 and TRV:NRC1 was included as controls. As expected, HR induced upon agroinfiltration of *Avr4* was compromised in TRV:Cf-4- and TRV:NRC1-infected plants. In addition, a reduced HR was found upon agroinfiltration of *Avr4* in plants silenced for *RAR1* and *SGT1*. *Cf-4*-mediated signaling also requires EDS1 and MEK2, as plants silenced for these genes displayed a less severe Avr4-induced HR (Figure 5C; orange circles). The Avr4-induced HR is not compromised in TRV:NDR1-infected plants (Figure 5C; red circles), indicating that NDR1 is not required for *Cf-4*-mediated signaling. Similarly, $NRC1^{D481V}$-induced HR was not compromised in TRV:NDR1-infected plants, whereas it was also not affected in TRV:Cf-4-infected plants. Interestingly, in contrast to Avr4, $NRC1^{D481V}$ still induces an HR in TRV:EDS1-infected plants, indicating that NRC1 exerts its activity downstream of EDS1 (Figure 5C; red circles). The $NRC1^{D481V}$-induced HR is compromised in plants silenced for *MEK2*, confirming that NRC1 requires a MAP-kinase cascade for HR signaling and that the protein is active upstream of these MAP-kinases. VIGS of *RAR1* and *SGT1* also compromises $NRC1^{D481V}$-induced HR (Figure 5C; orange circles), indicating that these chaperonin-like proteins are also required for NRC1 function. Possibly NRC1 is present in a downstream signaling complex that is stabilized by RAR1 and SGT1. As summarized in Figure 6, we have shown that NRC1 is required for HR signaling initiated by Cf-4 and that the protein is functional upstream of a MAPK cascade and downstream of EDS1.

Cf-4/Avr4

↓

EDS1

↓

NRC1

↓

MEK2

↓

HR
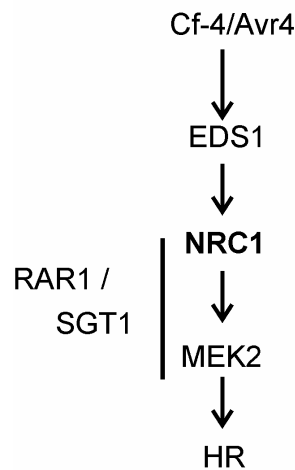
RAR1 /
SGT1

Figure 6. Model for NRC1-Mediated HR Signaling.

The model is based on epistasis experiments combining HR assays and VIGS in *N. benthamiana*. Cf-4/Avr4-induced HR requires EDS1, NRC1, MEK2 and SGT1/RAR1 (for details see text).

DISCUSSION

NRC1 Is a CC-NB-LRR Protein Required for *Cf-4*-Mediated HR and Resistance

In tomato seedlings expressing both *Avr4* and *Cf-4*, we previously identified *NRC1* as a slightly up-regulated *Cf-4/Avr4*-specific differential. The requirement of an *NRC1* orthologue for *Cf-4/Avr4*-induced HR in *N. benthamiana* was subsequently confirmed by VIGS (Gabriëls et al., 2006). *NRC1* of tomato encodes a CC-NB-LRR resistance protein analogue, suggesting that Cf proteins, which are extracellular RLPs, require a cytoplasmic NB-LRR protein to be functional. Indeed, also in *Cf-4*-containing tomato VIGS of *NRC1* compromises the Avr4-induced HR and resistance to *C. fulvum* (Figures 2C and 2D). The first NB-LRR protein that was found to function downstream in the defense signaling cascade initiated by a resistance protein is the CC-NB-LRR protein NRG1 of *N. benthamiana* (Peart et al., 2005). However, *NRG1* is not an orthologue of *NRC1* as there is no significant sequence homology and NRG1 is specifically involved in *N*-mediated defense and is for example not required for Inf1-induced HR. Interestingly, VIGS targeting *NRC1* appears to affect multiple HR signaling pathways.

Compromised resistance to *C. fulvum* was also observed when a TRV:NRC1-UTR construct was used for VIGS. Since the sequence of the 3'-untranslated region is very specific for *NRC1*, and

also no significant sequence similarity was found when searching the tomato TIGR (The Institute for Genomic Research) database, we expect to have specifically silenced *NRC1*. Furthermore, different VIGS constructs targeted to *NRC1* resulted in similar phenotypes in tomato. These observations, in combination with the single band observed on the high stringency tomato DNA gel blot (Figure 1B) render silencing of genes encoding potential homologous (CC)-NB-LRR proteins highly improbable. We confirmed silencing of *NRC1* in tomato by RT-PCR (Figure 2A). Silencing in tomato is less efficient when compared to *N. benthamiana* as it remains patchy (Rowland et al., 2005). Indeed, we always found residual *NRC1* transcripts, even when analyzing RNA isolated from single leaflets. Probably a phenotype such as a compromised HR or a compromised resistance is only manifested in localized areas where knock-down of *NRC1* transcription is nearly complete, whereas by RT-PCR residual transcripts in surrounding tissues will still be amplified.

VIGS in tomato using several TRV constructs targeted to *NRC1* results in slightly smaller plants as compared to TRV:00- and TRV:Cf-4-infected plants (Figure 2B). Furthermore, VIGS using TRV:NRC1 in *N. benthamiana* not only affects the HR but also alters plant morphology (Gabriëls et al., 2006). We can not exclude that VIGS using TRV:NRC1 triggers silencing of heterologous *N. benthamiana* genes having different functions, as a stretch of 23 nucleotides of complete identity is potentially sufficient to target additional genes (Thomas et al., 2001).

The HR Is an Essential Component of the Defense of Tomato to *C. fulvum*

Lu et al. (2003) identified a large set of cDNAs corresponding to genes required for *Pto*-mediated HR, whereas silencing of only some of them also compromised resistance to *P. syringae* expressing *AvrPto*. *Cf*-mediated resistance to avirulent strains of *C. fulvum* is typically associated with a microscopical HR (Rivas and Thomas, 2005). A role of NRC1 in both the HR and resistance does not only mean that this CC-NB-LRR protein is essential for defense signaling of tomato, but it also proves that the HR is an integral part of a successful resistance response of the plant to the invading fungus. Indeed, knock-down of *NRC1* affects the *Cf-4/Avr4*-induced HR and causes loss of full resistance to *C. fulvum*. Bendahmane et al. (1999) found that the HR is not required for *Rx*-mediated resistance to PVX. This was also observed for other virus resistance traits (Brommonschenkel et al., 2000; Cole et al., 2001; Hajimorad and Hill, 2001) and for *RPS2*-mediated resistance to *P. syringae* expressing AvrRpt2 (Yu et al., 1998). As speculated by Bendahmane et al. (1999) there are various levels of resistance in plants, ranging from extreme resistance without the development of an HR, as is the case for *Rx*-mediated resistance, to resistance which is associated with a systemic HR. These responses depend on various characteristics of the

elicitor-receptor interaction and *Cf*-mediated resistance could then classify as intermediate. Rowland et al. (2005) recently identified LeACIK1, a Ser/Thr protein kinase required for both *Cf/Avr*-induced HR as well as *Cf*-mediated resistance. This observation also indicates that the HR is an essential component in the defense response of tomato to *C. fulvum*. As LeACIK1 is specifically required for *Cf/Avr*-induced responses, this kinase is thought to act upstream in the signaling pathway (Rowland et al., 2005), whereas NRC1 should be more downstream. NRC1 seems not to be involved in basal resistance of tomato to *C. fulvum* since VIGS of the encoding gene in Cf0 tomato plants did not significantly increase the intensity of colonization by the fungus.

An NB-LRR Protein Involved in Multiple HR Signaling Pathways

To investigate whether NRC1 is a convergence point of multiple HR signaling pathways, we performed functional analysis of the gene by VIGS in transgenic *N. benthamiana* followed by agroinfiltration of matching *R* and *Avr* combinations (Baulcombe, 1999). In addition to its requirement for the HR induced by the Inf1 elicitin of *Phytophthora infestans* (Gabriëls et al., 2006), NRC1 is also required for *Cf-9/Avr9-*, *LeEix2/tvEix-*, *Pto/AvrPto-* and *Rx/CP*-initiated HR (Figure 3A). Thus, NRC1 is involved in HR pathways triggered by both extra- and intracellular R proteins which belong to different classes: RLPs (Cf-4, Cf-9 and LeEix2), the Ser/Thr protein kinase Pto and a CC-NB-LRR protein (Rx), which confer resistance to respectively fungi (*C. fulvum* and *Trichoderma viride*), a bacterium (*Pseudomonas syringae* pv. *tomato*), or a virus (PVX). NRC1 is involved in *Pto/AvrPto-* and *Rx/CP*-induced HR, but we did not observe compromised resistance to *P. syringae* and PVX in plants inoculated with VIGS constructs targeted to *NRC1*. Also *N*-mediated resistance to TMV was not affected, indicating that for resistance to these pathogens the HR is a secondary feature (Lu et al., 2003). Another possibility could be that due to functional redundancy or insufficient silencing of *NRC1,* resistance to these pathogens is not compromised.

A Model for a *Cf-4/Avr4*-Triggered HR Pathway

To determine where NRC1 exerts its activity in a *Cf-4/Avr4*-triggered HR pathway, we performed epistasis experiments in which we silenced genes required for HR signaling and concomitantly (over)expressed *NRC1* or *Avr4*. Agroinfiltration of *35S:NRC1* only resulted in an elicitor-independent HR upon co-expression with the gene encoding the p19 silencing inhibitor (Figure 5B). This inhibitor prevents the onset of Post-Transcriptional Gene Silencing (PTGS), allowing accumulation of higher amounts of the NRC1 protein (Voinnet et al., 2003). Since VIGS is also based on PTGS, co-agroinfiltration of *p19* is not compatible with our epistasis experiments.

Therefore we constructed a constitutively active mutant of NRC1, in a way similar to what was done for the CC-NB-LRR resistance proteins Rx and I-2 (Rx^{D460V} and I-2^{D495V}), by mutating the MHD motif (Bendahmane et al., 2002; Tameling et al., 2002; de la Fuente van Bentem et al., 2005). Indeed, agroinfiltration of constitutively active *NRC1^{D481V}* results in an elicitor-independent HR. *NRC1^{D481V}*-agroinfiltration does not result in an HR in SGT1-silenced plants. In addition, there is no HR upon agroinfiltration of the double mutant *NRC1$^{K191R/D481V}$*, in which the P-loop motif which is essential for functionality of the NB-LRR protein is mutated (Figure 5B) (Bendahmane et al., 2002; Tameling et al., 2002; de la Fuente van Bentem et al., 2005). Necrosis resulting from an aspecific response to the constitutively active protein would not be abolished in SGT1-silenced plants or by a P-loop mutation, indicating that the HR is a specific response and that *NRC1^{D481V}* can be used in epistasis experiments.

We agroinfiltrated *Avr4* in *N. benthamiana:Cf-4* silenced for several genes required for the HR and found that in all cases the responsiveness to Avr4 was decreased, except when *NDR1* was silenced (Figure 5C). Indeed, also NRC1^{D481V} induced a normal HR in TRV:NDR1-inoculated *N. benthamiana* (Figure 5C). We conclude that *Cf*-mediated signaling by NRC1 does not require NDR1, whereas it does require EDS1. Decreased Avr4 responsiveness indicated that, in addition to NRC1, Cf-4 indeed requires EDS1 and furthermore MEK2, RAR1 and SGT1 for HR signaling.

Interestingly, recently Hu et al. (2005) reported the possible requirement of EDS1 for *Ve*-mediated resistance. Ve is an extracellular RLP that is very similar to Cf proteins and provides resistance to the fungal pathogen *Verticillium dahliae*. Hu et al. (2005) found that in *Ve*-containing tomato plants carrying a mutation in *sun1* (suppressor of *N*), which was identified as an *EDS1* homologue, resistance to *V. dahliae* was compromised. It will be interesting to test whether VIGS of this *EDS1* homologue in tomato results in compromised resistance to *C. fulvum*.

We show that Avr4 responsiveness is suppressed in TRV:MEK2-inoculated *N. benthamiana:Cf-4* (Figure 5C). MAPKs have already been widely associated with the HR and/or disease resistance of plants (Pedley and Martin, 2005). Del Pozo et al. (2004) silenced *MAPKKKα*, which encodes the MAPKKK immediately upstream of MEK2 and found a clear reduction of the *Cf-9/Avr9*-induced HR, confirming that Cf proteins require a MAPK signaling cascade for the activation of the HR.

RAR1 and SGT1 are recruited by a large set of resistance proteins and are thought to play a role in the stabilization of R protein-containing recognition complexes and the regulation of downstream signaling (Muskett and Parker, 2003; Shirasu and Schulze-Lefert, 2003). Peart et al. (2002b) showed that in *N. benthamiana* silenced for *SGT1* both the *Cf-9/Avr9*- and the *Cf-4/Avr4*-induced HR is suppressed, an observation that was later confirmed for the Avr4 response in *N.*

benthamiana:Cf-4 (Gabriëls et al., 2006). Here we show similar results for VIGS using TRV:RAR1 (Figure 5C), indicating that indeed also for *Cf*-mediated defense signaling RAR1 and SGT1 play similar downstream roles. Agroinfiltration of constitutively active LeMEK2 and LeMAPKKKα (Yang et al., 2001; Del Pozo et al., 2004) in TRV:SGT1-inoculated *N. benthamiana* results in a reduced HR as compared to the controls (Figure 5A), indicating that indeed SGT1, and presumably also RAR1, act further downstream in defense signaling. RAR1 and SGT1 directly interact and could be involved in the assembly and stabilization of a signaling complex that amongst others, contains MAPK(K)(K)s and NRC1.

Agroinfiltration of constitutively active *NRC1^{D481V}* in parallel with *Avr4* in silenced *N. benthamiana:Cf-4* (Figure 5C) revealed that the response of TRV:EDS1-infected plants to Avr4 is suppressed, whereas these plants still mount an HR upon agroinfiltration with *NRC1^{D481V}*, thereby positioning NRC1 downstream of EDS1 (Figure 6). A similar observation was reported by Ekengren et al. (2003), who showed that resistance signaling by Pto, which requires the CC-NB-LRR protein Prf, is also NDR1-independent. Interestingly, also Peart et al. (2005) found that NRG1 does not require EDS1 to be functional, thereby proposing a role of NRG1 in signaling downstream of EDS1. The compromised HR upon agroinfiltration of *NRC1^{D481V}* in TRV:MEK2-inoculated plants indicates that in a linear signaling pathway NRC1 should be positioned above a MAP-kinase cascade. Indeed, in TRV:NRC1-infected plants the HR induced by both constitutively active LeMEK2 and LeMAPKKKα is not compromised (Figure 5A).

Possible Functions of NRC1 in HR Signaling

Our results indicate that NRC1 is a CC-NB-LRR protein required downstream in multiple HR signaling pathways. Its proposed downstream activity does not favor direct physical interaction with the Cf resistance proteins. Possibly, the protein is located at a convergence point and amplifies various R protein-specific HR signals. In mammals the regulator of apoptosis (a form of programmed cell death) is Apaf-1 (Apoptotic Protease Activating Factor 1) (Green, 2005). Apaf-1 belongs to the family of NOD (Nucleotide binding Oligomerization Domain) proteins (Inohara and Nunez, 2003) and contains an NB-ARC domain and WD-40 repeats (Jiang and Wang, 2000). Upon nucleotide binding NOD proteins are able to homo-oligomerize. In the case of Apaf-1, binding of cytochrome c, which is released from the mitochondria after perception of an apoptotic stimulus, and ATP induces oligomerization of the protein. Then caspase-9 is activated, followed by activation of executioner caspases, which eventually leads to programmed cell death (Green, 2005). Similarities between the structure of Apaf-1 and NB-LRR proteins allow speculations about similar

functions of these proteins in mammals and plants. Similar to NOD proteins, the NB-ARC domain of R proteins like I-2 and Mi is known to bind ATP after a conformational change upon ligand binding (Tameling et al., 2002). Possibly, also NB-LRRs oligomerize upon elicitor recognition and ATP binding. Indeed, recently N-oligomerization as a response to the p50 elicitor of TMV was reported (Mestre and Baulcombe, 2006). Maybe also in plants, components like cytochrome c interact with downstream NB-LRRs such as NRC1, thereby causing a conformational change, ATP binding and subsequent oligomerization. Finally, caspase-like proteins might become activated and execute cell death. Furthermore, downstream NB-LRRs might also function to form heterodimers with other NB-LRRs or R proteins. This could explain the convergence of several defense pathways and subsequent activation of a general downstream HR pathway.

METHODS

DNA Gel-Blot Analysis

Genomic DNA from *N. benthamiana* was isolated using the QIA-Gen DNA extraction protocol (Qiagen, Venlo, NL), whereas for tomato the standard protocol described by (Sambrook and Russell, 2001) was used. The DNA was digested with *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV or *Xba*I. The *N. benthamiana* gel-blot was hybridized with the ^{32}P-labeled (Prime-a-gene Labeling System, Promega, Madison, WI) 252 bp fragment present in the TRV:NRC1 vector and the DNA gel-blot of tomato was hybridized with a ^{32}P-labeled probe of 1293 bases corresponding to nucleotides 1876 to 3168 of the full-length NRC1 cDNA (Gabriëls et al., 2006). Sites for the restriction enzymes used are not present in the probes. Low stringency refers to washing at 55°C in 2x SSC and 0.5% SDS. High stringency conditions consist of washing at 65°C in 0.5x SSC and 0.5% SDS.

VIGS in tomato, HR and Disease Assays

For VIGS in tomato we used the pTRV-RNA1 and pTRV-RNA2 vectors described by Liu et al. (2002b). The *Cf-4* and *NRC1* fragments were excised from pTV00 by digestion with *Bam*H1/*Asp*718 and inserted into *Bam*H1/*Asp*718-digested pTRV-RNA2 (pYL156) (Liu et al., 2002a). To construct TRV:NRC1-UTR, part of the 3'-UTR of *NRC1* was amplified using primers 222-3'UTR-F (5'-GT<u>GGATCC</u>GCAGGTTCAACCAGCCTGGT-3'; *Bam*H1 site underlined) and 222-3'UTR-R (5'-GT<u>GGTACC</u>CAAGTGACTTGTTCTGCTGT-3'; *Asp*718 site underlined) and to construct TRV:NRC1-LRR, part of the *NRC1* region coding the LRRs was amplified using primers

222-LRR-F (5'-GT<u>GGATCC</u>GTTAAGAGGCTGCAATTTCT-3'; *Bam*H1 site underlined) and 222-LRR-R (5'-GT<u>GGTACC</u>GATCTTCTCAAGTTTATCAC-3'; *Asp*718 site underlined). The PCR fragments were *Bam*H1/*Asp*718-digested and inserted into *Bam*H1/*Asp*718-digested pTRV-RNA2. TRV:Prf construction has been described (Ekengren et al., 2003). All plasmids were transformed to *Agrobacterium tumefaciens* strain GV3101 by electroporation (Takken et al., 2000). To establish VIGS in tomato, cotyledons of ten- to twelve-day-old tomato seedlings were agroinfiltrated with a mixture of pTRV-RNA1 and the pTRV-RNA2-derived constructs (combined in a 1:1 ratio) (Liu et al., 2002a; Liu et al., 2002b). For each TRV construct either four *Cf-4*-containing tomato plants (Cf0 plants transformed with *Hcr9-4D* (*Cf-4*)) (Thomas et al., 1997), resistant to *C. fulvum* expressing *Avr4*, or four *Cf-9*-containing tomato plants (Cf0 plants transformed with *Hcr9-9C* (*Cf-9*)) (Jones et al., 1994), resistant to *C. fulvum* expressing *Avr9*, were used. As control Cf0 tomato plants (MM-Cf0), fully susceptible to *C. fulvum*, either TRV:00- or TRV:NRC1- inoculated were used. For disease assays, three weeks post TRV inoculation Cf0 and *Cf-4*-containing plants were inoculated with *C. fulvum* (De Wit, 1977). We used a *C. fulvum* race 5-*pGPD*::*GUS* (expressing *Avr4* and the β-glucuronidase gene under control of the constitutive Glyceraldehyde-3-Phosphate Dehydrogenase (*GPD)* promoter (Punt et al., 1988)). Colonization of the leaflets was assessed two weeks later by GUS staining. In parallel, leaflets of the second, third or fourth compound leaf were used for RT-PCR analysis to test for knock-down of the gene of interest (see below). For HR assays, leaflets of the third compound leaf of TRV-infected *Cf-4*- or *Cf-9*-containing plants were injected with Avr4 or Avr9, respectively. Both elicitors were injected into leaflets with a micro-syringe (Ito Corporation, Fuji, Japan). Avr4 was injected at a concentration of 10 μM, at ten sites per leaflet and four leaflets per plant. For Avr9, eight times diluted apoplastic fluid containing about 10μM of Avr9, isolated from a compatible interaction between race 5 of *C. fulvum* and Cf0 plants, was injected at eight sites per leaflet and four leaflets per plant. Resistance against *Pseudomonas syringae* pv. *tomato* was assayed in tomato RG-PtoR (*Pto/Pto, Prf/Prf*), inoculated with TRV:00, TRV:Prf or TRV:NRC1. The inoculation procedure and the determination of bacterial colonization of the leaves were performed as described previously (Ekengren et al., 2003)**.**

RT-PCRs

Four leaf discs (approximately 100 mg of fresh weight tissue in total) were collected from the second, third or fourth compound leaf of TRV-infected tomato plants. Total RNA was extracted using the QIA-Gen RNAeasy extraction protocol (Qiagen, Venlo, NL) and treated with RNase-Free

DNase (Bio-Rad, Veenendaal, NL). First strand cDNA was synthesized from 1 μg of total RNA using the Bio-Rad cDNA synthesis kit (Bio-Rad, Veenendaal, NL) and RT-PCR was performed using the following cycles: 95°C for 15 sec, 60°C for 45 sec and 72°C for 60 sec. The primers that were used (222F: 5'- TGAGGTATATTGCTTTCTCATCTGAC-3' and 222R: 5'- AGCTATTTTCCCACGGATGCCCAG-3') do not cover the fragment which is inserted in TRV:NRC1. Actin primers (ActinFnr182: 5'-TATGGAAACATTGTGCTCAGTGG-3' and ActinRnr183: 5'-CCAGATTCGTCATACTCTGCC-3') were used to check for the presence of equal amounts of cDNA in the PCR reactions.

VIGS in *N. benthamiana,* Agroinfiltration, HR and Disease Assays

Four-week-old *N. benthamiana* plants were agroinfiltrated with a 1:1 mixture of pTV00-derived constructs (binary TRV RNA2 vector) and pBintra6 (binary TRV RNA1 vector) (Ratcliff et al., 2001), or a 1:1 mixture of pTRV-RNA2-derived constructs and pTRV-RNA1 (Liu et al., 2002a; Liu et al., 2002b). We used the following TRV constructs: TRV:NRC1, TRV:Cf-4 (Gabriëls et al., 2006) and TRV:SGT1 (Peart et al., 2002b), all in the TRV vector described by Ratcliff et al. (2001) and TRV:EDS1, TRV:MEK2, TRV:RAR1 and TRV:NDR1 (Ekengren et al., 2003), all in the TRV vector described by Liu et al. (2002b). For the various TRV constructs in each experiment four plants were used. We agroinfiltrated *AvrPto* and *CP* in TRV-infected *N. benthamiana* expressing the resistance gene *Pto* (*N. benthamiana:Pto*, line 38-12 (Rommens et al., 1995)) (Pedley and Martin, 2003) and *Rx* (*N. benthamiana:Rx*, line Rx-18) (Bendahmane et al., 1999), respectively. In all other cases agroinfiltration was performed in *N. benthamiana* expressing the resistance gene *Cf-4* (*N. benthamiana:Cf-4*) (Gabriëls et al., 2006). Three weeks post TRV inoculation the third, fourth and fifth leaf above the inoculated leaves were challenged with *A. tumefaciens* that directs expression of *AvrPto* (OD_{600}=0.06) (Tang et al., 1996), *CP* (OD_{600}=0.12) (Bendahmane et al., 1999), *Avr4* (OD_{600}=0.03), *Cf-9* and *Avr9* (mixed in a 1:1 ratio, OD_{600}=0.2) (Van der Hoorn et al., 2000), *LeEix*2 and *tvEix* (mixed in a 1:1 ratio, OD_{600}=1.0) (Ron and Avni, 2004), the β-glucuronidase (*GUS*) gene (OD_{600}=2.0) (Van der Hoorn et al., 2000), *NRC1* and *p19* (mixed in a 1:1 ratio, OD_{600}=1.0) (Voinnet et al., 2003), the constitutively active $NRC1^{D481V}$ or the inactive $NRC1^{K191R/D481V}$ double mutant (OD_{600}=2.0), $LeMAPKKK\alpha^{KD}$, $LeMAPKKK\alpha^{KD-}$ (Del Pozo et al., 2004) (both at OD_{600}=0.12) or *LeMEK2DD* and *LeMEK2* (Del Pozo et al., 2004) (both at OD_{600}=0.25). Two days post infiltration of $LeMAPKKK\alpha^{KD}$, $LeMAPKKK\alpha^{KD-}$, *LeMEK2DD* or *LeMEK2* the leaves were sprayed with a 7.5 μM solution of 17-β-estradiol in water, containing silwet (4 μl/100 ml) (Del Pozo et al., 2004). For protein injections, Avr4-HIS-FLAG-tagged protein

was treated with enterokinase EK-max according to the manufacturer's recommendations (Invitrogen, Breda, NL) and 5 µM Avr4 protein in water, supplemented with 0.2% tween (v/v) was used for injections. Two to five days post agroinfiltration or protein injection the leaves were examined for the development of an HR, or assayed for β-glucuronidase (GUS) activity.

TMV:GFP- and PVX:GFP-based viral vectors were inoculated onto *N. benthamiana* plants either by *in vitro* RNA transcripts (TMV:GFP) or by agroinfiltration (PVX:GFP) (Donson et al., 1991; Jones et al., 1999). Systemically infected leaves were collected and homogenized in 0.1M phosphate buffer, pH 7 and the homogenate was used to inoculate *N. benthamiana:N* (line 310A (Peart et al., 2002a)) or *N. benthamiana:Rx* (line Rx-18 (Bendahmane et al., 1999), that had been inoculated with various recombinant TRV silencing constructs (Peart et al., 2002a). Two weeks post TMV:GFP or PVX:GFP inoculation putative systemically infected leaves were analyzed for GFP fluorescence using UV light illumination.

Binary *35S:NRC1* Vector Construction and Mutagenesis

Full-length *NRC1* cDNA was PCR-amplified using primers 222-Start-F (5'-GGGATCCATGGTTGATGTAGGGGTTGA-3') and 222-Stop-R (5'-GTCACTGCAGACCTTTCTAAGAAGCTGTCTG-3'), thereby introducing *Nco*I and *Pst*I restriction sites, respectively (restriction sites underlined). The PCR fragment was *Nco*I/*Pst*I-digested and inserted into *Nco*I/*Pst*I-digested pRH80 (Van der Hoorn et al., 2000). Subsequently, the construct was *Xba*I/*Kpn*I-digested and the resulting fragment containing the 35S promoter, the *NRC1* open reading frame and the NOS terminator (tNOS), was cloned into the *Xba*I/*Kpn*I-digested pMOG800 binary vector (Honée et al., 1998) to create plasmid NRC1(wt). To create constitutively active binary *NRC1^{D481V}*, the D481V mutation was introduced by overlap extension PCR (Higuchi et al., 1988) using the *NRC1wt* plasmid as a template and flanking primers 222-Start-F and 222-Stop-R and mismatch primers 222MHD-F (5'-CAAAACTTGTCGTGTTCATGTCATGTTGTATGAG-3') and 222MHD-R (5'-CCAGCAAAACTCATACAACATGACATGAACACGAC-3') (mutation underlined). The fragment was *Nco*I/*Pst*I-digested, inserted into pRH80 and the *35S-NRC1^{D481V}-tNOS* fragment was excised and subsequently inserted into pMOG800 as described above. In a similar way the P-loop mutant *NRC1^{K191R}* and the inactive double mutant *NRC1^{K191R/D481V}* were created. Here, the K191R mutation was introduced using mismatch primers 222Ploop-F (5'-GGAATGCCTGGTCTTGGCAGAACTACACTAGC-3') and 222Ploop-R (5'-GCTAGTGTAGTTCTGCCAAGACCAGGCATTCC-3') (mutation underlined) with respectively plasmid

NRC1(wt) and *NRC1^{D481V}* as a template. All constructs were sequence-verified and transformed to *A. tumefaciens* strain GV3101.

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CHAPTER 5

General Discussion

GENERAL DISCUSSION

The research described in this thesis focuses on the identification and functional analysis of tomato genes involved in resistance to *Cladosporium fulvum*. As a method to identify genes involved in resistance, a synchronized *Cf-4/Avr4* "dying seedling system" in combination with cDNA-AFLP analysis was employed. This allowed us to identify 442 differentially expressed transcripts correlated with the induction of plant defense. To investigate whether the corresponding Avr4-Responsive Tomato (*ART*) genes are indeed required for defense, we performed high-throughput functional analysis using Virus-Induced Gene Silencing (VIGS). 192 out of the 442 *ART* fragments were selected to examine the role of the corresponding genes in the establishment of an Hypersensitive Response (HR) upon VIGS in *Nicotiana benthamiana* (chapter 2)*.* In the mean time, VIGS in tomato was optimized and for the same set of *ART* fragments the function in resistance to *C. fulvum* was investigated. *ART* fragment 222 was found to be required for both *Cf-4*-mediated HR and resistance (chapters 2, 3 and 4). The gene corresponding to *ART* fragment 222 encodes an NB-LRR protein. We decided to focus on this NB-LRR-encoding gene and found that it is also involved in *LeEix2-, Pto-* and *Rx*-mediated HR. Therefore the encoded protein was designated NRC1*,* for NB-LRR Required for HR-associated Cell death 1. Overexpression of a constitutively active *NRC1* mutant results in an HR, which proved to be dependent on MEK2 (a MAPKK), RAR1 (Required for Mla12 resistance 1) and SGT1 (Suppressor of G2 allele 1) but independent of EDS1 (Enhanced Disease Susceptibility) and NDR1 (Non-specific Disease Resistance) (chapter 4)*.* In this chapter resistance mechanisms of plants and mammals, possibly related to defense pathways in which *NRC1* is involved, will be discussed.

Defense Mechanisms Operating in Plants

Resistance mechanisms in plants are composed of several defense layers. Passive non-host resistance is based on the presence of physical barriers like the cell wall, a waxy cuticle, or antimicrobial compounds. In addition, active non-host defense mechanisms are induced upon recognition of essential and conserved Pathogen-Associated Molecular Patterns (PAMPs) (Thordal-Christensen, 2003; Nürnberger et al., 2004). Receptors recognizing PAMPs are thought to be conserved and PAMP-induced resistance is likely to be broad and durable. Therefore, research on plant defense mechanisms related to recognition of PAMPs currently receives much attention. Research on non-host resistance mechanisms revealed that *Arabidopsis thaliana* knock-outs of *PEN1, PEN2* and/or *PEN3* are disabled in non-host resistance to *Pseudomonas syringe* pv.

phaseolicola and two powdery mildew fungi *Blumeria graminis* f. sp. *hordei* (*Bgh*) and *Erysiphe pisi,* that colonize grass and pea species, respectively. PEN1, PEN2 and/or PEN3 proved to be involved in inhibiting pathogen penetration (Nürnberger et al., 2004; Lipka et al., 2005). Further, it is known that *PEN1* encodes a membrane-associated syntaxin, possibly involved in membrane fusion and secretion events. *PEN2* encodes a glycosyl hydrolase localized to peroxisomes that accumulate at pathogen penetration sites. Recently it was reported that *PEN3* encodes an <u>A</u>TP <u>B</u>inding <u>C</u>assette (ABC)-transporter protein (Stein et al., 2006). PEN3 is localized at the plasma membrane and accumulates at the penetration site during pathogen infection. This indicates that pre-invasion defense barriers exist, which may be induced upon pathogen detection by PAMP receptors. Furthermore, the lipase-like EDS1 protein, and its sequence-related interaction partners PAD4 (<u>P</u>hyto<u>a</u>lexin <u>D</u>eficient 4) and SAG101 (<u>S</u>enescence-<u>A</u>ssociated <u>G</u>ene 101) are involved in non-host resistance (Feys et al., 2005). Since non-host resistance to *Bgh* is decreased in *eds1, pad4,* and *sag101* mutants, but the pathogen penetration rates are unaltered, it is suggested that unlike the PEN proteins which function in pre-invasion non-host resistance, the EDS1, PAD4 and SAG101 proteins function in post-invasion non-host resistance (Lipka et al., 2005).

In contrast to most general pathogen patterns (PAMPs), specific pathogen-derived elicitors, like the elicitin Inf1, which is a small elicitor protein from *Phytophthora infestans*, induce an HR on non-host plants like *N. benthamiana* (Kamoun et al., 1998). This HR-based non-host resistance is referred to as type II non-host resistance (Mysore and Ryu, 2004). *Phytophthora* species elicit an HR on a range of plants, suggesting these plants specifically recognize these elicitins. In an attempt to isolate an elicitin receptor, *Brassica rapa* lines were treated with elicitin from *Phytophthora cinnamomi* and subsequently divided into elicitin-responsive and non-responsive lines. Agroinfiltration of a constitutively active form of the MAPKK MEK4, showed a similar differential response. This indicates that the crucial protein(s) that determine the difference in responsiveness are downstream of the MAP-kinases and can not be an elicitin receptor. In addition, the lines that do respond with a macroscopic HR to elicitin and MEK4, also show faster disease development upon inoculation with the necrotrophic pathogen *Alternaria brassicola.* Probably, in responsive plants the presence of an enhancer, or the absence of a suppressor of cell death, acts downstream of the MAP-kinase pathway (Takemoto et al., 2005). We found NRC1 to be a positive regulator of multiple HR pathways. However, the enhancer of elicitin-induced cell death is not likely to be an NRC1 orthologue, since NRC1 proved to function upstream of a MAP-kinase pathway (chapter 4). Furthermore, NRC1 is required for both *Inf1-* and *Cf-4/Avr4*-induced HR, suggesting that this protein is involved in both type II non-host resistance and host-specific "gene-for-gene" based resistance (chapter 2).

Host-resistance is induced in plant cultivars containing resistance (R) proteins that (directly or indirectly) recognize the matching avirulence factor (Avr) of a pathogen. Direct interaction between a resistance protein and its matching Avr has been described for the rice resistance protein Pita with AVR-Pita from the rice blast fungus *Magnoporthe grisae* (Jia et al., 2000). For most other interactions it has been hypothesized that R proteins are able to monitor (guard) a modification of the virulence target (guardee) by the matching Avr (Van der Biezen and Jones, 1998). In the absence of the cognate R proteins, the avirulence proteins modify a virulence target, thereby suppressing resistance responses and facilitating growth of the pathogen.

Proteins Involved in Downstream Plant Defense Signaling

Several components, which can be either negative or positive regulators, of defense pathways have been identified already. Examples are calcium-dependent signal molecules, NDR1, EDS1, kinases and their substrates, RAR1 and proteins involved in the proteasome machinery, and even NB-LRR proteins.

Calcium-dependent signal molecules often contain calmodulin domains, enabling binding of Ca^{2+} and subsequent activation of downstream pathways. Indeed, Calcium-Dependent Protein Kinases (CDPKs) are induced in Avr4-challenged cell suspensions (Romeis et al., 2000) and based on silencing experiments a gene encoding a calmodulin-like protein proved to be required for resistance of tomato to *P. syringae* pv. *tomato* (Chiasson et al., 2005).

NDR1 encodes a small putative membrane protein and Arabidopsis *NDR1* mutants are susceptible to avirulent strains of *P. syringae* DC3000 and to *Hyaloperonospora parasitica* (Century et al., 1995; Century et al., 1997). In general, NDR1 is involved in CC-NB-LRR-triggered resistance pathways, while EDS1 is required for TIR-NB-LRR-mediated resistance (Aarts et al., 1998). Although there are some exceptions, like the CC-NB-LRR proteins Rx and Prf which do not require NDR1 (Peart et al., 2002a). Interestingly, the CC-NB-LRR protein NRC1 also appeared to be independent of NDR1 (chapter 4).

EDS1 is involved in both basal resistance and TIR-NB-LRR-mediated resistance (Aarts et al., 1998). In addition to EDS1 requirement by the Receptor Like Protein (RLP) Ve (conferring resistance to *Verticillium dahliae*) (Hu et al., 2005), EDS1 was also found to be required for resistance mediated by the RLP Cf-4 (chapter 4). These results indicate that EDS1 is involved in both TIR-NB-LRR- and RLP-mediated defense.

Several kinases required for defense signaling have been described, for example ACIK1 (Avr-Cf Induced Kinase 1), which is involved in *Cf*-mediated HR and resistance (Rowland et al., 2005).

Activation of kinases causes phosphorylation of downstream targets or substrates. A protein phosphorylated early in *Cf*-mediated defense is the plasmamembrane-localized syntaxin Ntsyp121 (Heese et al., 2005). Syntaxins form part of the SNARE complex required for the fusion of incoming transport vesicles and might thus facilitate membrane trafficking or signaling through the plasmamembrane. Another plasma-membrane localized syntaxin is PEN1. As mentioned earlier, mutations in *PEN1* increased the penetration frequency of *B. graminis* f. sp. *hordei* into the non-host Arabidopsis (Collins et al., 2003; Lipka et al., 2005). Interestingly, a yeast two-hybrid analysis of Cf-9 in *N. plumbaginifolia* resulted in the identification of a VAP27-encoding gene, which is also involved in SNARE complexes (Laurent et al., 2000). VIGS of genes encoding syntaxins or *VAP27* in tomato might reveal their function in pathogen resistance.

Several proteins related to ubiquitination and the proteasome machinery are involved in plant defense. Examples are components of the SCF (<u>S</u>kip1-<u>C</u>ullin-F box) ubiquitin ligase complex, like the <u>A</u>vr9-<u>C</u>f9-induced <u>F</u>-box protein (ACF) (Durrant et al., 2000) which is involved in *Cf*-mediated resistance to *C. fulvum* (H. van den Burg pers. comm.). Another example is SGT1, which was originally identified in yeast to interact with SKP1 (<u>S</u>-phase <u>K</u>inase associated <u>P</u>rotein 1) (Kitagawa et al., 1999). VIGS in *N. benthamiana* revealed that SGT1 is involved in both host- and non-host resistance to *P. syringae* pv. *maculicola* (which is a pathogen of Brassicaceae), and *Xanthomonas axonopodis* pv. *vesicatoria* (which is a pathogen of pepper) (Peart et al., 2002b). Proteins involved in ubiquitination and the proteasome machinery might regulate the degradation of negative regulators of defense, thereby activating defense responses (Liu et al., 2002; Suty et al., 2003).

HSP90 (<u>H</u>eat <u>S</u>hock <u>P</u>rotein 90), SGT1 and RAR1 seem to be involved in many R pathways. This indicates that these proteins function either downstream in a common signaling pathway, or that they function as chaperones or co-chaperones to interact with and stabilize R proteins. Indeed, HSP90 interacts with RAR1 and SGT1, which might function as co-chaperones to stabilize R proteins or R-protein complexes (Hubert et al., 2003; Takahashi et al., 2003; Liu et al., 2004). We found that HSP90 is also required for Cf-4/Avr4-induced HR. Since Cf-4 is an extracellular resistance protein it might be that the intracellularly located HSP90 stabilizes other, downstream signaling components.

Recently, the first NB-LRR protein located downstream of a resistance protein was identified and entitled NRG1 (<u>N</u> <u>R</u>equirement <u>G</u>ene 1), as VIGS of *NRG1* in *N. benthamiana* compromised *N*-mediated resistance to TMV (Peart et al., 2005). We found a similar type of NB-LRR protein, designated NRC1, which is involved in multiple HR pathways and *Cf-4*-mediated resistance to *C. fulvum.* Given the requirement for HSP90 by many NB-LRR resistance proteins, it is tempting to speculate that HSP90 may be involved in the stabilization of NRC1.

Defense Mechanisms Operating in Mammals

In mammals a set of pathogen recognition receptors recognize PAMPs from different classes of pathogens. This recognition is referred to as innate immunity, in contrast to adaptive immunity by which somatically generated, adaptive immune responses are delivered to the sites of infection (Inohara and Nunez, 2003; Jones and Takemoto, 2004). Plants do not have an adaptive immune system, but comparison of mammalian innate immunity with innate immune responses of plants might increase our understanding of the molecular mechanisms operating in plant defense as well as in mammalian immunity.

Mammalian pathogen recognition receptors are for example the plasma membrane Toll-Like Receptors (TLRs) which contain an intracellular TIR domain or the cytosolic NOD (Nucleotide-binding Oligomerization Domain) proteins (Figure 1). TLRs are glycoproteins located in the plasmamembrane or in intracellular membranes of compartments where the LRRs face the lumen (Bell et al., 2003). NOD proteins contain an N-terminal effector, a central NB and an LRR-ligand - binding domain, similar to intracellular plant NB-LRR proteins. NOD proteins are not only involved in pathogen-induced apoptosis (programmed cell death) but also in apoptosis induced by other stress responses. Upon stress, the ligand binding domains can bind to apoptosis-inducing ligands from the organism itself, or compounds derived from pathogens. For example, the ligand binding domains of mammalian TLR5 and NOD1 interact with flagellin and peptidoglycans from bacteria, respectively. Flagellin is a protein subunit of the bacterial flagellum, whereas peptidoglycans provide the shape and rigidity to bacteria. Both are conserved essential patterns and can be classified as PAMPs. Apaf-1 is a member of the NOD protein family and is a regulator of apoptosis. After perception of an apoptotic stimulus, cytochrome-c is released from the mitochondria and subsequently binds to Apaf-1 and stimulates binding of ATP and subsequent oligomerization of Apaf-1. The induced proximity due to oligomerization allows recruitment and activation of caspase-9, followed by activation of executioner caspases, finally leading to programmed cell death (Green, 2005). Similarities between the structure of Apaf-1 of mammals and NB-LRR proteins of plants allow us to speculate about the possibility of similar functions for these proteins in plants.

Possibly, also plant NB-LRR proteins oligomerize upon elicitor recognition. Indeed, transient expression of two differently tagged N proteins, followed by immunoprecipitation revealed that the two tagged forms of N co-immunoprecipitate in the presence of the P50 elicitor of TMV. This N-oligomerization is an early event in defense signaling since silencing of EDS1 and NRG1 has no effect on N-oligomerization. Furthermore, in SGT1-silenced plants the N protein was hardly detected, indicating that SGT1 plays a role in stabilization of this resistance protein (Mestre and Baulcombe, 2006). Similar to mammalian innate immune responses, oligomerization of the N protein might cause activation of caspase-like proteins and execution of cell death in plants. Furthermore, downstream NB-LRRs might form heterodimers with other NB-LRRs or R proteins. This could explain the convergence of several defense pathways and subsequent activation of a general downstream cell death pathway.
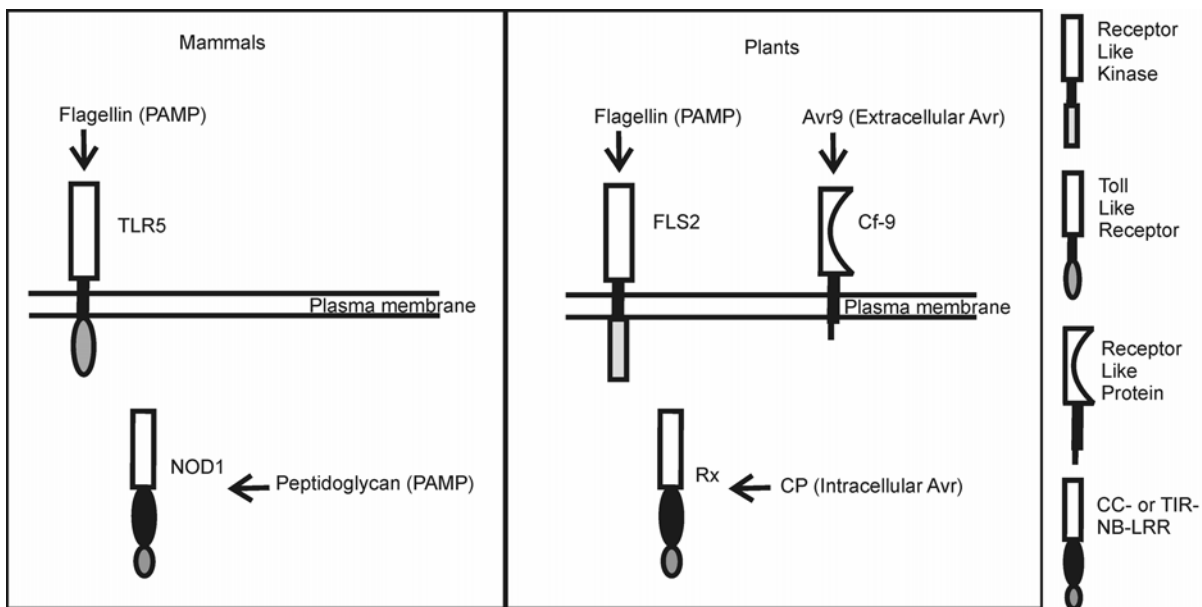
Figure 1. Schematic Representation of Receptors in Mammals and Plants (partly adapted from Jones and Takemoto (2004)).

The mammalian toll-like receptor TLR5 recognizes the PAMP flagellin. The mammalian NB-LRR protein NOD1 facilitates peptidoglycan recognition.

In plants the receptor-like kinase FLS2 induces non-host defense responses upon recognition of flagellin. The receptor-like protein Cf-9 induces host specific defense responses upon indirect recognition of the extracellular avirulence protein Avr9, while NB-LRR proteins (like Rx) induce these defense responses upon recognition of intracellular avirulence proteins like the Coat Protein (CP) from potato virus X.

VIGS in *N. benthamiana* and Tomato Reveals New Genes Involved in Plant Defense Signaling

Nowadays, identification of large sets of genes or proteins putatively involved in defense signaling pathways is possible using approaches based on transcriptomics or proteomics, respectively. However, irrespective of the approach, functional analysis is required to confirm the function of the corresponding genes or proteins *in vivo*. To determine whether the genes corresponding to the identified *ART* fragments are involved in resistance, 192 of them were silenced and scored for loss of HR and resistance using VIGS. VIGS in *N. benthamiana* proved to be very efficient, and *N. benthamiana* is suitable for agroinfiltration and subsequent scoring for decreased or increased HR. Although tomato cDNA fragments might not in all cases be sufficiently homologous to knock-down endogenous *N. benthamiana* genes by VIGS (Thomas et al., 2001), VIGS using 20 of the 192 *ART* fragments resulted in a decreased *Cf-4/Avr4-* or *Inf1*-induced HR, of which five strongly comprised this response. For these five *ART* fragments corresponding full-length cDNAs were isolated and two of the five *ARTs* encode an L19 ribosomal protein. The other three encode HSP90, a nuclear GTPase and a CC-NB-LRR protein, that we designated NRC1 (chapter 2). 26% of the 442 differentially expressed *ART* genes appeared to be down-regulated in *Cf-4/Avr4* compared to *Cf-4* control seedlings. Although among all 192 *ART* fragments used for VIGS, approximately 25% correspond to genes down-regulated in *Cf-4/Avr4,* an increased or spontaneous HR was never observed. Genes down-regulated during defense are possibly negative regulators of defense. Tight control of negative regulators of HR is important to prevent uncontrolled cell death. It might be possible that absence of a phenotype after silencing of negative regulators is due to redundancy, which is required for tightly regulated processes.

Although VIGS in tomato is less efficient, in the sense that it is less homogeneous than VIGS in *N. benthamiana,* it is essential to investigate the function of *ART* genes in resistance of tomato to *C. fulvum.* VIGS in tomato using *ART* transcripts with homology to (I) an unknown protein, (II) a histon H3 protein, (III) the NB-LRR protein designated NRC1, and (IV) a ribosomal protein L1 reproducibly resulted in loss of full resistance to *C. fulvum* (chapter 3). Quantification of growth in planta of a *C. fulvum pGPD*::*GUS* strain is hampered due to epiphytic growth before penetration. Inoculation with a *C. fulvum pAvr9::GUS* strain would enable detection of apoplastic growth, as the *Avr9* promoter is only induced *in planta*. Currently, inoculations are being performed with *C. fulvum pGPD::GFP,* enabling monitoring of the whole plant for growth of *C. fulvum,* using UV-light, and subsequent confirmation of intercellular fungal growth in silenced leaflets with confocal microscopy (J.H. Vossen, pers. comm.).

A disadvantage of VIGS is that it never results in a complete gene knock-out and it therefore may not be sufficiently efficient to compromise highly expressed genes (Rowland et al., 2005). Another implication of VIGS is the possibility that whole gene families with sufficient homology are knocked-down (Thomas et al., 2001). Thus, the function of one specific gene can only be studied by targeting particular downstream domains of a gene that are unique for the individual members of a gene family. Therefore, we performed VIGS of *NRC1* using fragments of the NB-domain, the LRR-domain and the 3'- UTR- domain (chapter 4), and found similar phenotypes.

In conclusion, VIGS is a high-throughput system that can be used as a pre-screen to identify genes required for HR and resistance. In future studies stable RNAi- or overexpressing transformants should provide more information on the function of the identified genes.

NRC1, A CC-NB-LRR-Encoding Gene With a Major Role in Plant Defense

VIGS in *N. benthamiana* and tomato identified *NRC1* as a key gene required for *Cf*-mediated HR and resistance. This is the first report of an NB-LRR protein required for *Cf*-mediated defense. Moreover, VIGS using *NRC1* revealed that it is also required for *LeEix2*-, *Rx*- and *Pto*-mediated defense, confirming its importance in different defense pathways. However, silencing of *NRC1* did not compromise resistance to TMV:GFP, PVX:GFP or *P. syringae* pv. *tomato* (chapter 4). It could be that resistance signaling pathways are more redundant as compared to HR signaling pathways, thereby minimizing the effect of knock-down of *NRC1*. In the case of resistance to PVX, absence of an effect of *NRC1*-silencing might be due to the fact that resistance to PVX is extreme and independent of an HR (Yu et al., 1998), and as a consequence also independent of NRC1. Another explanation might be that VIGS of *NRC1* was not sufficient to compromise resistance to these pathogens. In future experiments the generation of plants in which *NRC1* is stably knocked-down would facilitate a study of its effect and specificity on resistance to a range of pathogens.

Blast analysis on the Arabidopsis TAIR (The Arabidopsis Information Resource) database using different fragments of *NRC1* revealed putative Arabidopsis orthologs. Challenging Arabidopsis knock-outs for five of these individual genes with *H. parasitica* did not affect resistance to this pathogen (B. Thomma, pers. comm.), suggesting that they are not true Arabidopsis *NRC1* orthologs, or there is redundancy, or *NRC1* is not required for resistance to *H. parasitica.*

Transient expression of a constitutively active mutant of NRC1 under control of the 35S promoter resulted in an elicitor-independent HR in *N. benthamiana*. Epistasis experiments revealed that this NRC1-induced HR is dependent on MEK2, SGT1 and RAR1 but is downstream of EDS1 and independent of NDR1 (chapter 4).

Conclusions and Perspectives

Identifying *NRC1* as a gene required for *Cf*-mediated resistance fills a small gap in our understanding of this defense signaling pathway. We have attempted to position NRC1 in a known plant defense signaling pathway by performing epistasis experiments, which were limited to VIGS of the "defense components" *Cf-4, EDS1, NDR1, MEK2, RAR1* and *SGT1,* and allowed us to position NRC1 downstream of Cf-4 and EDS1 and upstream of MEK2. RAR1 and SGT1 proved to be required for overall functionality of the various signaling components. In the future these experiments could be extended by silencing additional components such as ACIK1 (Peart et al., 2002a; Rowland et al., 2005).

Identification of genes in defense pathways is crucial as it will reveal components required for HR-induction and resistance. In addition, these genes could be applied in resistance breeding to obtain durable resistance. First of all, genes downstream of the *R* genes itself will be less affected by adaptation of pathogens with respect to their *Avr* genes. Furthermore, genes involved in defense signaling might be required for resistance to multiple pathogens. Yeast two-hybrid analysis might reveal proteins interacting with and/or stabilizing NRC1. Identification of these proteins most likely leads to the identification of new genes involved in multiple defense pathways. Transcriptomics comparing expression of *NRC1* stable knock-downs with wild-type *NRC1* control plants will identify genes that are down- or up-regulated due to the absence or presence of *NRC1*. To engineer disease resistant plants, positive regulators of defense, like *NRC1,* might be expressed under control of a pathogen-inducible promoter. Subsequently, attack by pathogens would trigger *NRC1* gene expression thereby inducing resistance to multiple (hemi)-biotrophic pathogens. Contrary to *NRC1*-expression, knock-down of *NRC1* might abolish the induction of necrosis which could subsequently result in resistance to necrotrophic pathogens.

Furthermore, genes down-regulated in the *Cf-4/Avr4* seedlings as compared to *Cf-4* and *Cf0/Avr4* control seedlings might be screened for increased resistance to various pathogens upon VIGS in susceptible tomato plants. Increased resistance suggests silencing of a negative regulator of defense. Subsequently, plants with stable knock-downs or knock-outs of such a gene could be generated and would be expected to provide resistance to specific or even a broad range of pathogens.

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SUMMARY

Plant diseases caused by pathogenic micro-organisms can result in severe crop losses and are a threat for global food and feed production and quality. Resistance breeding based on the introduction of a single dominant resistance gene is often not durable, as under selection pressure pathogens are able to overcome resistance by circumventing recognition by the host. After perception of a pathogen by a plant many different defense signaling pathways are activated, which are likely more difficult to avoid by the pathogen than the initial recognition event. Furthermore, these downstream responses are probably conserved among different plant species and are effective against different pathogens. The goal of the present study is to employ the tomato-*Cladosporium fulvum* interaction as a model to identify genes involved in defense signaling pathways. Identification of these genes increases our understanding of cellular responses of plants resistant to pathogens. This knowledge should eventually be exploited to develop durable resistant plants, either by marker-assisted breeding or by transgenesis.

C. fulvum is a foliar, biotrophic fungal pathogen which causes leaf mold disease of tomato. In resistant tomato plants, within one to two days post penetration a microscopic Hypersensitive Response (HR) is observed preventing further growth of the biotrophic fungus. Tomato is the only host for *C. fulvum* and recognition of specific avirulence (Avr) factors encoded by *Avr* genes of the fungus is mediated by matching resistance proteins encoded by *Cf* genes of the plant, following the gene-for-gene model. Besides this host-specific and gene-for-gene-based resistance, mechanisms providing passive- and active- non-host resistance and some of the downstream defense responses are presented in a short overview **(chapter 1).**

To identify genes required for *Cf/Avr*-induced defense pathways, we used a system that allows synchronized induction of defense responses by one *Cf/Avr* gene pair without the interference of additional fungal genes or proteins, referred to as the "dying seedling system". Therefore, a near-isogenic tomato line carrying *Cf-4* was crossed to tomato transgenic for the *Avr4* gene (and lacking functional *Cf* genes), resulting in offspring carrying the *Cf-4/Avr4* gene pair. When such plants are grown at 33ºC the HR is suppressed. Subsequent shifting these plants to room temperature results in a synchronized HR. RNA was isolated from *Cf-4* controls and *Cf-4/Avr4* seedlings at 0, 30, 60 and 90 minutes after the temperature shift and subsequently cDNA-AFLP analysis was performed. This resulted in 442 differentially expressed cDNA-AFLP fragments corresponding to genes putatively involved in plant defense, designated Avr4-Responsive Tomato

(*ART*) genes. To determine the requirement of the corresponding genes for plant defense <u>V</u>irus-<u>I</u>nduced <u>G</u>ene <u>S</u>ilencing (VIGS), a technique that allows generating fast transient individual "knock-downs" of relatively large sets of genes, was used. We first generated knock-downs for a set of 192 selected *ART* fragments in *Nicotiana benthamiana* transgenic for *Cf-4*. VIGS of *ART* genes encoding a heat shock protein 90, a nuclear GTPase, an L19 ribosomal protein and, most interestingly, an NB-LRR protein clearly affected both *Cf-4/Avr4-* and *Inf1*-induced HR. The latter is an elicitor of *Phytophthora infestans* on its non-host *N. benthamiana*. Since VIGS using the *ART* fragment encoding the NB-LRR protein affected both the *Cf-4/Avr4* and *Inf1*-induced HR in *N. benthamiana,* the protein was designated NRC1 (<u>N</u>B-LRR <u>R</u>equired for HR-associated <u>C</u>ell death 1) **(chapter 2)**.

Chapter 3 describes the high-throughput functional analysis using VIGS in tomato to score for genes required for resistance to *C. fulvum.* VIGS was optimized in tomato using a fragment of the phytoene desaturase (*PDS*) gene. Successful silencing of *PDS* is visible as white tissue, thus providing a visual marker for silencing. Subsequently, VIGS of the resistance gene *Cf-4* and a gene required for *Cf-2*-mediated resistance, *Rcr3* was performed and in addition, a method to sensitively detect growth of *C. fulvum* in tomato leaves was optimized. Finally, we performed VIGS using the 192 selected *ART* fragments followed by inoculation with the fungus. VIGS using four of the selected 192 *ART* fragments, encoding a histon H3 protein, a ribosomal protein L1, an unknown protein and the NB-LRR protein, resulted in decreased resistance of tomato to *C. fulvum.*

The research described in **chapter 4** is focused on the *NRC1* gene, which is the first NB-LRR encoding gene required for *Cf*-mediated defense. Although *NRC1* proved to be required for multiple HR pathways, silencing of the gene only affected resistance to *C. fulvum*. Transient expression of a constitutively active mutant of NRC1 resulted in an elicitor-independent HR. Epistasis experiments revealed that the NRC1-induced HR is dependent on MEK2, SGT1 and RAR1, but independent of NDR1. It is hypothesized that NRC1, required for multiple defense pathways and exerting its activity downstream of EDS1 and upstream of MEK2, is a key regulator of defense.

In **chapter 5** different mechanisms generating resistance to pathogens, that operate in plants and in mammals, are discussed. Knowledge of defense pathways and the identification of the genes involved might reveal components required for resistance to many different pathogens.

SAMENVATTING

Plantenziekten veroorzaakt door pathogene micro-organismen kunnen resulteren in substantiële schade aan gewassen en zo een bedreiging vormen voor de produktie en kwaliteit van voedsel en veevoer. Resistentieveredeling gebaseerd op het introduceren van een enkel dominant resistentiegen is vaak niet duurzaam. Onder selectiedruk kunnen pathogenen namelijk herkenning door hun waardplant omzeilen en zo resistentie doorbreken. Het mechanisme waarmee een plant een pathogeen herkent is specifiek. Zodra de plant een pathogeen herkent worden verschillende afweerreacties geactiveerd, die uiteindelijk leiden tot resistentie. Waarschijnlijk zijn delen van deze afweerreacties geconserveerd bij verschillende plantensoorten en werkzaam tegen verschillende pathogenen. In het hier beschreven onderzoek wordt de interactie tussen tomaat en de pathogene schimmel *Cladosporium fulvum* gebruikt als een model om plantengenen betrokken bij afweerreacties te identificeren. Uiteindelijk proberen we langs deze weg afweerreacties beter te begrijpen en het verkregen inzicht te gebruiken om duurzaam-resistente planten te ontwikkelen, hetzij middels merkergestuurde veredeling, hetzij middels het maken van transgene planten.

C. fulvum is een schimmel die in levend bladweefsel van tomaat groeit en de bladvlekkenziekte veroorzaakt. In resistente planten is binnen één tot twee dagen na penetratie van de schimmel een microscopische overgevoeligheidsreactie te zien, die bestaat uit een vorm van geprogrammeerde celdood. Deze lokale celdood, welke gepaard gaat met diverse andere afweerreacties, voorkomt verdere groei van de biotrofe schimmel. Tomaat is de enige waardplant voor *C. fulvum*. Sommige genotypen van tomaat missen de juiste *Cf*-resistentiegenen en zijn niet in staat om de door *C. fulvum* geproduceerde avirulentie (Avr) eiwitten te herkennen, waardoor ze vatbaar zijn voor deze schimmel. De tomatenplanten die door de aanwezigheid van *Cf*-resistentiegenen *C. fulvum* met de corresponderende avirulentiegenen wel kunnen herkennen zijn resistent. Dit resistentiemechanisme is gebaseerd op het zogenaamde "gen-om-gen" model. Naast deze waardplant-specifieke, "gen-om-gen"-gebaseerde resistentie worden ook de zogenaamde passieve- en actieve "niet waardplant-specifieke" afweerreacties in **hoofdstuk 1** kort besproken.

Om de genen betrokken bij de afweerreacties van tomaat, geïnduceerd na herkenning van de door *C. fulvum* geproduceerde Avr eiwitten te identificeren, is een zogenaamd "doodgaande zaailingen" systeem gebruikt. Hiervoor zijn tomatenplanten met het resistentiegen *Cf-4* gekruist met transgene tomaten die het avirulentiegen *Avr4* van *C. fulvum* tot expressie brengen, resulterend in nakomelingen met zowel het *Cf-4* resistentiegen als het corresponderende *Avr4* avirulentiegen.

Deze nakomelingen overleven bij 33°C, een temperatuur waarbij de overgevoeligheidsreactie wordt onderdrukt. Dit betekent dat de overgevoeligheidsreactie synchroon en systemisch geactiveerd kan worden door deze zaailingen op te laten groeien bij 33°C en vervolgens te verplaatsen naar kamertemperatuur. Om veranderingen in genexpressie te bestuderen is RNA geïsoleerd, van zowel de *Cf-4* controle lijnen als van de *Cf-4/Avr4* nakomelingen op 0, 30, 60 en 90 minuten na het overplaatsen van de planten van 33°C naar kamertemperatuur, dat vervolgens werd gebruikt voor cDNA-AFLP analyse. Op deze manier zijn 442 verschillende cDNA-AFLP fragmenten geïdentificeerd corresponderend met genen die mogelijk betrokken zijn bij de afweer van de plant. Deze genen werden Avr4-Responderende-Tomaat (*ART*) genen genoemd. Om te bepalen of deze genen ook werkelijk noodzakelijk zijn voor de overgevoeligheidsreactie en/of resistentie is Virus-Geïnduceerde Gen Silencing (VIGS) gebruikt, een techniek waarmee de expressie van een bepaald gen snel en vrijwel geheel onderdrukt kan worden. In eerste instantie is de expressie van genen corresponderend met 192 van de geïdentificeerde cDNA-AFLP fragmenten onderdrukt in tabaksplanten (*Nicotiana benthamiana*) die getransformeerd zijn met het resistentiegen *Cf-4.* Vervolgens is de reactie van deze planten op Avr4 getest door middel van agroinfiltratie van het *Avr4* gen. Bovendien is de reactie op Inf1, een elicitor van *Phytophthora infestans* bekeken. Het onderdrukken van de expressie van genen coderend voor een "heat shock eiwit", een "nucleair GTPase", een "L19 ribosomaal eiwit" en een erg interessant NB-LRR eiwit, onderdrukte de overgevoeligheidsreactie geïnduceerd door agroinfiltratie van *Avr4* en *Inf1*. Aangezien VIGS van het *ART* fragment coderend voor het NB-LRR eiwit in *N. benthamiana* de *Cf-4/Avr4* en *Inf1*-geïnduceerde overgevoeligheidsreactie onderdrukte, is dit gen *NRC1* (NB-LRR nodig (required) voor de overgevoeligheidsreactie-geïnduceerde celdood 1) genoemd (**hoofdstuk 2**).

In **Hoofdstuk 3** is beschreven hoe, met behulp van VIGS, de expressie van genen overeenkomend met de bovengenoemde 192 cDNA-AFLP fragmenten onderdrukt kon worden in tomaat. Eerst is VIGS in tomaat geoptimaliseerd door de expressie van het phytoeen desaturase (*PDS*) gen te onderdrukken. Het succesvol onderdrukken van de *PDS*-genexpressie is zichtbaar in de vorm van verbleking van het bladweefsel. Vervolgens is de expressie van het resistentiegen *Cf-4,* en het gen, dat naast *Cf-2* nodig is voor herkenning van Avr2 van *C. fulvum, Rcr3,* onderdrukt. Daarnaast is er een zeer gevoelige methode ontwikkeld om de groei van *C. fulvum* in tomatenbladeren te detecteren. Uiteindelijk zijn de 192 geselecteerde *ART* fragmenten gebruikt voor VIGS in tomaat, om daarna deze tomatenplanten te inoculeren met *C. fulvum*. VIGS met vier van de 192 *ART* fragmenten (coderend voor een histon H3 eiwit, een L1 ribosomaal eiwit, een onbekend eiwit en weer het NB-LRR eiwit) resulteerde in verminderde resistentie van tomaat tegen *C. fulvum*.

Hoofdstuk 4 handelt hoofdzakelijk over het *NRC1* gen, aangezien dit het eerste NB-LRR-coderende gen is dat nodig is voor resistentie tegen *C. fulvum* en bovendien betrokken lijkt te zijn bij de overgevoeligheidsreactie geïnduceerd door elicitoren van verschillende pathogenen. Overexpressie van een constitutief actieve mutant van NRC1 resulteerde in een elicitor-onafhankelijke overgevoeligheidsreactie, hetgeen mogelijkheden bood tot het doen van "epistase" experimenten. Deze experimenten lieten zien dat de NRC1-geïnduceerde overgevoeligheidsreactie afhankelijk is van MEK2, SGT1 en RAR1, maar onafhankelijk van NDR1. Één van de mogelijke conclusies uit al deze experimenten is dat NRC1 een sleutelrol speelt bij verschillende afweerreacties en "downstream" van EDS1 en "upstream" van MEK2 functioneert.

In **hoofdstuk 5** worden de verschillende afweerreacties in planten en zoogdieren bediscussieerd. Inzicht in afweerreacties en de identificatie van genen betrokken bij deze reacties zou kunnen leiden tot de ontdekking van componenten die nodig zijn voor resistentie tegen verschillende pathogenen.

DANKWOORD

nodig had, voor het meedenken en voor de mooie Unifarm-sfeer! Naast het werk in de kas en in het lab vond ik het altijd erg leuk om naar symposia en congressen te gaan. Ali, maar ook Hans en Ria bedankt dat ik altijd binnen kon lopen voor hulp bij het papierwerk en de financiën.

Aan het begin van mijn AIO-periode deelde ik de kamer met Rianne en zag al van een afstandje hoe het is om met "de laatste loodjes" bezig te zijn. In het lab zat ik naast Bas, die mij *Cladosporium* inoculaties leerde of grapjes maakte als ik met een rood hoofd en een bril vol A. tum spetters terugkwam uit de kas. Helaas verlieten Camiel, Rianne, Nienke en later ook Bas het Clado-lab. Gelukkig bleven John, Renier, Marco en Iris. Een paar dagen voor het Gordon Conference met John en Renier kamperen, met z'n drieën in één tent, was erg geslaagd (en zoals John zou zeggen "don't make any assumptions"...). John, jij had altijd allerlei praktische tips, niet alleen op het lab, maar ook voor bijvoorbeeld de geocache opdracht. Erg gaaf om zo een echte schat op La Gomera te vinden! Renier, als kamergenoot kon ik je altijd alles vragen. Ondanks jou advies om te focussen wilde ik toch graag die grote VIGS-screen in tomaat doen. Toen er later echt keuzes gemaakt moesten worden, heb ik je gelukkig nog vaak om raad kunnen vragen. Bedankt! Op het moment dat Ralf zijn eerste afstudeervak bij mij begon werd ik zelf opeens diegene die adviezen mocht geven. Ralf, bedankt voor de leuke samenwerking en al je hulp bij de agroinfiltraties in *Nicotiana benthamiana* (beschreven in hoofdstuk 2). Marco, ik volgde jou op als jongste "Clado-AIO" en al vanaf het begin had jij altijd allerlei tips voor mij. Jij bent iemand die anderen altijd helpt en de gezelligheid erin houdt, of het nu is door te praten of te zingen. Ik vond het echt heel leuk om samen met jou en Mieke, en later ook met Peter vB de fyto-weekenden te organiseren. Leuke weekenden waarin veel gelachen werd met onder andere Rob, Jos, Wilco, Martijn, Rays, Maita, Sander en Corrie in Zuid-Limburg, of in het donkere bos met de "Witte Wieven", maar later ook tijdens de karaoke avond met onder andere Peter vE, Pieter, Klaas, Yaite, Iris, Emily, Ursula en Irene. De maandag erna werden alle gebeurtenissen als de mooiste verhalen verteld. Ik zal het missen!

Halverwege mijn AIO-periode kwam niet alleen Jack onze groep versterken. Bart begon als postdoc al snel zijn eigen groepje. Met nieuwe projecten en nieuwe AIO's en postdocs, zoals Peter vE, Ursula, Melvin, Emily en Ioannis, zorgde hij ervoor dat Iris en ik niet meer de jongste Clado-AIO's waren. Bart, bij jou kon (en kan) ik altijd langs komen voor adviezen. Heel erg bedankt voor je luisterend oor en je goede suggesties. Verder kwam Grardy ons versterken. Ze zeggen wel eens "moeders zijn de beste managers". Grardy is daar het bewijs van. Zij doet proeven voor drie verschillende mensen, alle cloneringen in die grote TRV vector lukken haar en naast alle proeven regelt zij alles op en voor het lab. Grardy, dankjewel voor de leuke samenwerking!

Waar ik ook achter ben gekomen is dat we in plaats van keuzes maken liever beide opties tegelijk doen, oftewel "op 1 gen focussen" en tegelijk "met alle 192 doorgaan". En dat werd

mogelijk toen Ahmed ons kwam versterken. Ahmed, als jonge enthousiaste analist zocht jij alles tot in de puntjes uit, vroeg ons "het hemd van het lijf" en tijdens het agroinfiltreren of clado-inoculeren in de kas hielden we hele discussies. Heel erg bedankt voor je goede vragen, enthousiasme, leuke samenwerking en natuurlijk voor alle RT-PCRs!

Een extra ervaring binnen dit AIO project was de samenwerking met Keygene. Het was even wennen om alle posters en abstracts etcetera goed te laten keuren, maar ik vond het een leuke uitdaging om mijn werk ieder half jaar te mogen presenteren aan de projectcommissie bij Keygene. Bij deze wil ik dan ook alle mensen van de BSP betrokken bij dit "Cladosporium-project" bedanken voor alle input. Verder wil ik ook alle mensen van Keygene zoals onder andere Lianne, Gert-Jan, Jenny, Antoine maar vooral ook Ludvik en Hanneke bedanken voor de samenwerking. Gelukkig zijn al onze agroinfiltraties niet voor niets geweest en is hoofdstuk twee nu geaccepteerd voor publicatie in MPMI!

Frank, jij hebt mij tijdens mijn eerste afstudeervak de beginselen van de moleculaire fytopathologie geleerd en mij er enthousiast voor gemaakt. Ik vind het mooi dat ik ook tijdens andere afstudeervakken, tijdens mijn AIO periode en eigenlijk nu nog steeds bij jou terecht kan voor goede en simpele oplossingen. Dankjewel!

Ik zal de akker van fytopathologie missen. Het was mooi, gezellig en vrij op deze, voor mij nu zo vertrouwde akker. Natuurlijk vind ik het jammer dit land te verlaten, maar ik ga niet ver weg en hoop nog af en toe op bezoek te mogen komen.

En nu...nu komt een van Jos z'n voorspellingen uit...ik ga in het bedrijfsleven werken. Nu liggen er nieuwe akkers voor me. Sommige hebben stukken land die overlappen met de voor mij vertrouwde fyto-akker, andere zijn nog nieuw en vol geheimen voor mij. Ik vind het een uitdaging om mijn ervaringen met die van nieuwe collega's te delen en zo de geheimen van de Keygene-akkers te ontrafelen en naar een goede oogst toe te werken. Gelukkig hoef ik dit niet alleen te doen. Michiel, graag wil ik je bedanken voor al je kritische vragen die in eerste instantie de structuur van "mijn" fyto-akker verstoorden, waarna we deze, mede dankzij jouw input en leuke discussies gelukkig weer goed konden herstellen.

Gelukkig weet ik dat ik naast collega's altijd op familie en vrienden kan rekenen. Iris en Judy, jullie ken ik al 9 en 11 jaar. We hebben samen bij muziekvereniging "De Ontzetting" gespeeld, en in commissies en/of bestuur gezeten. Iris was natuurlijk sinds september 2002 ook collega en kamergenoot. Dat betekende dat we alle positieve en negatieve resultaten, presentaties, etcetera konden bespreken, meestal eindigend met de conclusie "het komt wel goed". En zo is het! Judy, tijdens de wandelingen met jou en het groepje "oud-ontzetters" kon ik altijd mijn verhaal kwijt. Deze wandelingen, de weekendjes met de CAID, maar ook de repetities met "De Ontzetting" en de

basketbalwedstrijden waren altijd een leuke afleiding en gaven me nieuwe energie om verder te gaan.

Natuurlijk wil ik ook mijn (schoon)familie bedanken. Jan, Gerd en Pieter, heel erg bedankt voor alle gezelligheid en belangstelling. Pap en mam, maar ook Jeroen en Miriam, jullie leven altijd met me mee en ik kijk altijd weer uit naar een vertrouwd en gezellig weekend "in os Rooy". Pap en mam, het geeft een goed gevoel dat, wat er ook gebeurt, waar we ook zijn, jullie altijd achter ons staan. "Meer als je best kun je niet doen", en "de aanhouder wint" zeggen jullie altijd. Mede dankzij jullie steun en met deze motto's in het achterhoofd is het dan eindelijk gelukt en is mijn boekje af. Eine dikke Merci!

Lieve Maarten, natuurlijk kom jij in het dankwoord, en niet alleen voor al het werk aan de lay-out, ook voor je steun, de momenten waarop je mij motiveerde als het even tegen zat, je relativerend vermogen en voor het moois dat we altijd op elkaar kunnen rekenen! En nu...nu is het boekje echt af, nu verheug ik me op spannende en ontspannende wandelingen in Wageningen, de Veluwe, Limburg, de Ardennen, de Pyreneeën...waar ook ter wereld, maar samen met jou!

Susan

CURRICULUM VITAE

Suzan Gabriëls werd geboren op 13 september 1977 te Weert en groeide op in het naburig gelegen Stramproy. In 1995 behaalde zij haar VWO diploma aan het Bisschoppelijk College te Weert. Hetzelfde jaar begon ze aan de opleiding Plantenveredeling en Gewasbescherming aan de Wageningen Universiteit. In 1998 mocht ze met vijf andere studenten de algemene introductie dagen organiseren. Na nog een jaar vakken volgen begon ze in 1999 aan een afstudeervak moleculaire fytopathologie aan de Wageningen Universiteit. In 2000 ging ze naar Australië voor een afstudeervak moleculaire virologie aan de Queensland University in samenwerking met het Queensland Agricultural Biotechnology Centre. Terug in Wageningen deed ze nog een afstudeervak moleculaire plantenveredeling bij Plant Research International waarna ze in september 2001 haar studie *cum laude* kon afronden.

In september 2001 begon Suzan aan haar promotieonderzoek bij de vakgroep Fytopathologie aan de Wageningen Universiteit, onder leiding van prof. dr. ir. Pierre de Wit en dr. ir. Matthieu Joosten. Dit promotieonderzoek werd uitgevoerd in samenwerking met Keygene, en werd gefinancierd door de onderzoekschool EPS. Vanaf januari 2006 heeft zij een aanstelling als toegevoegd onderzoeker bij Keygene.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School
EXPERIMENTAL PLANT SCIENCES
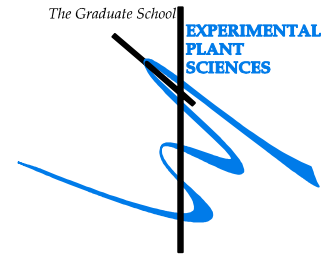
Issued to: Suzan Gabriëls
Date: 15 May 2006
Group: Laboratory of Phytopathology, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Functional genomics as a tool to identify key genes mediating the hypersensitive response	07 Dec 2001
Subtotal Start-up Phase	1.5 credits*

2) Scientific Exposure	*date*
► **EPS PhD student days**	2002 through 2005
► **EPS theme symposia**	2001 through 2005
► **NWO Lunteren days and other National Platforms**	
Symposia Lunteren (2-days meetings)	2001 through 2005
Genomics meetings (2x 1-day)	2001
WCS days (1-day)	2003 and 2004
Geneyous symposia	Jan 2004
► **Seminars (series), workshops and symposia**	
Workshop Programmed Cell Death (1-day)	Dec 2002
Seminars "Frontiers in Plant Development" (2x)	2002
Flying seminars (2x)	2003
Symposium on Signal Transduction	18 Mar 2003
► **International symposia and congresses**	
Gordon Research Conference "Plant Molecular Biology", Plymouth, New Hampshire (USA)	07-12 Jul 2002
XI International Congress on "Molecular Plant-Microbe Interactions", St. Petersburg (Russia)	18-26 Jul 2003
XII International Congress on "Molecular Plant-Microbe Interactions", Mérida (Mexico)	14-19 Dec 2005
► **Presentations**	
Several poster presentations from in total 3 different posters	2002 through 2005
Presentation at the Autumn School "Disease Resistance in Plants"	Oct 2002
Presentation at the EPS PhD Student Day 2003	Mar 2003
Presentation at Lunteren 2003	Apr 2003
Presentation at XI International Congress on Molecular Plant-Microbe Interactions 2003	Jul 2003
Presentation at the Summer school "Environmental Signaling: Arabidopsis as a model"	Aug 2005
► **IAB interview**	04 Jun 2004
Subtotal Scientific Exposure	21.6 credits*

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Autumn School "Disease Resistance in Plants"	14-16 Oct 2002
Summer School "Functional Genomics"	25-28 Aug 2003
Bioinformation Technology-1	8-16 Nov 2004
Summer school "Environmental Signaling: Arabidopsis as a model	22-24 Aug 2005
► **Individual research training**	
Subtotal In-Depth Studies	4.2 credits*

4) Personal development	*date*
► **Skill training courses**	
Endnote	2001
Scientific Art Work	2002
Career Orientation	2004
Techniques for writing and presenting a scientific paper	22-15 Nov 2004
► **Organization of PhD students day, course or conference**	
Organization of PhD student day	03 Jun 2004
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	5.1 credits*

TOTAL NUMBER OF CREDIT POINTS*	**32.4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

* A credit represents a normative study load of 28 hours of study

Front cover:

Silencing of *PDS* in tomato causes photobleaching of fruits.

Back cover:

Cladosporium fulvum-inoculated plants stained with trypan blue (first row).

Response of Cf9 tomato plants upon injection of apoplastic fluid (second row).