DETERMINANTS OF NONHOST RESISTANCE TO PHYTOPHTHORA INFESTANS

DISSETATION

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ABSTRACT

Phytophthora infestans, a plant pathogenic oomycete, causes late blight on potato and tomato resulting in devastating economic losses each year. During infection, P. infestans secretes a diverse set of effector molecules that aim to reprogram the host into a susceptible state. Recognition of effector molecules however, leads to induction of the hypersensitive response (HR) a form of programmed cell death and resistance. Interestingly, both resistant host and nonhost plants display localized HR upon P. infestans ingress, indicating mechanistic similarities or overlap between resistance types. In this dissertation, I investigated the molecular determinants of nonhost resistance and cell death in plants. In order to characterize nonhost resistance, we explored Arabidopsis thaliana-P. infestans associations as a model system. Cytological investigations as well as gene expression analysis revealed that activation of HR and induction of defense responses occurs during P. infestans infection. I investigated the role of P. infestans effector proteins in nonhost resistance and cell death. I showed that INF2A and INF2B induced HR-like cell death in Nicotiana and that INF2A-induced cell death requires the ubiquitin-ligase associated protein SGT1. Consequently, I proposed a role for these genes in avirulence or incompatibility
in *Nicotiana*. I investigated the involvement of cell death in *P. infestans*-host interactions. I characterized PINPP1.1 and the CRN family and speculated that they represent classes of virulence genes. Comparisons between PINPP1.1, CRN2 and INF1 induced cell death revealed that at least two multiple signaling pathways exist. These differences could reflect functional roles for the CRNs and PINPP1.1 during pathogenesis. Overall I conclude that *P. infestans*-plant associations are characterized by extensive cellular reprogramming. Characterizations of *P. infestans* effector genes and understanding the perturbations they facilitate in plants will greatly enhance our understanding of *P. infestans* virulence and host specificity.
Dedicated to Jorunn
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CHAPTER 1

1.1 INTRODUCTION

The oomycetes include some of the most devastating pathogens of plants (Erwin and Ribeiro, 1996). Despite their filamentous growth habit, the oomycetes represent a eukaryotic lineage that is distinct from true fungi. Early biochemical as well as recent DNA sequence analysis indicate relatedness to brown algae and diatoms (Stramenopiles) rather than fungi (Baldauf et al., 2000). Consequently, plant parasitic oomycetes are thought to have acquired pathogenicity mechanisms independently from fungi (Kamoun, 2001; 2003; Huitema et al., 2004).

*Phytophthora infestans*, a plant pathogenic oomycete, causes late blight on potato and tomato resulting in devastating economic losses each year (Erwin and Ribeiro, 1996). The infection cycle starts when motile zoospores splash onto plant surfaces, encyst and penetrate their substrate through the formation of a penetration peg (Hardham et al., 2001; van West et al., 2002). Penetration is aided by the formation of an appressorium and occurs into cells directly.
Pathogen ingress is followed by rapid intercellular hyphal growth and colonization of apoplastic spaces, occasionally forming intracellular feeding structures (haustoria) (Hardham et al., 2001). In the late stages of infection, profuse sporulation occurs when sporangiophores baring sporangia are formed. These sporangia release zoospores under wet and low temperature conditions or penetrate directly to start a new infection cycle. *P. infestans* is marked by two distinct growth phases. In the early stage, local infection occurs in the absence of notable cellular responses to pathogen invasion (biotrophy) whereas extensive necrotic lesions develop in the late stages of the interaction (necrotrophy) (Kamoun, 2003). For this reason, *P. infestans* is referred to as a hemibiotrophic pathogen.

During infection, *P. infestans* secretes a diverse set of effector molecules. These effectors can be divided into two classes based on their predicted localization during infection. Members of the first class target the plant extracellular space (apoplastic effectors) where they can break down physical and biochemical barriers or inhibit defense related molecules. Members of the second class translocate into the plant cell where they localize to distinct subcellular compartments (cytoplasmic effectors) where they could alter host cellular metabolism or cell signaling (Jia et al., 2000; Tyler, 2002). The function of both effector classes is to reprogram the host into a susceptible state or to increase virulence (Huijtema et al., 2004). In *Phytophthora*, gene families from both classes have been identified that have anticipated roles in virulence. Many of these genes encode cell wall degrading enzymes and other hydrolytic
enzymes as well as inhibitory molecules. A substantial group of genes however, encode proteins with unknown function. The challenge therefore remains to fully understand their functions in plants and contributions to virulence (Huitema et al., 2004).

Several *P. infestans* effectors have been implicated as activators of plant cell death. INF1, a member of the elicitin family, induces the hypersensitive response (HR), a form of programmed cell death, in most members of the *Nicotiana* genus (Kamoun et al., 1998). Isolates that do not express *inf1* were found to exhibit increased virulence on *Nicotiana benthamiana*, indicating a role for INF1 as a species-specific avirulence factor in *P. infestans-Nicotiana* interactions (Kamoun et al., 1998).

More recently, *crn2* and *PiNPP1.1* were identified as genes encoding necrosis inducing proteins from *P. infestans* (Torto et al., 2003; this thesis). In contrast to INF1, both CRN2 and PINPP1.1 induce cell death in a wide range of *Solanaceous* species including potato and tomato (Torto et al., 2003; this thesis). These findings have led researchers to hypothesize that cell death plays an ambiguous role in *Phytophthora*-plant associations and ask questions pertaining to the role of cell death inducing effectors in both resistant and susceptible interactions.

The hypersensitive response (HR) is associated with resistance to *P. infestans*. In resistant hosts, attempted pathogen infection is met by a rapid and localized induction of cell death and defense responses, via receptor (*R* gene) mediated recognition of pathogen effector molecules (avirulence gene) or their
activity (Dangl and Jones, 2001). The outcome of early defense induction is abolishment or interruption of disease progression and ultimately resistance (Dangl and Jones, 2001). Interestingly, associations between *P. infestans* and nonhost plant species also result in early HR induction, suggesting that the mechanisms are principally similar to those that govern host resistance (Kamoun *et al*., 1999; Vleeshouwers *et al*., 2000; Huitema *et al*., 2003). Consequently, recognition of multiple or indispensable pathogen molecules is hypothesized to result in activation of HR and defense responses which in turn confer nonhost resistance (Kamoun *et al*., 1999).

Much is known about the mechanisms that mediate signal perception and subsequent transduction. Resistance proteins (encoded by *R* genes) are thought to survey both the intercellular as well as the intracellular spaces for foreign (effector) molecules or the perturbations they mediate (Dangl and Jones, 2001; Martin *et al*., 2003). An increasing number of *R* genes are identified and allow further genetic and biochemical investigations of signal perception and transduction in plants. Recognition of pathogen molecules initiates a set of discrete signaling steps that lead to the activation of HR or defense responses (Dangl and Jones, 2001). Recent studies showed that multi-protein complexes (signalosomes) regulate signal transduction via targeted degradation of signal molecules (Liu *et al*., 2002; 2004; Takahashi *et al*., 2003). SGT1, HSP90 and RAR1 were found to be prominent members of signalosomes, presumably enhancing complex stability thereby facilitating signaling (Liu *et al*., 2004). In addition, downstream protein kinase pathways and transcriptional regulators are
thought to ensure amplification and fine-tuning of perceived signals and initiate defense responses. Despite the vast number of \( R \) genes and corresponding \( Avr \) genes, only a limited number of signaling components have been identified, suggesting either substantial convergence or an intricate web of signaling cascades. The identification of perceived pathogen signals as well as the signaling pathways they initiate or perturb should prove essential to enhance our understanding of \( P. \) infestans-plant associations.

In this dissertation, I investigated the molecular determinants of nonhost resistance and cell death in plants. With the emergence of genomics as an important branch of molecular genetics research, both computational and robust functional techniques have become essential tools in the quest towards understanding \( P. \) infestans-plant interactions. Recent successful applications of new technologies have led to the identification of effector molecules and therefore, have provided a precedent for using a set of pre-determined criteria in selecting candidate gene families. In chapter 2, I provide an overview of emerging themes in \textit{Phytophthora} and plant-microbe interaction research. In addition, I elaborate on the available technologies that can be employed to undertake comprehensive investigations of plant-microbe interactions. These tools and the questions they will help answer are expected to significantly impact our understanding of virulence and resistance mechanisms in \( P. \) infestans-plant associations.

In chapter 3, I characterized and explored \textit{Arabidopsis thaliana} as a model for \( P. \) infestans-nonhost associations. Inoculations of \textit{Arabidopsis} accessions
with *P. infestans* zoospores resulted in resistance outcomes. Development of disease lesions were never observed in inoculation experiments of Arabidopsis ecotypes with a range of *P. infestans* isolates. Subsequently, we performed cytological investigations of the interaction, measured *P. infestans* biomass fluctuations during nonhost associations and used microarray gene expression profiling to characterize *P. infestans*-induced transcriptional changes. Results presented in this chapter show the activation of HR as well as induction of active defense responses upon attempted infection of *Arabidopsis*.

In chapter 4, I describe the characterization of INF2A and INF2B, two closely related members of the elicitin gene family. Expression of both genes together with the previously identified elicitin INF1 in *N. benthamiana* and tobacco, revealed differences in intensity and specificity of cell death induction. Furthermore, INF2 induced HR-like cell death was followed by induction of moderate defense responses in tobacco. Finally, INF2A-induced cell death required the ubiquitin-ligase associated protein SGT1, indicating signal transduction is required for cell death execution. Altogether, these results suggested that INF2 helps confer *P. infestans* avirulence on *Nicotiana*.

Whether *P. infestans* is a successful pathogen on a plant species or not, cell death is always associated with the interaction. In chapter 5, I present the identification of PINPP1.1, an inducer of cell death in tomato and *N. benthamiana*. The identification of cell death inducers in host plants raises pertinent questions about their role in *P. infestans*-plant associations. Towards an attempt to deduce the role of PINPP1.1, I investigated the requirements of
PINPP1.1-induced cell death in *Nicotiana benthamiana*. I employed virus induced gene silencing (VIGS) to silence known disease signaling components and test their involvement in PINPP1.1 as well as INF1 induced cell death. Using this approach, I showed that SGT1 and HSP90 are both required for INF1 and PINPP1.1 induced cell death and identified four signaling components that affected PINPP1.1 induced cell death only. These results suggested the existence of separate signaling pathways. Co-infiltration of PINPP and INF1 resulted in synergistic induction of cell death. Altogether, this chapter suggests the existence of two interacting pathways and raise the possibility that PINPP1.1 has a distinct role in virulence.

In chapter 6, I characterized the complex CRN gene family. The *P. infestans* *crn1* and *crn2* genes were originally identified in an integrated computational and functional screening approach and a large gene family was subsequently discovered in *Phytophthora* (Torto et al., 2003). Here, I investigated the evolutionary history of the CRN protein family and characterized 13 of its members *in planta*. Sequence and subsequent comparative analysis showed that the CRN family: 1) is diverse and contains a mosaic of conserved sequences, 2) bares a conserved motif on the N-terminal end, 3) contains two main clusters of genes and 4) Exhibits patterns of reticulate evolution in one cluster of CRN genes. To further understand the functional role of the CRN gene family, we used potato virus X (PVX) to ectopically express its members *in planta*. Most but not all *crn* genes were found to induce cell death in *N. benthamiana* and *Solanum* spp. I expanded the functional analysis with microarray gene expression profiling.
experiments and found that CRN1 and CRN2 induced defense responses in tomato. In addition, CRN2 expression in Arabidopsis led to cell death indicating the presence of a conserved target or ancient perception machinery. Finally, VIGS analysis showed that CRN2 induced responses markedly differ from INF1 induced cell death. These results were confirmed in co-expression assays. This chapter contributes to our understanding of effector evolution and function in P. infestans-host interactions.

I conclude that P. infestans-plant associations are characterized by extensive cellular reprogramming. In nonhost interactions, rapid and local induction of HR is accompanied by induction of defense responses. Identification of effector molecules in P. infestans has allowed detailed analysis of perception and signal transduction. INF2A and INF2B were identified as inducers of cell death in N. benthamiana and tobacco and consequently, I proposed a role for these genes in avirulence or incompatibility in Nicotiana.

I investigated the involvement of cell death in P. infestans-host interactions. I characterized PINPP1.1 and the CRN family and speculated that they represent classes of virulence genes. Comparisons between PINPP1.1, CRN2 and INF1 induced cell death revealed that at least two signaling pathways exists. These results could reflect distinct functional roles for the CRNs and PINPP1.1 during pathogenesis.
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1.3 REFERENCES


CHAPTER 2

LINKING SEQUENCE TO PHENOTYPE IN PHYTOPHTHORA-PLANT INTERACTIONS

2.1 ABSTRACT

Oomycetes such as Phytophthora spp., establish pathogenic interactions with a diversity of plants and animals, but the molecular mechanisms underlying these diseases remain poorly characterized. However, research on Phytophthora pathosystems has accelerated significantly with ongoing advances in microbial and plant genomics and the resulting resources. A variety of functional analyses are being used to associate gene sequences with key processes that regulate interactions between these important pathogens and their hosts. Data from such analyses are starting to shed light on the relationship between pathogen molecules that manipulate host cell structure and function and innate defense responses of plants.
2.2 INTRODUCTION

Oomycetes such as members of the genus *Phytophthora*, establish close interactions with a diversity of plants resulting in important diseases on crop, ornamental, and native plants (Erwin and Ribeiro, 1996). Despite superficial morphological similarities with the fungi, oomycetes form a distinct group of eukaryotic organisms that are more closely related to brown algae and diatoms (Baldauf et al., 2000). Until recently, *Phytophthora* species were chronically understudied at the molecular level. With the continuing advances in genomics and the resulting resources, research on *Phytophthora* has accelerated significantly and is facing a new phase (Birch and Whisson; Kamoun, 2003). Genome sequencing projects are under way for the potato and tomato late blight pathogen *Phytophthora infestans*, the soybean root rot pathogen *Phytophthora sojae*, and the sudden oak death pathogen *Phytophthora ramorum*. Numerous expressed sequence tags (ESTs) are also increasingly available for *Phytophthora* spp. and other oomycetes. In parallel, genome sequence resources have been accumulating for several economically important host plants of *Phytophthora*, such as tomato, potato, and soybean, as well as experimental hosts, such as *Arabidopsis thaliana* and *Nicotiana* spp. The goal in this post-genomics era is to link sequences to phenotypes in a rapid and efficient manner. To meet this challenge, the *Phytophthora* and plant research communities have embarked on a diversity of functional analyses to associate gene sequences with key processes that regulate interactions between these pathogens and their hosts.
Molecular cross-talk between *Phytophthora* and plants involves a multitude of signal exchanges. The pathogen produces effectors; these are molecules that manipulate host cell structure and function by facilitating infection (virulence factors) or triggering defense responses (avirulence factors or specific elicitors). They interact directly or indirectly with components of the defense response pathways of plants, which can be resistance proteins or various other plant molecules generally termed virulence targets (Figure 2.1). Functional genetic analyses of *Phytophthora*-plant interactions involve identification and characterization of these various molecules and the processes they control. With the availability of genome sequences, novel functional genomics strategies to link sequences to phenotypes in a robust and efficient manner have become available (see Figures 2.2-2.4 for an illustration of some of the methodologies incorporated in these strategies). Here, we review recent advances in classical and genome-scale functional genetic analyses of *Phytophthora* pathosystems (the biological system that comprises pathogen and host) and provide an outlook on how functional genomics can impact these analyses.

### 2.3 PATHOGEN EFFECTOR GENES

*Phytophthora spp.* produce motile spores, or zoospores, that reach leaf or root surfaces, encyst, germinate, and penetrate plant tissue (Hardham, 2001; van West *et al.*, 2002; 2003). Germinating cysts produce germ tubes, which swell to form appressoria or appressorium-like structures that facilitate adhesion and penetration of plant surfaces. In root infecting species, penetration can occur between cells without the aid of an appressorium (Hardham, 2001). *Phytophthora*
species are thought to have an arsenal of genes that facilitate or contribute to these early infection events. The Car genes of P. infestans (GenBank accessions AF061186 and AF061185) are up-regulated in germinating cysts and appressoria shortly before penetration of plant tissue (Gornhardt et al., 2000). They encode extracellular mucin-like proteins that have been suggested to facilitate adhesion (Gornhardt et al., 2000). More recently, The CBEL gene (cellulose binding and elicitor, GenBank accession X97205), which encodes a cellulose binding protein of Phytophthora parasitica, was shown to be essential in adhesion to cellulosic substrates (Gaulin et al., 2002). However, although P. parasitica strains silenced for the CBEL gene were impaired in their ability to attach to cellophane membranes, they remained able to infect tobacco plants (Gaulin et al., 2002).

Several genes with significant similarity to degradative enzymes such as cutinases, proteases, endo- and exo-glucanases, and chitinases have been identified in EST libraries and are thought to facilitate infection by breaking down physical barriers in the plant (Kamoun et al., 1999; Qutob et al., 2000; McLeod et al., 2002). A handful of Phytophthora genes encoding degradative enzymes have been characterized in detail, including phospholipases (Nespoulous et al., 1999), a beta-glucosidase/xylosidase (Brunner et al., 2002), exo-1,3-beta-glucanases (McLeod et al., 2002), an endo-1,3-beta-glucanase (McLeod et al., 2002), and endopolygalacturonases (endoPGs) (Gotesson et al., 2002; Torto et al., 2002). The endoPG family is remarkable in many respects. In P. cinnamomi, endoPGs form a major family with at least 19 members (Gotesson et al., 2002). Birth-and-death evolution, reticulate evolution, and diversifying selection were detected in this gene family and might have contributed to the evolution of this structurally
diverse and complex class of enzymes (Gotesson et al., 2002). Phylogenetic analyses indicated that *Phytophthora* endoPGs, exo-1,3-beta-glucanases, and an endo-1,3-beta-glucanase are more similar to fungal genes than to their plant and bacterial counterparts (McLeod et al., 2002; Gotesson et al., 2002; Torto et al., 2002). These observations are in sharp contrast with phylogenies constructed from ribosomal sequences or compiled protein sequences from mitochondrial and housekeeping chromosomal genes, which consistently group oomycetes with brown algae and diatoms (Baldauf et al., 2000; 2003; Lang et al., 1999). The apparent discrepancies between these phylogenies could reflect a convergent evolution in the arsenal of hydrolytic enzymes between oomycetes and fungi, two distantly related groups of filamentous pathogenic microbes that have similar life strategies and hosts (Gotesson et al., 2002; Torto et al., 2002; Latijnhouwers et al., 2003). These observations suggest that there are common mechanisms of infection among filamentous microbes. In the future, comparative genomics analyses between plant pathogenic oomycetes and fungi will help define a common set of virulence genes.

Following infection, plants exhibit complex defense responses, for example through the production of the pathogen-inducible antimicrobial enzymes glucanases and proteases that degrade microbial cell walls and proteins. *Phytophthora* can suppress these defense responses by producing inhibitory molecules that target host enzymes (Kamoun, 2003; Ham et al., 1997; Sanchez et al., 1992; Yoshioka et al., 1995). In *P. infestans*, water-soluble glucans have been reported to suppress host defenses in a plant cultivar-specific manner (Sanchez et al., 1992; Yoshioka et al., 1995). Recently, genes encoding secreted
proteins that inhibit soybean endo-beta-1,3 glucanase have been cloned from *P. sojae* (Rose *et al.*, 2003). These proteins, termed glucanase inhibitor proteins (GIPs, GenBank accessions AF406607-AF406609), share significant structural similarity to the trypsin class of serine proteases, but bear mutated catalytic residues and are proteolytically nonfunctional as a consequence (Rose *et al.*, 2002). GIPs are thought to function as counterdefensive molecules that inhibit the degradation of beta-1,3/1,6 glucans in the pathogen cell wall and/or the release of defense-eliciting oligosaccharides by host endo-beta-1,3 glucanases (Rose *et al.*, 2002). Data mining of *P. infestans* ESTs revealed an additional class of secreted inhibitory proteins that contain domains typical of Kazal serine protease inhibitors. One of these proteins, EPI1 from *P. infestans*, was found to inhibit and physically interact with tomato proteases suggesting a novel type of defense-counterdefense mechanism between plants and *Phytophthora* (M. Tian and S. Kamoun, XXII Fungal Genetics Conference, Asilomar, CA, 2003, http://www.fgsc.net/asil2003/asil2003abs.htm).

Several *Phytophthora* effector molecules are known to induce a variety of cellular defense responses in plants (Fellbrich *et al.*, 2002; Kamoun *et al.*, 1998; Orsomando *et al.*, 2001; Qutob *et al.*, 2002; 2003; Sacks *et al.*, 1995; Torto *et al.*, 2003; Villalba *et al.*, 1997). Some of these effectors induce defense responses in both susceptible and resistant plants and are referred to as general elicitors. Other effectors induce defense responses specifically in resistant plants, and are known as specific elicitors. However, in many cases the contribution of elicitors to the infection process remains ambiguous due to the lack of direct evidence obtained from knock-out or overexpression *Phytophthora* mutants. General
elicitors have been likened to pathogen-associated molecular patterns (PAMPs) of animal pathogens, which are surface-derived molecules that induce the expression of defense response genes and the production of antimicrobial compounds in host cells (Nurnberger et al., 2002; Gomez-Gomez and Boller, 2002). Nonetheless, it cannot be ruled out that some elicitors might function as toxins that facilitate colonization of host tissue during the late phase of Phytophthora infections when host tissue collapses and turns necrotic (Kamoun, 2003; Qutob et al., 2002).

Originally, Phytophthora elicitors were identified and purified using biochemical methods and later cloned using reverse genetics and hybridization methods. This classical strategy led to the identification of genes encoding INF1 elicitin from P. infestans (GenBank accession U50844) (Kamoun et al., 1997), cryptogein from Phytophthora cryptogea (GenBank accession Z34459) (Panabieres et al., 1995), PsojNIP and GPE1 from P. sojae (GenBank accessions AF320326 and U10471) (Qutob et al., 2002; Sacks et al., 1995), NPP1 and CBEL from P. parasitica (GenBank accessions AF352031 and X97205) (Fellbrich et al., 2002; Villalba et al., 1997), and PcF from Phytophthora cactorum (GenBank accession AF354650) (Orsomando et al., 2001). More recently, elicitors from Phytophthora have been identified using functional genomic strategies (Qutob et al., 2002; 2003; Torto et al., 2003). These approaches typically involve two steps: (1) data mining for genes fulfilling particular criteria, and (2) functional genetic analyses to identify the desired gene(s) among the selected candidates.
Examples of computational tools that were developed to mine sequence data sets include PexFinder (with Pex standing for *Phytophthora* extracellular protein), an algorithm based on SignalP v2.0 (Nielsen *et al.*, 1999) and designed to identify putative secreted or membrane associated proteins from ESTs (Qutob *et al.*, 2002; Torto *et al.*, 2003). Other data mining tools are embedded into EST analysis pipelines, such as the XGI system of the National Center for Genome Resources (NCGR, http://www.ncgr.org) (Inman *et al.*, 2000; Waugh *et al.*, 2000), which performs automated BLAST searches (Altschul *et al.*, 1997) against a variety of target databases, BLIMP searches of the BLOCKS+ protein motif database (Henikoff *et al.*, 2000), PexFinder analyses (Torto *et al.*, 2003), and eight separate analyses as part of InterProScan searches (Zdobnov *et al.*, 2001) against the InterPro database (Apweiler., 2001) (see the *Phytophthora* Functional Genomics Database (PFGD, www.pfgd.org) as an example). The XGI system also performs automated assignment of Gene Ontology annotations based on high-scoring homologies with Swiss-Prot data as well as curated Gene Ontology (GO, http://www.geneontology.org/) annotations available through InterPro.

Rapid functional assays for expressing *Phytophthora* genes in planta are well established and are ideal for discovering genes with elicitor function (Kamoun *et al.*, 2002) (Figures 2.2 and 2.3). Using an *Agrobacterium tumefaciens* binary vector carrying the potato virus X (PVX) genome, Torto *et al.* (2003) screened 63 candidate Pex cDNAs for elicitor activity in planta and recovered two novel necrosis-inducing cDNAs, crn1 and crn2 (GenBank accessions AF424675 and AF424677). These cDNAs encode extracellular proteins that belong to a large and complex protein family in *Phytophthora*. The
crn genes are expressed in *P. infestans* during colonization of the host plant tomato and crn2 induces defense response genes in tomato. Qutob *et al.* (2002) also used the PVX vector to express 16 *Pex* cDNAs from *P. sojae* in *Nicotiana benthamiana*. One of these cDNAs encodes PsojNIP, a 26-kDa protein that is similar in sequence to necrosis-inducing proteins from various eukaryotic and prokaryotic species. PsojNIP induces necrosis and cell death in tobacco and the host plant soybean. Interestingly, Fellbrich *et al.* (2002) independently identified an ortholog of the *PsojNIP* gene, by purification of a necrosis-inducing protein, NPP1, from culture filtrates of *P. parasitica*, and cloning by reverse genetics. These two studies, which were published side-by-side, offer a comparison between classical and functional genomics approaches to elicitor discovery.

Bos *et al.* (2003) described another functional genomics strategy that combines data mining with intraspecific comparative genomics and functional analyses to identify novel avirulence genes from *Phytophthora*. This approach provides a rapid and efficient alternative to classical positional cloning strategies for isolating avirulence genes that match known disease resistance gene (*R* genes) and has the potential to uncover "orphan" avirulence genes for which corresponding *R* genes have not been previously identified (Bos *et al.*, 2003).
2.4 PLANT DEFENSE GENES

A number of $R$ genes that target *Phytophthora* spp. have been genetically defined. Cloning of these $R$ genes has been achieved in a few cases. Eleven late blight $R$ genes were introgressed into potato from the Mexican wild species *Solanum demissum* using classical breeding. One of these genes, $R1$ (GenBank accession AF447489), was cloned using a combination of positional cloning and candidate gene approach (Ballvora *et al.*, 2002). The $R1$ gene is predicted to encode a polypeptide of 1293 amino acids that belongs to the CC-NBS-LRR (coiled coil motif, nucleotide binding site, and leucine-rich repeat domain) class of plant $R$ genes (Dangl and Jones, 2001; Meyers *et al.*, 2003). Another late blight $R$ gene, $RB$ (GenBank accession AAP45164), was recently cloned from the wild diploid potato species *Solanum bulbocastanum* using a combination of map-based cloning and long-range-polymerase chain reaction (Song *et al.*, 2003). $RB$ is predicted to encode a protein of 970 amino acids that also belongs to the CC-NBS-LRR class (Song *et al.*, 2003). As with several other $R$ genes, both $R1$ and $RB$ belong to complex loci that carry several $R$ gene analogs (RGAs) of the CC-NBS-LRR class (Ballvora *et al.*, 2002; Song *et al.*, 2003). Unlike $R1$, which is only effective against races of *P. infestans* that carry Avr1 (Ballvora *et al.*, 2002), $RB$ is effective against all tested races of the pathogen and holds great promise to help achieve sustainable management of late blight (Song *et al.*, 2003; Helgeson *et al.*, 1998). In fact, somatic hybrids between potato and the parental *S. bulbocastanum* clone PT29, as well as a number of backcrossed progenies, exhibited broad spectrum and persistent resistance in a variety of field trials over several years (Song *et al.*, 1998; Helgeson *et al.*, 1998). Whether this phenotype
is solely due to RB, or whether additional resistance genes are involved remains to be determined.

The P. infestans Avr (avirulence) genes that trigger R1- and RB-mediated resistance responses are unknown. Sequence analyses suggest that the R1 and RB proteins are localized inside the plant cell (Ballvora et al., 2002; Song et al., 2003). How these proteins perceive molecular signals from the avirulent pathogen is one of the trying research questions in Phytophthora-plant interactions. The most prevalent theory maintains that Phytophthora and other oomycete and fungal pathogens deliver effector molecules into plant cells through specialized structures, such as haustoria, even though the exact mechanism of how these molecules would cross the plant plasma membrane remains unknown (Jia et al., 2000; Tyler, 2002). Cloning and functional analysis of Avr-R gene pairs in Phytophthora pathosystems will provide insight into this issue.

The model plant Arabidopsis thaliana is emerging as an experimental system for studies of resistance to Phytophthora. Arabidopsis exhibits both host resistance (against Phytophthora spp. that can cause disease on this plant), and nonhost resistance (against Phytophthora spp. that cannot cause disease on Arabidopsis). Two Phytophthora species were reported to infect Arabidopsis providing pathosystems that are more amenable to genetic analysis than agronomically important Phytophthora diseases. Cabbage isolates of Phytophthora brassicae (previously known as Phytophthora porri) (Roetschi et al., 2001; Si-Ammour et al., 2003), and several isolates of Phytophthora cinnamomi (Robinson and Cahill, 2003) can infect and extensively colonize
Arabidopsis. Both species exhibit marked variation in the responses they induce on different Arabidopsis ecotypes. Most Arabidopsis defense mutants did not show any alteration in their resistance to P. brassicae. However, pad2, a mutant with reduced production of the phytoalexin camalexin, was heavily colonized resulting in a hyper-susceptibility phenotype (Roetschi et al., 2001). As of now, the molecular identity of pad2 has not been reported.

Other Phytophthora species, such as P. infestans and P. sojae cannot colonize and cause disease on Arabidopsis resulting in nonhost interactions (Takemoto et al., 2003; Huitema et al., 2003). Cytological and molecular analyses indicate that nonhost resistance of Arabidopsis to these species is associated with the hypersensitive response (HR) and other active defense responses (Takemoto et al., 2003; Huitema et al., 2003). Considering the impressive genetic and functional genomic resources that are available, Arabidopsis offers good prospects for dissecting the complex interactions between nonhost plants and Phytophthora. For example, pen2, an Arabidopsis mutant deficient in a cell wall glycosyl hydrolase, was recently shown to allow enhanced penetration and HR in response to P. infestans (P. Schulze-Lefert, European Plant Science Organization Conference, Brunnen, Switzerland, 2002, http://www.epsoweb.org/catalog/Conf2002.htm.

Research on genetic dissection of defense response pathways in plants is greatly benefiting from the emergence of N. benthamiana as an alternative and complementary model system to Arabidopsis. N. benthamiana allows rapid and high-throughput analysis of gene function using virus-induced gene silencing (VIGS) (Baulcombe, 1999) (Figure 2.4). Using VIGS, a loss-of-function
phenotype can be generated for a given candidate gene within a month. VIGS can also be used in forward genetic screens. For example, Lu et al. (2003) identified 79 *N. benthamiana* cDNAs to be required for HR against the bacterial pathogen *Pseudomonas syringae* by screening 4992 cDNAs randomly picked from a normalized library.

VIGS is also facilitating the genetic dissection of the HR of *N. benthamiana* to *P. infestans* and the elicitin INF1. Yoshioka et al. (2003) showed that two respiratory burst oxidase homologs of *Nicotiana benthamiana*, NbrbohA and NbrbohB (GenBank accessions BAC56864 and BAC56865), are required for 

\[ \text{H}_2\text{O}_2 \]

accumulation and resistance to *P. infestans*. VIGS of the *Nbrboh* genes also led to a reduction of HR cell death caused by INF1 suggesting that the oxidative burst is required for full development of the HR. Kanzaki et al. (2003) reported that two cytosolic molecular chaperones proteins NbHSP90c-1 and NbHSP70c-1 of *N. benthamiana* (Genbank accessions AB105429 and AB105430) are required for INF1-mediated HR and non-host resistance to the bacterial pathogen *Pseudomonas cichorii*. Cytosolic HSP90 proteins were also recently implicated in a variety of *R* gene mediated responses to bacterial, fungal, and viral pathogens (Lu et al., 2003; Takahashi et al., 2003).

NbHSP90c-1 interacts with NbSIPK (GenBank accession AB098730), a mitogen-activated protein (MAP) kinase that is activated during INF1-mediated HR (Sharma et al., 2003). However, VIGS of NbSIPK and another MAPK kinase (NbWIPK, GenBank accession AB098729) did not result in a loss of HR to INF1 suggesting that activation of these MAPK kinase cascades is not required for response to INF1 (Sharma et al., 2003). Peart et al. (2002) also used VIGS to
show that the response of *N. benthamiana* to INF1 was dependent on the ubiquitin ligase associated protein NbSGT1 (Genbank accession AF516180 and AF516181), which is also required for nonhost resistance to bacterial plant pathogens. Takahashi *et al.* (2003) reported that cytosolic HSP90 interacts with SGT1 and another resistance signaling protein RAR1 (Genbank accession AY438026) suggesting that these proteins might function as co-chaperones that together with HSP90 regulate the activity and stability of substrate proteins that are essential for disease resistance signaling.

### 2.5 DATABASES FOR *PHYTOPHTHORA*-PLANT GENOMICS

A critical component of functional genomics is the dissemination of large data sets to the research community through public databases as a more effective alternative to traditional publications. Ideally, a functional genomics database should be iterative and interactive, allowing users to develop hypotheses, query the database with specific questions, collect information, test hypotheses, and finally revisit the database to refine their queries or deposit new information.

In recent years, several genome databases have emerged for *Phytophthora* spp. and their host plants, mainly through projects funded by the NSF Plant Genome Research Program (Table 2.1). The *Phytophthora* Functional Genomics Database (PFGD) is a publicly accessible resource that interrelates functional and sequence data. PFGD builds upon cDNA and genome sequences derived from other databases, including the former *Phytophthora* Genome Consortium (PGC) database and other public *P. infestans* sequence data.
Sequences are screened, clustered, analyzed and annotated using NCGR’s XGI system (Inman et al., 2000; Waugh et al., 2000). PFGD also includes functional annotation of *P. infestans* candidate effector genes. To allow an integrated analysis of solanaceous sequences with those of *P. infestans*, the Solanaceae Genomics Database (SolGD) has been developed as a sister database for PFGD. Currently, SolGD contains unigenes derived from ESTs of solanaceous plants including tomato, potato, *N. benthamiana*, *Nicotiana tabacum*, and *Nicotiana otophora*. Interlinkage between PFGD and SolGD facilitates analysis of the 'interaction transcriptome', which consists of host and pathogen genes whose expression might be regulated during infection (Birch and Kamoun, 2000).

Other resources include the Solanaceae Genomics Network (SGN), which provides genomic information about solanaceous species, including comparative maps, marker sequences, ESTs, and phylogenetic information. In addition, comprehensive resources on tomato and potato ESTs are available via The Institute for Genome Research (TIGR) Tomato Gene Index and Potato Gene Index, respectively. Further, the Solanaceae Gene Expression Database (SGED) stores gene expression data obtained through TIGR expression profiling service of 10,000-clone potato cDNA microarrays, and the Tomato Expression Database (TED) contains basic information about tomato ESTs and cDNA microarray data. Finally, the Solanaceae *R* Gene Sequences Database (SOLAR) archives genomic resources centered on *R* genes from various solanaceous plants, such as wild potato, tomato and pepper. SOLAR includes physical and genetic maps, genomic sequences of *R* gene loci and candidate late blight *R* genes, and
evolutionary models and bioinformatic tools for R gene analysis. The web addresses of the databases described here are listed in Table 2.1.

2.6 FUTURE PERSPECTIVES

Functional genomics of Phytophthora-plant interactions is a promising area of research. There is already a respectable set of data mining and functional genomics tools that have been incorporated into expanding databases. In addition, the field is now embracing comparative genomics as a result of the exponential increases in sequence data for oomycetes and their hosts. Nevertheless, this field is in its infancy and requires additional technical developments in bioinformatics and functional analysis. Here, we summarize some of the key themes that will immediately impact the development of functional genomics of Phytophthora pathosystems. These are:

- Completion of genome sequencing of Phytophthora and host species.
- Identification of candidate effector and defense genes using bioinformatics and wet lab approaches.
- Proteomics approaches to molecular interactions at the infection interface.
- High-throughput functional analyses of Phytophthora effector genes.
- High-throughput functional analyses of plant defense genes using Arabidopsis genetics and VIGS.
- Development of databases that incorporate functional and sequence data.
These and other advances in genome-scale functional analyses of *Phytophthora* pathosystems will lead to a greater understanding of the basic molecular mechanisms underlying the interactions between these economically important pathogens and their plant hosts. Ultimately, this will allow the identification of novel genetic targets for efficient pathogen management and improvement of crops.

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A modified version of this has been published as:

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Figure 2.1. A simplified view of molecular interactions between *Phytophthora* and plants. *Phytophthora* secretes effector proteins (green half circles) that interact with plant molecules known as virulence targets (purple crescents). These virulence targets are thought to be components of the plant defense response that are being inactivated by pathogen effectors. In susceptible plants, the interaction between effectors and virulence targets results in molecular events that facilitate colonization, such as suppression of defense responses, enhanced disease susceptibility, and elicitation of disease symptoms. In resistant plants, plant resistance (R) proteins recognize the effector-virulence target complex resulting in the activation of the hypersensitive response (HR). The objective of molecular studies of *Phytophthora*-plant interactions is to identify and functionally characterize these various molecular players. The symbol o-o depicts protein-protein interactions that are thought to be crucial for the outcome of the infection.
Figure 2.2. Agroinfiltration: transient gene expression in plants using Agrobacterium tumefaciens-mediated transformation. (a) Strategy for agroinfiltration. The gene of interest (candidate gene) is cloned in a plant gene expression cassette in a T-DNA binary vector, and the recombinant plasmid is transformed into A. tumefaciens. Then, the A. tumefaciens strain carrying the binary plasmid is cultured, washed, and incubated in a solution that contains acetosyringone to induce the bacteria’s vir genes. Finally, the bacterial solution is infiltrated with a syringe into leaf panels of a mature plant, such as Nicotiana benthamiana. Within 48 hours, most of the plant cells within the infiltrated area are transiently transformed with the T-DNA and express the candidate gene. (b) Symptoms observed on leaves of N. benthamiana following infiltration with A. tumefaciens strain containing a binary vector expressing the hypersensitive response (HR)-inducing gene inf1 of Phytophthora infestans. Inoculated leaves were photographed 5 days after inoculation with A. tumefaciens containing the binary vector p35S-INF1 (bottom left and top right sides of the leaf), and the negative control pGUSi (top left and bottom right). (c-d) UV autofluorescence observed in N. benthamiana leaf panels corresponding to the negative control (c) and the p35S-INF1 (d) infiltration areas. Cell-death associated fluorescence is observed in panel (d), whereas background red fluorescence is caused by chloroplasts.
Figure 2.3. Agroinfection: transient gene expression in plants using *Agrobacterium tumefaciens* carrying a binary potato virus X (PVX) vector (a) Strategy for agroinfection. The gene of interest (candidate gene) is cloned in a binary PVX vector and the recombinant plasmid is transformed into *Agrobacterium tumefaciens*. Then, the *A. tumefaciens* strain carrying the binary PVX plasmid is inoculated by wounding plant leaves with a toothpick. T-DNA will be transferred to a few cells surrounding the wounded area resulting in expression of PVX, systemic spreading of the virus in the plant, and expression of the candidate gene in plant tissue. (b-e) Symptoms observed in *Nicotiana benthamiana* following inoculation with *A. tumefaciens* carrying a binary PVX vector expressing the hypersensitive response (HR)-inducing gene *inf1* of *Phytophthora infestans*. Leaves were photographed 8 days after inoculation with *A. tumefaciens* containing a binary PVX vector (b, d), and a PVX:INF1 construct (c, e). The arrows indicate the sites of inoculation. (f-g) Large scale agroinfection assays on tobacco leaves. (f) Inoculation of a tobacco leaf with a 96-needle colony replicator dipped in a microtiter plate containing *A. tumefaciens* strains carrying recombinant PVX plasmids. (g) Symptoms observed on a tobacco leaf 10 days after inoculation. The necrotic lesions correspond to sites inoculated with PVX:INF1. For clarity, only 40 of the 96 inoculations are shown.
Figure 2.4. Virus-induced gene silencing (VIGS). (a) Strategy for VIGS using *Agrobacterium tumefaciens* binary vectors carrying Tobacco Rattle Virus (TRV) RNA1 and RNA 2 genomes. The plant gene of interest (gene X) is cloned into a binary TRV RNA 2 vector and transformed into *A. tumefaciens*. Then, *A. tumefaciens* strains carrying TRV RNA 2 plasmid RNA 1 genome are cultured, washed in inducing buffer, and mixed 1:1 prior to infiltration into leaf panels. TRV is allowed to infect the plant resulting in systemic gene silencing (3 weeks). (b-c) Symptoms in *Nicotiana benthamiana* silenced for the ubiquitin ligase-associated gene SGT1 following inoculation with *A. tumefaciens* carrying a binary PVX vector expressing inf1 of *Phytophthora infestans*. Leaves were photographed 8 days after the secondary PVX challenge. Note the absence of necrotic lesion in the SGT1-silenced leaf (c) suggesting that SGT1 is required for INF1-mediated HR (Peart *et al.*, 2002). (d-g) UV autofluorescence on leaf panels from TRV control plants challenged with PVX (d), PVX:INF1 (e), or the TRV:SGT1-silenced plant challenged with PVX (f) or PVX:INF1 (g). Cell-death associated fluorescence is observed in panel (e), whereas background red fluorescence is caused by chloroplasts.
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<td>Solanaceae Genomics Network (SGN)</td>
<td><a href="http://www.sgn.cornell.edu/">http://www.sgn.cornell.edu/</a></td>
<td>Lycopersicon esculentum, L. pinninelliifolium, L. hirsutum, L. pennellii, Solanum tuberosum, S. melongena</td>
<td>Comparative genetic maps, marker sequences, ESTs, phylogenetic information, a tomato-Arabidopsis synteny map, and a mutant phenotype database named &quot;The Genes That Make Tomatoes&quot; Annotated unigene sequences</td>
</tr>
<tr>
<td>Solanaceae Genome Database (SolGD)</td>
<td><a href="http://www.solgd.org/">http://www.solgd.org/</a></td>
<td>L. esculentum, S. tuberosum, Nicotiana tabacum, N. benthamiana, N. otophora</td>
<td>Genetic maps and marker information</td>
</tr>
<tr>
<td>NSF Potato Genome Project (UC Berkeley)</td>
<td><a href="http://www.potatogenome.org">http://www.potatogenome.org</a></td>
<td>Mainly Solanum spp.</td>
<td>Genome sequences and resources in the Solanaceae R Gene Sequences Database (SOLAR)</td>
</tr>
<tr>
<td>NSF Potato Functional Genomics (TIGR)</td>
<td><a href="http://www.tigr.org/tdb/potato/">http://www.tigr.org/tdb/potato/</a></td>
<td>S. tuberosum</td>
<td>Annotated and assembled ESTs, DNA microarray gene expression profiling data</td>
</tr>
<tr>
<td>Solanaceae Gene Expression Database (SGED)</td>
<td><a href="http://www.tigr.org/tdb/potato/SGED_index2.shtml">http://www.tigr.org/tdb/potato/SGED_index2.shtml</a></td>
<td>S. tuberosum</td>
<td>Annotated and assembled ESTs, DNA microarray gene expression profiling data</td>
</tr>
</tbody>
</table>

Table continues next page
Table 2.1. Major public databases and genomic resources for *Phytophthora* spp. and their host plants\(^1\).

\(^1\)The plant list focuses on two major botanical families, the solanaceae and the leguminae, which are hosts to two major *Phytophthora* pathogens, *P. infestans*, *P. sojae*. 

<table>
<thead>
<tr>
<th>Database/Resource</th>
<th>Website</th>
<th>Organism</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>C.M. Rick Tomato Genetics Resource Center (TGRC)</td>
<td><a href="http://tgrc.ucdavis.edu/">http://tgrc.ucdavis.edu/</a></td>
<td><em>Lycopersicon</em> spp.</td>
<td>Seed bank of tomato germplasm</td>
</tr>
<tr>
<td>Tomato TIGR Gene Index</td>
<td><a href="http://www.tigr.org/tdb/tgi/lgi/">http://www.tigr.org/tdb/tgi/lgi/</a></td>
<td><em>L. esculentum</em></td>
<td>Annotated and assembled ESTs DNA microarray gene expression profiling data</td>
</tr>
<tr>
<td>Tomato Expression Database (TED)</td>
<td><a href="http://ted.bti.cornell.edu/">http://ted.bti.cornell.edu/</a></td>
<td><em>L. esculentum</em></td>
<td>DNA microarray gene expression profiling data</td>
</tr>
<tr>
<td>Transcriptome Analysis of BY-2 (TAB)</td>
<td><a href="http://mrg.psc.riken.go.jp/strc/">http://mrg.psc.riken.go.jp/strc/</a></td>
<td><em>N. tabacum</em></td>
<td>ESTs from tobacco BY-2 cell culture</td>
</tr>
<tr>
<td>Legume Information System (LIS)</td>
<td><a href="http://www.comparative-legumes.org/">http://www.comparative-legumes.org/</a></td>
<td><em>Medicago truncatula</em> and various leguminae</td>
<td>Annotated EST unigenes and genome sequences</td>
</tr>
<tr>
<td>SoyBase</td>
<td><a href="http://www.soybase.org/">http://www.soybase.org/</a></td>
<td><em>Glycine max</em> and various leguminae</td>
<td>Genetic maps and marker information</td>
</tr>
</tbody>
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CHAPTER 3

ACTIVE DEFENSE RESPONSES ASSOCIATED WITH NONHOST RESISTANCE OF
ARABIDOPSIS THALIANA TO THE OOMYCETE PATHOGEN PHYTOPHTHORA INFESTANS

3.1 ABSTRACT

The molecular basis of nonhost resistance, or species-specific resistance, remains one of the major unknowns in the study of plant-microbe interactions. Here, we describe the characterization of a nonhost pathosystem involving the model plant Arabidopsis thaliana and the economically important and destructive oomycete pathogen Phytophthora infestans. Cytological investigations into the early stages of this interaction revealed germination of P. infestans cysts on Arabidopsis leaves, direct penetration of epidermal cells, formation of infection vesicles and occasionally secondary hyphae, followed by a typical hypersensitive response. P. infestans biomass dynamics during infection of Arabidopsis was monitored using kinetic PCR, revealing an increase of biomass during the first 24 hours after inoculation, followed by a decrease in later stages.
Transgenic reporter lines and RNA blot analyses were used to characterize the defense responses induced upon *P. infestans* infection. Significant induction of *PDF1.2* was observed at 48 hours after inoculation, whereas elevated levels of *PR* gene expression were detected three days after inoculation. To further characterize this defense response, DNA microarray analyses were carried out to determine the expression profiles for ca. 11,000 *Arabidopsis* cDNAs 16 hours after infection. These analyses revealed significant overlap between *Arabidopsis* nonhost response and other defense-related treatments described in the literature. In particular, nonhost response to *P. infestans* was clearly associated with the activation of the jasmonate pathway. The described *Arabidopsis*-*P. infestans* pathosystem offers excellent prospects for improving our understanding of nonhost resistance.

**3.2 INTRODUCTION**

Plants are challenged by numerous pathogens throughout their life cycles and yet are able to fend off most infections. Indeed, in interactions between plants and microbial pathogens, resistance is the rule and disease the exception. This phenomenon is known as nonhost resistance or species-specific resistance and is thought to explain why a pathogen can cause disease in particular plant species but not in others. Understanding the molecular basis of nonhost resistance remains one of the elusive quests in the study of plant-microbe interactions. Preformed barriers and compounds such as saponins are ubiquitous in plants and play an important role in nonhost resistance to filamentous fungi.
(Morrissey and Osbourn, 1999; Osbourn, 1996). However, most contemporary models of nonhost resistance evoke a complex overlay of specific resistance and nonspecific defense responses (Gomez-Gomez and Boller, 2002; Heath, 2000; Kamoun et al., 1999; Kamoun, 2001; Nurnberger and Brunner, 2002). Specific resistance has been extensively studied in host pathosystems and typically follows Flor's gene-for-gene model. In this model, resistance is determined by the simultaneous expression of a pathogen avirulence (Avr) gene with the corresponding plant resistance (R) gene leading to the hypersensitive response (HR), a general defense response of plants that includes apoptotic cell death (Dangl and Jones, 1998; Flor, 1971; Staskawicz et al., 1995). The extent to which the gene-for-gene model can be expanded to nonhost interactions remains unclear. However, we and others have speculated that in many pathosystems nonhost resistance can be explained by the occurrence of an arsenal of R genes that recognize multiple or essential Avr genes (Heath, 2000; Kamoun et al., 1998; Kamoun et al., 1999; Kamoun, 2001; Staskawicz et al., 1995).

The oomycetes represent a diverse and phylogenetically unique branch of eukaryotic microbes that includes many important pathogens of plants (Baldauf et al., 2000; Margulis and Schwartz, 2000; Sogin, 1998). The most notorious oomycetes are Phytophthora species, arguably the most devastating pathogens of dicot plants (Erwin and Ribeiro, 1996; Kamoun, 2000; Kamoun, 2003). For example, Phytophthora infestans causes late blight, a devastating disease that results in multibillion-dollar losses in potato and tomato production (Fry and Goodwin, 1997a; Fry and Goodwin, 1997b; Garelik, 2002; Smart and Fry, 2001).
Most plants, such as weeds and various crops, are resistant to *P. infestans*, and grow unimpaired in or next to fields with high incidence of late blight (Colon *et al*., 1992; Kamoun *et al*., 1999). Understanding the molecular basis of nonhost resistance to *P. infestans* will provide insight into a key molecular process and will offer novel perspectives for engineering durable late blight resistance in crop plants.

A first insight into the basis of nonhost resistance to *Phytophthora infestans* came through detailed cytological analyses. Microscopic observations revealed penetration of epidermal cells by *P. infestans* in all examined interactions, including those with plant species unrelated to the solanaceous hosts (Gross *et al*., 1993; Kamoun *et al*., 1998; Kamoun *et al*., 1999; Naton *et al*., 1996; Schmelzer *et al*., 1995; Vleeshouwers *et al*., 2000). Fully resistant plants, such as the nonhosts *Solanum nigrum*, parsley, and tobacco display a typical localized HR at all infection sites (Colon *et al*., 1992; Kamoun *et al*., 1998; Kamoun *et al*., 1999; Naton *et al*., 1996; Schmelzer *et al*., 1995; Vleeshouwers *et al*., 2000). The HR can be highly localized to a single epidermal cell or can affect a group of cells surrounding the penetrating hyphae depending on the interaction examined (Kamoun *et al*., 1998; Vleeshouwers *et al*., 2000). The view that has emerged from these studies is that the HR, perhaps mediated by *R* genes, is associated with all known forms of genetic resistance to *P. infestans* including nonhost resistance (Kamoun *et al*., 1999; Kamoun, 2001).

Some of the *Phytophthora* molecules that trigger the HR or other defense responses in nonhost plants are known. Species-specific elicitors have been
described in *P. infestans* and other *Phytophthora* species and can trigger defense responses in nonhost plants. For example, an extracellular transglutaminase that is conserved in *P. infestans* and other *Phytophthora* species induces defense responses in the nonhost parsley (Brunner *et al*., 2003; Nurnberger and Brunner, 2002). Members of the INF elicitin family induce the HR and related biochemical changes specifically in *Nicotiana* (Kamoun *et al*., 1997; Kamoun *et al*., 1998; Sasabe *et al*., 2000). *P. infestans* strains deficient in the elicitin INF1 induce disease lesions on *Nicotiana benthamiana*, suggesting that INF1 functions as an *Avr* factor that conditions resistance in this species (Kamoun *et al*., 1998). Using gene silencing, Peart *et al*. (2002) recently showed that the response of *N. benthamiana* to INF1 was dependent on the ubiquitin ligase-associated protein SGT1, which is also required for nonhost resistance to bacterial plant pathogens. The *N. benthamiana* pathosystem holds great promise for dissecting elicitor response and resistance to *P. infestans* since this plant is amenable to high-throughput functional assays using virus-induced gene silencing (VIGS) (Baulcombe, 1999). Nevertheless, the *N. benthamiana*-*P. infestans* interaction does not qualify as a strict nonhost pathosystem since some wild-type isolates of *P. infestans* were recently found to infect this plant (F Govers, personal communication; C Smart and WE Fry, personal communication) (Kamoun, 2001).

We elected to employ *Arabidopsis thaliana* as a model for understanding nonhost resistance to oomycete pathogens. Several biotrophic oomycetes, such as *Peronospora parasitica* and *Albugo candida*, are known to infect *Arabidopsis*
(Holub et al., 1995; Parker et al., 1996; Rehmany et al., 2000; Reignault et al., 1996). Cabbage isolates of *Phytophthora brassicae* (previously known as *Phytophthora porri*) (Roetschi et al., 2001) and several isolates of *Phytophthora cinnamomi* (Robinson and Cahill, 2003) can also infect *Arabidopsis* and these pathosystems are expected facilitate the study of host infection by *Phytophthora*. However, most *Phytophthora* species, such as *P. infestans*, and the root pathogen *Phytophthora sojae* cannot infect *Arabidopsis* suggesting that this plant forms an untapped source of resistance to *Phytophthora* (Kamoun et al., 1999; Kamoun, 2001; Takemoto et al. 2003). Considering the impressive set of functional genomic resources that are available, *Arabidopsis* offers good prospects for dissecting the complex interactions that take place between a nonhost plant and an oomycete pathogen and forms both an alternative and a complementary system to ongoing work on resistance of *Nicotiana* to *P. infestans*. In this study, we describe the characterization of a nonhost pathosystem involving *Arabidopsis* and an economically important *Phytophthora* species. Using cytological and molecular analyses, as well as microarray gene expression profiling, we obtained an overview of the active defense responses associated with nonhost resistance of *A. thaliana* to *P. infestans*.
3.3 MATERIALS AND METHODS

Plant growth conditions

*Arabidopsis* (Col-3) seeds were routinely surface sterilized in 70% EtOH for 30 seconds, followed by incubation in 50% bleach solution for 10 minutes. Seeds were then washed multiple times in dH$_2$O before plating onto MS-Phytagar sucrose plates (1XMS salts, 2% w/v sucrose, 0.8% w/v PHYTAGAR). Plated seeds were incubated at 4°C for three to four days prior to germination. Seven-to ten day old seedlings were transferred to potting media and grown under controlled conditions (22°C, 8 hr photoperiod). Mature non-bolting plants at the rosette stage (4-5 weeks) were used for infection experiments.

*Phytophthora infestans* culturing and infection assays

Cultures of *P. infestans* isolate 90128 (A2 mating type, race 1.3.4.7.8.9.10.11, isolated from potato in the Netherlands in 1990), were routinely grown on rye agar medium supplemented with 2% sucrose (Caten and Jinks, 1968). Zoospores were produced by flooding 11-14 day-old cultures with dH$_2$O, followed by incubation at 4°C for 1-3 hrs. Rosette leaves of 4-5 week-old *Arabidopsis* (Col-3) plants, were inoculated with 10-µl droplets of zoospore suspensions for microscopy. For the DNA microarray and Northern time course experiments, complete rosette stage plants were sprayed with zoospore suspensions. Concentrations ranging from 200,000 to 500,000 zoospores per ml were used for all experiments. Deionized water was used as a negative control in all relevant experiments.
Microscopic observations

Leaf discs containing the inoculum were excised at various times after inoculation and examined by microscopy for plant response and growth of *P. infestans*. Lactophenol-trypan blue staining and destaining with chloral hydrate were performed as described earlier (Colon et al., 1992; Wilson and Coffey, 1980). The discs were examined using a Zeiss Axiophot microscope equipped with a high-pressure mercury vapor lamp. Autofluorescence was observed with a G365 excitation filter, FT395 interference beam splitter and LP420 barrier filters.

GUS staining procedure

Complete *Arabidopsis* leaves were immersed in a GUS staining solution (2mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) (Rose Scientific Ltd, Edmonton Alberta, Canada) in 0.2% Triton X-100, 50mM NaHPO4 Buffer (pH7.2), 2mM Potassium Ferrocyanide, 2mM Potassium Ferricyanide). Vacuum was applied for 10 minutes and was then gently released over several minutes. Leaves were incubated at 37°C for 24 hours and examined macroscopically for staining patterns.

Kinetic PCR quantification of *P. infestans*

For kinetic (real-time) PCR quantification of *P. infestans* biomass in plant tissue, four samples were taken for every experimental treatment or time point, with each sample consisting of a pool of four leaf discs corresponding to four independent inoculation sites. Total DNA was extracted from each pool of four
leaf discs using the Qiagen Plant DNA extraction kit following the manufacturer instructions. DNA was quantified using the picogreen ds DNA quantification kit (Molecular Probes, Eugene, OR) and checked by electrophoresis. Dilutions were made using volumes of 10 µl or larger to minimize pipeting errors. Kinetic PCR was performed on a Roche Lightcycler (Roche, Indianapolis, IN) using Lightcycler-FastStart DNA Master SYBR Green I reagents, primers J-08-3 and J-08-4 previously described by Judelson and Tooley (2000), and 2-10 ng of total DNA as template. PCR conditions were 45 cycles of 1 sec at 95°C, 5 sec at 50°C, 10 sec at 72°C with a temperature transition rate of 20°C per sec. Phytophthora infestans relative DNA amounts were estimated using a standard curve generated using 100, 10, 1 and 0.1 pg of purified P. infestans total DNA. Natural log (LN) transformation was applied on the data to obtain distributions that approached normality. Subsequently, ANOVA was performed on the normalized data using both treatment and replication in the model as fixed effects using the SAS statistical software package version 8 (SAS Institute Inc., Cary, NC, USA). Least significant difference mean separation was used to detect significant differences between the time points assayed.

RNA manipulations and Northern blot analysis

RNA was extracted using the Trizol reagent (Gibco-BRL, Bethesda, MD) using manufacturer's directions. RNA samples were checked for purity and integrity using spectrophotometry and electrophoresis. When necessary, samples were cleaned further by adding 1 volume of phenol:chloroform:isoamyl-
alcohol (125:24:1, pH 6.7) to the sample, high-speed centrifugation (14,000 rpm, 15 minutes) and subsequent collection of the aqueous phase. This step was then followed by another extraction using equal volumes of chloroform. RNA was ethanol precipitated from the aqueous phase and dissolved in dH$_2$O. Membranes for Northern blot analysis were prepared using a modified method from McMaster and Carmichael (1977) as described in Sambrook et al. (1989). 15-20 µg of RNA per sample was used and RNA was blotted on Hybond N$^+$ membranes (Amersham, Piscataway, NJ) following standard procedures and instructions from the manufacturer.

Hybridization probes were first prepared using Polymerase Chain Reaction (PCR) amplifications. Primers for amplification of PDF1.2, BGL2 and UBQ5 were described elsewhere (Glazebrook et al., 1996; Penninckx et al., 1996; Xiao et al., 2000). Primers for amplification of the selected genes for microarray validation were: At1g21400-F (5'-GAGAAGTCGATATGGACATGATAC-3') and At1g21400-R (5'-AACGGATGGTGGAGTGAGGAAG-3'); At5g25350-F (5'-CTTCACAATTGCTGGAATCTTCA-3') and At5G25350-R (5'-CGAAGTTCTCAATTGAGACCAG-3'); At2g40000-F (5'-CTTGAATATTGTCTGGAATCTTCA-3') and At2g40000-R (5'-GGAACATCCCAACAAACGGA-3'); At3g43740-F (5'-GAGGAAGATGGTATCATCAG-3') and At3g43740-R (5'-TCCCATTACCGGTGGTGATG-3'). Amplified fragments were purified from TAE-agarose gels and sequenced using an ABI Prism 377 automated sequencer.
Similarity searches were used to confirm amplification of the correct fragments.

All probes were labeled with $\alpha$-$^{32}$P-dCTP using a random primer labeling kit (Gibco-BRL, Bethesda, MD). All Northern blot hybridizations were carried out at 65 °C in Modified Church Buffer (0.36M Na2HPO4, 0.14M NaH2PO4, 1mM EDTA and 7%SDS). Membranes were washed for 15 minutes in 1XSSC/0.5%SDS, and 0.5X SSC /0.5%SDS at 65 °C, followed by 1 wash in 0.5X SSC /0.5% SDS for one minute at room temperature. Membranes were exposed to a phosphor imager screen for 24-48 hours and scanned using a Molecular Dynamics Storm 840 Phosphor Imager.

**DNA microarray hybridizations**

Total RNA samples were subjected to standard probe preparations and microarray hybridization procedures as described in the protocol section on the Arabidopsis Functional Genomics Consortium (AFGC) website (http://afgc.stanford.edu/afgc.html/site2.htm). Briefly, two purified mRNA samples were used for synthesis of probes labeled with either CY3 or CY5 fluorescent dye. Four separately labeled cDNA samples were generated using a dye swap to distinguish replicates of the same treatment. These were hybridized to two slides in a dye-swap experimental setup (technical replication). Hybridized slides were scanned using the Scan Array 3000 (GSI Lumonics, Billerica, MA). Two data points were obtained for each spot on one slide.
Microarray data analysis

The data generated were normalized by the default method specified by the AFGC (http://afgc.stanford.edu/afgc_html/site2.htm). All data analysis and processing steps were done using the SAS statistical software package version 8 (SAS Institute Inc., Cary, NC, USA). For analysis purposes, normalized but 'raw' expression values were downloaded for both hybridized slides (technical replicates) from the AFGC web site. The natural log of every expression value was calculated to obtain a data set with distributions that approached normality. Transformed data points were then used for comparisons applying a regression analysis/outlier detection technique. In short, for every slide, normalized and transformed expression values of both treatments were plotted against each other (X-axis, expression values of mock treatment, Y-axis, expression values of *P. infestans* treatment). A 99% confidence interval (CI) was generated and used to select cDNAs that correspond to data points falling outside of the 99% CI, representing significant differential expression. Lists of identifiers representing the cDNA spots that were detected were compiled. Only cDNAs identified as outliers in both slides were considered significantly differentially expressed and were used for further analysis. The cDNA identifiers were used to retrieve annotated locus names from the *Arabidopsis* Information Resource (TAIR) database (http://www.Arabidopsis.org). A non-redundant data set was generated and was used to construct cluster data files.
Data compilation and cluster analysis

To identify overlap between the genes identified in this study and published data sets, we first compiled files containing nucleotide sequences for the differentially regulated genes reported in the DNA microarray studies of Schenk et al. (2000) and Maleck et al. (2000). We then used amino acid sequences corresponding to the 54 differentially expressed genes (Table 3.1), to perform a tBLASTN search against the generated sequence files. E-value scores below $10^{-12}$ were considered significant. Data matrices were constructed by retrieving and combining expression data of the genes common in either relevant data set. Expression ratios for each gene and treatment were calculated using the inherent control of each treatment as the denominator. Two data matrices were used for cluster analysis using the CLUSTER analysis software package (Eisen et al., 1998). Self-Organizing Map (SOM) analysis was first used to generate classes of genes based on expression ratios across all treatments. Output files generated in this procedure were then used to order the input file for cluster analysis. Complete average linkage hierarchy clustering was used for both comparisons presented. Generated results were visualized and evaluated using TreeView (Eisen et al., 1998).

3.4 RESULTS

Interaction between Arabidopsis and P. infestans

To characterize the interaction between Arabidopsis and P. infestans, we performed repeated inoculations of Arabidopsis with P. infestans zoospores. We
tested numerous inoculation parameters, including *Arabidopsis* leaves at the seedling or rosette stage, multiple combinations of *Arabidopsis* ecotypes and *P. infestans* strains, detached vs. attached leaves, and drop vs. spray inoculations. In all treatments, late blight lesions and sporulation were never observed, whereas infection of the host plant tomato was observed under most of the conditions tested. Normally, no macroscopic symptoms could be detected on *Arabidopsis*, but occasionally, discrete necrotic specks typical of the HR could be observed at the inoculation site, particularly when highly concentrated zoospore solutions were used.

**Cellular responses of *Arabidopsis* to *P. infestans*** (contributed by Vivianne Vleeshouwers)

To determine the cellular responses of *Arabidopsis* to *P. infestans*, we performed microscopic examinations of leaves inoculated at the rosette stage with droplets of zoospores. These analyses revealed penetration of *Arabidopsis* epidermal cells in multiple independent infection sites. Cyst germination, penetration of epidermal cells, and the formation of infection vesicles occurred as early as 46 hours after inoculation, and in some cases was followed by the formation of a short secondary hyphae (Figure 3.1). Penetrated epidermal cells displayed features typical of the HR, including granulated cell cytoplasm, thickened cell walls, condensed nuclei near the penetration site, and autofluorescence under UV light (Figure 3.1). These responses were typically limited to the penetrated epidermal cell.
**Phytophthora infestans** biomass dynamics during infection of *Arabidopsis*

We used quantitative PCR technology (kinetic PCR) to examine changes in *P. infestans* biomass during the interaction with *Arabidopsis* and the host plant tomato (Figure 3.2). Primers specific to highly repetitive sequences (>10,000 x) in the *P. infestans* genome were previously used to quantify relative levels of *P. infestans* DNA in infected plant tissue, and were found to reflect an accurate and sensitive estimate of *P. infestans* biomass (Judelson and Tooley, 2000). We performed kinetic PCR on DNA extracted from discs excised from *Arabidopsis* leaves infected with droplets of *P. infestans* zoospores at successive time points (0, 16, 24, 48, and 72 hours after inoculation). Control treatments included inoculated leaves from tomato (host), and inoculum incubated in water in the absence of plant tissue (no-host). In both *Arabidopsis* and tomato, significant increases in *P. infestans* biomass were observed in the initial 16 hours. *Phytophthora infestans* biomass continued to increase over the three-day period on tomato, whereas it steadily declined on *Arabidopsis* to reach the lowest level at 72 hours after inoculation (Figure 3.2). In contrast, no notable changes in biomass were observed over the three-day period for inoculum incubated in the absence of plant tissue (Figure 3.2). Statistical analyses using ANOVA were performed for each time point and suggested that the changes in biomass observed on *Arabidopsis* are statistically significant (P = 0.0011). Based on a protected mean separation, biomass increased significantly during the initial stages of *Arabidopsis* infection followed by a significant decrease in later stages.
of the interaction (P<0.05). Independent repetitions of the time course and the kinetic PCR experiments demonstrated that these biomass changes are reproducible (data not shown), however, the extent of the decrease in biomass observed at the later stages varied between experiments.

**Local induction of PR1 and BGL2 genes during P. infestans infection**

We used two transgenic *Arabidopsis* lines carrying fusions between the *PR1* and *BGL2* promoters to the β-glucuronidase (GUS) reporter gene (Cao *et al*., 1997; Manners *et al*., 1998; Stone *et al*., 2000) to examine expression of these defense genes during *P. infestans* infection. The transgenic lines were inoculated at the rosette stage with either *P. infestans* zoospores or water droplets (mock treatment) and leaves were excised and stained with X-Gluc at successive time points after inoculation. In both lines, elevated levels of *PR*-gene expression was detected three days after inoculation, as GUS staining around the inoculation sites (Figure 3.3). No GUS expression was detected around mock-inoculated sites. Similar results were obtained from a series of independent experiments. Occasionally, light GUS staining was observed at some sites as early as two days after inoculation, but in most cases GUS staining was only observed three days after inoculation or later.

**Induction of PDF1.2 during P. infestans infection**

We assayed the expression of known defense genes during infection of *Arabidopsis* by *P. infestans* using Northern blot time-course analyses. Rosette
leaves of Arabidopsis plants were sprayed with either water or P. infestans zoospore suspensions, and used for RNA extraction at 0, 16, 24, 48 and 72 hours after inoculation. Northern blot hybridizations were performed with the defense-response genes PDF1.2, BGL2, and the constitutive gene UBQ5 (Figure 3.4). Significant induction of PDF1.2 was observed at 48 hours after inoculation. No induction of BGL2 was observed under the Northern blot conditions over the three-day period. No changes in transcript levels were observed in mock-inoculated plants. Independent replications of the time course suggested that the induction of PDF1.2 is significant and reproducible (data not shown), however, the timing of PDF1.2 induction varied between 16 and 48 hours depending on the experiment.

DNA microarray gene-expression profiling of Arabidopsis nonhost response to P. infestans

To further investigate Arabidopsis nonhost response to P. infestans, we used DNA microarray analyses to determine expression profiles for ca. 11,000 Arabidopsis cDNAs using the Arabidopsis Functional Genomics Consortium (AFGC) Microarray Facility (Wisman and Ohlrogge, 2000). In two hybridization experiments, RNA populations derived from mock treated and P. infestans-infected leaves were compared against each other using a dye-swap approach. Normalized data were subjected to regression analysis and subsequent outlier detection. Lists of outliers extracted from both hybridization data sets were compared to each other and a list containing an overlapping set of cDNAs was
generated. A total of 89 cDNAs were identified that fall outside a 99% confidence interval (CI) in both hybridizations (supplementary data at http://www.oardc.ohio-state.edu/phytophthora/supp.htm). The cDNA identifiers were used to retrieve their respective predicted loci using the TAIR annotation database (http://www.arabidopsis.org) resulting in 54 annotated and non-redundant Arabidopsis genes (Table 3.1). A total of 11 genes were represented by multiple cDNAs (range 2-12) that were identified independently as being differentially expressed in both experiments. This suggests that the experiments and analyses we employed are reliable and robust.

Of the 54 Arabidopsis genes identified as differentially expressed during the nonhost interaction with P. infestans, 52 were up-regulated and 2 were down-regulated. The 54 genes were classified into 7 functional categories that included physiological states related to metabolism, cell wall modification, development, as well as defense and stress responses. Genes that had known functions but that could not be placed in a particular functional category, were compiled in a separate class (Table 3.1).

Validation of DNA microarray analysis using Northern blot analysis

We validated the analysis of the microarray data by regression approach using Northern Blot hybridizations (Figure 3.5). A Northern blot containing the RNA samples that were used in the microarray experiment was hybridized with probes from two genes, At1g21400 and At5g25350, that were selected as differentially expressed (Table 3.1), and two genes, At2g40000 and At3g43740,
that were not. In addition, probes for PDF1.2 and the constitutive gene UBQ5 were included as controls (Figure 3.5). The signals obtained with the various probes were quantified using a phosphor imager, normalized to the UBQ5 signal, and used to calculate induction ratios. The induction levels obtained by Northern blot and microarray hybridization correlated well (Figure 3.5). At1g21400 and At5g25350 were at least 2-fold induced in the Northern blot experiment versus 3 to 5-fold in the microarray hybridizations. In contrast, At2g40000 and At3g43740 showed no significant differential expression by Northern blot hybridization. We also validated the microarray data by Northern Blot analysis using RNA isolated from an independently performed experiment (biological replicate). In this experiment, At1g21400 and At5g25350 were induced 2.8- and 2.4-fold relative to the UBQ5 gene (data not shown). Overall, these results suggest that the microarray experiment and data processing by regression analyses are reliable.

Comparison of Arabidopsis nonhost response to P. infestans to other defense-related treatments

Transcriptional changes observed during P. infestans infection were compared with those reported in two recent microarray analyses of defense-related treatments (Maleck et al., 2000; Schenk et al., 2000). We used tBlastN searches to compare the data set of differentially expressed genes from our study to those published previously (see Material and Methods). Of the 54 Arabidopsis genes represented in our data set, 15 matched cDNAs identified in the study performed by Maleck et al. (2000) and 15 cDNAs identified by Schenk
et al. (2000) (Table 3.1). The expression ratios of the respective genes were extracted from the data sets provided with the two studies and used for building two data matrices. Cluster analysis of the two matrices generated an overview of relatedness between the various treatments and *P. infestans* infection (Figure 3.6). Patterns of defense responses induced by MeJA treatment were found more similar to responses induced by *P. infestans* infection, whereas ethylene, SA and *Alternaria* inducing conditions resulted in less similar defense-induction profiles (Figure 3.6A). Similar comparisons to SAR-related treatments as described by Maleck et al. (2000) were made. Cluster analysis revealed a notable resemblance of our expression data to gene expression profiles in *cim11* mutant genotypes, as well as gene expression changes 48 hrs after treatment with the salicylate analog benzothiadiazole (BTH). In addition, *P. infestans* defense responses shared some similarity to those induced by *nim1* over expression, compatible and incompatible *Peronospora parasitica* interactions, *Pseudomonas syringae* (*AvrRpt2*) infection, and early response after BTH treatment (4hrs) (Figure 3.6B). Experimental treatments involving plant genotypes containing the *nahG* gene showed least similarity together with *cim6* and *cim7* and various double-mutant genotypes.

### 3.5 DISCUSSION

We performed cytological and molecular characterization of a nonhost pathosystem involving the model crucifer plant *A. thaliana* and the destructive and economically important oomycete pathogen *P. infestans*. Our cytological
observations confirm previous work by Vleeshouwers et al. (2000) showing that the interaction of *P. infestans* with nonhost plants, including those that are phylogenetically distant from the solanaceous hosts, is typically associated with penetration of plant tissue and the HR. However, a more detailed cytological investigation needs to be performed to determine whether the proportion of successful penetration events and the level of HR induction upon penetration differ between host and nonhost interactions. Nevertheless, our results suggest that recognition of *P. infestans* by *Arabidopsis* takes place and may form one important barrier in nonhost resistance. Therefore, a model that evokes an arsenal of *Arabidopsis R* genes that recognize multiple or essential *P. infestans Avr* genes is sufficient to explain nonhost resistance in this pathosystem, but it cannot be ruled out that additional layers of nonspecific defense responses occur. With extensive genetic and genomic resources available, the described *Arabidopsis-P. infestans* pathosystem offers excellent prospects for dissecting the complex layers that may form nonhost resistance.

In addition to cytological analyses, we used kinetic PCR to monitor relative levels of *P. infestans* DNA and consequently biomass during infection (Figure 3.2). Previously, DNA and RNA blot hybridizations have been used to estimate the biomass of pathogenic oomycetes in plant tissue (Kamoun et al., 1998; Rairdan et al., 2001). However, these techniques are not sensitive enough to monitor the small changes in *P. infestans* biomass that are expected to occur on nonhost plants. In contrast, kinetic PCR is highly sensitive, quantitative, objective and should prove ideal for nonhost pathosystems. To enhance the sensitivity of
the kinetic PCR quantification, we used primers corresponding to highly repetitive (>10,000 x) sequences from the *P. infestans* genome that allow amplification of as little as 10 fg of *P. infestans* DNA (Judelson and Tooley, 2000). The sensitivity of these primers is obvious since we routinely obtained quantifiable signals from inoculation sites bearing as little as 1000 zoospores.

*Phytophthora infestans* exhibited dynamic changes in biomass over a three-day infection of *Arabidopsis* (Figure 3.2). Over the first 16 hours, a significant increase in biomass was observed that was similar to the increase observed on the host tomato. This early increase may correspond to germination of cysts, penetration of plant epidermis, and formation of infection vesicles and short secondary hyphae as determined by cytology on both *Arabidopsis* and host plants. This suggests that some level of growth and nuclear division, perhaps in the infection vesicles or secondary hyphae, occurs in *P. infestans* during early infection of *Arabidopsis*. Subsequently, a gradual but significant decrease in *P. infestans* biomass was observed from 24 to 72 hours after inoculation of *Arabidopsis* and contrasted sharply with the steady increase observed on tomato. This decrease may reflect death and degradation of *P. infestans* hyphae caused by the HR and correlates with the termination of pathogen ingress determined by cytology. Interestingly, the dynamic changes of *P. infestans* biomass observed on *Arabidopsis* contrasted with the constant level of biomass observed for *P. infestans* cysts germinating in water in the absence of plant tissue. These results support the interpretation that successful penetration of the plant epidermis rather than surface growth is required for the biomass increase.
we observed in the early stages of the interaction. Altogether, these results indicate that *P. infestans* is able to successfully initiate an infection on *Arabidopsis* and complement the cytological analyses.

To gain a first insight into the molecular aspects of *Arabidopsis* nonhost response to *P. infestans*, we examined changes in the expression of defense genes using Northern blot hybridizations with probes for *PDF1.2* and *BGL2* (Figure 3.4), and the transgenic lines *PR1::GUS* and *BGL2::GUS* (Figure 3.3). Although no induction of *PR1* and *BGL2* was detected by Northern blot analyses, localized expression of both genes was detected around inoculation sites in the transgenic reporter lines starting from three days after inoculation. Since we found *PR1* and *BGL2* to be locally induced at the very late stages of the interaction, the discrepancy between the two methods may point to a difference in sensitivity. Considering that most of the cells in the inoculated leaves are not infected, a dilution effect may have reduced the sensitivity of the Northern analysis.

*PDF1.2* is a marker for the jasmonate (JA)/ethylene (ET)-mediated defense-response pathways and its up-regulation has been associated with numerous pathogen or defense-related treatments (Glazebrook, 2001). On the other hand, *PR1* and *BGL2* are marker genes for the salicylate (SA)-mediated defense pathway that is typically induced following infection by necrotizing pathogens or the HR, and during systemic acquired resistance (SAR) (Glazebrook, 2001; Ryals *et al.*, 1996). Altogether, our data suggest sequential induction of the JA/ET pathway followed by the SA pathway during nonhost
response of *Arabidopsis* to *P. infestans*. Studies in other *Arabidopsis* pathosystems suggest significant cross talk and co-regulation of both SA and JA/ET mediated defense pathways (Clarke *et al.*, 2000; Ellis *et al.*, 2002; Glazebrook, 2001; Schenk *et al.*, 2000). In addition, these pathways have been shown to work antagonistically as well as in concert to confer enhanced resistance to fungal, bacterial and oomycete pathogens (Cohn *et al.*, 2001; Ellis *et al.*, 2002; van Wees *et al.*, 2000). The direct role of JA, ET and SA signaling in nonhost resistance to *P. infestans* remains to be determined. Quantitative assays using *Arabidopsis* mutant genotypes covering the various branches of known defense pathways are currently underway to address this question.

To gain a better understanding of *Arabidopsis* nonhost responses to *P. infestans*, we performed DNA microarray experiments comparing responses of *Arabidopsis* plants inoculated with *P. infestans* to mock inoculated counterparts. Despite the harsh selection conditions imposed on the data set, a remarkably high level of redundancy was found amongst the positive cDNAs (see supplementary data at http://www.oardc.ohio-state.edu/phytophthora/supp.htm). Subsequent Northern blot analysis validated our regression analysis strategy. There was a clear correlation in induction levels between the microarray experiment and Northern blot hybridizations using two selected genes and two non-selected genes (Figure 3.5). In addition, about one third of the cDNAs that were identified in our experiments overlapped with cDNAs identified in the defense response gene expression profiling studies of Maleck *et al.* (2000) and Schenk *et al.* (2000) (Table 3.1).
We classified plant responses using cluster analyses of gene expression profiles across the *P. infestans* treatments and other defense related treatments (Figure 3.6). *P. infestans*-induced defense responses were most similar to gene-expression changes after MeJA treatment. From the 15 genes that were used for these comparisons, 14 genes were also induced by MeJA according to Schenk et al. (2000), suggesting that nonhost defense responses to *P. infestans* are associated with the activation of the JA response pathway (Figure 3.6A). In contrast, expression profiles of all other treatments, such as ethylene, *Alternaria* and SA, had less similarity to our data set. Similarly, patterns of overlap were found in the comparison of SAR-related treatments (Maleck et al., 2000) to our data set (Figure 3.6B). Cluster analysis of expression profiles indicated similarity between treatments and the occurrence of two general groups of *Arabidopsis* genes within the set of *P. infestans*-up-regulated genes. Two genes were upregulated 48 hours after BTH treatment and had higher expression levels in the *cim11* (constitutive immunity) mutant background. A second and larger group of seven genes was commonly up-regulated in our treatment and during *Peronospora parasitica* compatible and incompatible interactions, BTH treatment (4 hrs), plants over expressing *NIM1*, and *Pseudomonas syringae* infection. Transcript levels of the genes used in this comparison were either unchanged or lowered in all NahG plants and NahG-containing mutants (*cim11*NahG and *cim6*NahG), *cim6* and *cim7* mutant genotypes and other related treatments.

Some notable genes that are induced in the defense-related treatments examined by Schenk et al. (2000) and Maleck et al. (2000) were identified in this
study (Table 3.1). Among these, Rap2.3 (At3g16770), which encodes an AP2 domain transcription factor, was up-regulated during nonhost resistance and many other defense responses suggesting that it may mediate common regulatory steps in defense pathway activation or modulation. Another gene, Cyp83B1 (At4g31500), is a member of a large family of cytochrome P450 genes, and is involved in the production of indole-glucosinolates as well as the plant hormone IAA (auxin) (Bak and Feyereisen, 2001). The expression of Cyp83B1 and related members of this gene family was found to be elevated upon SA and MeJA treatments and were associated with an increase in indole-glucosinolates (Mikkelsen et al., 2003). Smolen et al. (2002) identified a non-functional mutant of Cyp83B1 that showed a lesion-mimic phenotype. Altogether, these data indicate a possible involvement of Cyp83B1, and perhaps indole-glucosinolates, in defense responses and possibly regulation of the HR.

Many of the Arabidopsis genes identified as up-regulated during nonhost response to P. infestans could be related to cellular aspects of signaling and defense. For instance, four glycosyl hydrolase genes (At3g13750, At5g49360, At5g56870 and At5g64570), that are possibly involved in modifications of cell wall components were up-regulated two- to five-fold (Table 3.1). During cell stress and pathogen attack, cell wall modifications are commonly observed (Heath, 1998; Nicholson and Hammerschmidt, 1992; Vleeshouwers et al., 2000) which is also illustrated by the cell wall depositions described in figure 3.1. Therefore, cell wall alterations are likely to form a major barrier in nonhost resistance. Other notable genes that were upregulated include genes related to
oxidative stress such as catalase (At1g20620), glutathione transferases (At2g30860 and At4g02520), and peroxidase (At2g37130). The occurrence of these genes during nonhost HR is not surprising since the role of oxidative stress and production of active oxygen species (AOS) during the HR is well documented (Delledonne et al., 2001; Levine et al., 1994; Sasabe et al., 2000). Since the HR is observed in the Arabidopsis-P. infestans interaction, induction of these genes in concert with early defense is plausible. Overall, these data supports the concept that defense responses induced by P. infestans involve the HR as well as JA-mediated signaling and defense.

In addition to genes in common between nonhost and various host defense treatments, some genes uniquely up-regulated during the P. infestans interaction were identified (Table 3.1). The function of these genes in nonhost resistance remains unclear, but they represent attractive candidates for functioning in processes unique to nonhost resistance to P. infestans, and perhaps, other nonhost pathogens.

In this study, we characterized the interaction between P. infestans, and the nonhost plant A. thaliana. An integrated multifaceted approach has enhanced our understanding of this interaction and is helping us devise future research strategies. Based on the diversity of molecular genetic tools and genomic resources available for Arabidopsis and Phytophthora, we expect this nonhost pathosystem to become of key importance in studies on molecular plant-microbe interactions. Further research on this pathosystem will provide significant insight into key molecular processes regulating nonhost resistance to an economically
important pathogen. The knowledge gained will result in immediate biotechnological applications and will offer novel perspectives for engineering durable resistance in crop plants.

**Microarray data availability**

The entire data set can be freely obtained and searched at the AFGC website (http://afgc.stanford.edu/afgc_html/site2.htm). Data for the differentially regulated genes is also provided as a supplementary file (http://www.oardc.ohio-state.edu/phytophthora/supp.htm).

**A modified version of this chapter has been published as:**

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3.7 REFERENCES


Figure 3.1. Hypersensitive response (HR) in *Arabidopsis* 46 hours after inoculation with *Phytophthora infestans*. Upon penetration of *P. infestans*, an infection vesicle and a secondary hypha were formed, and the HR was induced in a single epidermal cell. Characteristics of the HR cell are: (A) granular structure of the cytoplasm noted with DIC optics, (B) fluorescence of cytoplasm and cell wall with UV illumination and thickening of the cell wall. cw, thickened cell wall; h, hyphae; HR, HR cell; iv, infection vesicle; n, nucleus, bar =15 μm.
Figure 3.2. Kinetic PCR quantification of Phytophthora infestans biomass upon germination and penetration of Arabidopsis (nonhost, open squares), tomato (host, solid squares) and in the absence of a plant substrate (no-host, open triangles). Zoospore suspensions of P. infestans were used to inoculate the respective plants. Four samples, each containing 4 leaf discs, were harvested at 0, 16, 24, 48, and 72 hrs after inoculation and used for DNA extraction. Four uninoculated leaf discs were added to the no-host treatment upon harvesting. Two ng of total DNA was used for every sample as template for the PCR amplifications. DNA quantities were estimated after a natural log transformation of the obtained values. Arbitrary units of P. infestans DNA were used.
Figure 3.3. Induction of Arabidopsis PR1::GUS (A) and BGL2::GUS (B) expression by Phytophthora infestans. Transgenic lines were drop inoculated with P. infestans zoospore suspensions. Leaves were harvested and stained with X-Gluc three days after inoculation with a droplet of P. infestans zoospores. Local expression of GUS was detected in both transgenic lines, three days after inoculation. Mock-inoculated sites did not show any detectable staining (Left side of leaf in Panel A).
**Figure 3.4.** Time course Northern blot analysis of genes expressed in *Arabidopsis* rosette leaves 0, 16, 24, 48 and 72 hrs after inoculation with *Phytophthora infestans* zoospores or mock inoculation with water. The probes corresponded to *PDF1.2*, a marker gene for the jasmonic acid pathway, and *BGL2*, a marker gene for the salicylic acid-mediated defense pathway. As a loading control, a probe for the constitutive ubiquitin 5 (*UBQ5*) gene was used.
Figure 3.5. Validation of microarray analyses using Northern blot hybridization. RNA samples obtained from Arabidopsis rosettes 16 hours after inoculation with Phytophthora infestans (Inf) or mock inoculation with water (H₂O) were blotted and hybridized with probes for At1g21400 and At5g25350, that were selected as differentially expressed based on microarray data analysis, and At2g40000 and At3g43740, that were not selected. PDF1.2 and UBQ5 were used as a positive control and a loading control, respectively. The numbers on the right correspond to the induction levels based on the Northern blot (RNA blot), and the two microarray experiments (Slide I & II).
Figure 3.6. Cluster analysis illustrating relatedness of transcriptional changes between *Phytophthora infestans* and other defense-related treatments. The *P. infestans* data sets corresponding to two microarray experiments (*P. infestans* I and II) were combined with overlapping data from the transcriptional profiling experiments reported by Schenk *et al.* (2000) (A) and Maleck *et al.* (2000) (B). The gene numbers are indicated on top, and the defense treatments were described in Schenk *et al.* (2000) and Maleck *et al.* (2000). Red color corresponds to up-regulated genes, whereas green color represents down-regulated genes.
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Table 3.1. Arabidopsis gene loci and number of representative cDNAs found differentially expressed during Phytophthora infection by micro-array analysis. 11,000 cDNAs were used as targets for labeled cDNA from the two treatments. Genes were selected or considered significantly differentially expressed when data points fell out of a 99% confidence interval during regression analysis in two separate comparisons (slide 1 and slide 2). Genes that were found in previous studies (Maleck et al. 2000; and Schenk et al. 2000) are indicated.
CHAPTER 4

DIFFERENCES IN INTENSITY AND SPECIFICITY OF HYPERSENSITIVE RESPONSE INDUCTION IN NICOTIANA BY INF1, INF2A AND INF2B OF PHYTOPHTHORA INFESTANS

4.1 ABSTRACT

Elicitins form a family of structurally related proteins that induce the hypersensitive response (HR) in plants, particularly Nicotiana spp. The elicitin family is composed of several classes. Most species of the plant pathogenic oomycete genus Phytophthora produce the well characterized 10-kDa canonical elicitins (class I), such as INF1 of the potato and tomato pathogen Phytophthora infestans. Two genes, inf2A and inf2B, encode a distinct class (class III) of elicitin-like proteins and also occur in P. infestans. Unlike secreted class I elicitins, class III elicitins are thought to be cell-surface anchored polypeptides. Molecular characterization of the inf2 genes indicated that they are widespread in Phytophthora species and occur as a small gene family.
Southern blot and Northern blot hybridizations using gene-specific probes showed that *inf2A* and *inf2B* genes and transcripts can be detected in seventeen different *P. infestans* isolates. Functional secreted expression in plant cells of the elicitin domain of the *inf1* and both *inf2* genes was conducted using a binary potato virus X (PVX) vector (agroinfection) and *Agrobacterium tumefaciens* transient transformation assays (agroinfiltration), and resulted in HR-like necrotic symptoms and induction of defense response genes in tobacco. However, comparative analyses of elicitor activity of INF1, INF2A and INF2B revealed significant differences in intensity, specificity and consistency of HR induction. Whereas INF1 induced the HR in *Nicotiana benthamiana*, INF2A induced weak symptoms, and INF2B induced no symptoms on this plant. Nonetheless, similar to INF1, HR induction by INF2A in *N. benthamiana* required the ubiquitin ligase-associated protein SGT1. These results suggest that variation in the resistance of *Nicotiana* to *P. infestans* is a reflection of variation in the response to INF elicitins. The ability of *N. tabacum*, but not *N. benthamiana*, to respond to INF2B could explain differences in resistance to *P. infestans* observed for these two species.

### 4.2 INTRODUCTION

Specific recognition events are well established as the functional basis of numerous incompatible (resistance) interactions between plants and pathogens, particularly those occurring at the subspecific or varietal level. This is also known as race or cultivar- specific resistance. Recognition is defined by the direct or
indirect perception of pathogen signal molecules by plant receptors (Dangl and Jones 2001; Staskawicz et al. 1995). The pathogen signal molecules are commonly referred to as elicitors, encoded by avirulence (Avr) genes, whereas the plant receptors are often though to be resistance proteins encoded by R genes. Recognition results in the induction of signal transduction pathways leading to the expression of complex defense responses including the hypersensitive response (HR), a form of programmed cell death often associated with disease resistance in plants (Dangl et al. 1996). Numerous examples of race/cultivar specific interactions follow the Avr-R gene model; however, the extent to which recognition events are involved in incompatible interactions occurring at the species or genus level (nonhost resistance) remains unclear (Heath 2000; Kamoun 2001; Kamoun et al. 1999a).

The oomycete plant pathogen Phytophthora infestans causes late blight, a devastating and re-emerging disease of potato and tomato (Birch and Whisson 2001; Fry and Goodwin 1997a; Fry and Goodwin 1997b; Schiermeier 2001; Shattock 2002; Smart and Fry 2001). In contrast to host plants, nonhosts, such as tobacco and other species of the genus Nicotiana, are typically resistant to P. infestans. Cytological analyses of leaves of several Nicotiana species inoculated with P. infestans showed that penetration of epidermal cells always occurred (Kamoun et al. 1998a). This was followed by the HR that varied between different Nicotiana species in timing, severity and number of affected cells. In Nicotiana tabacum (tobacco), P. infestans was blocked early in the infection following penetration of epidermal cells and secondary intercellular hyphae were not
observed. In contrast, in *Nicotiana benthamiana*, secondary hyphae with haustoria were formed and some level of mesophyll colonization occurred. The plant response reached a climax three days post inoculation with clusters of HR cells engulfing the invading hyphae. These observations suggest that several layers of resistance to *P. infestans* occur with various degrees of effectiveness in the different *Nicotiana* species (Kamoun 2001; Kamoun *et al*. 1998a; Kamoun *et al*. 1999a).

*P. infestans* as well as other *Phytophthora* species express a family of structurally related extracellular proteins, known as elicits, which induce the HR and other biochemical changes associated with defense responses in *Nicotiana*, but not in potato and tomato (Kamoun *et al*. 1993; Kamoun *et al*. 1997a; Ponchet *et al*. 1999; Ricci *et al*. 1989; Sasabe *et al*. 2000). *P. infestans* strains deficient in the elicitin INF1 induced disease lesions in *N. benthamiana*, suggesting that INF1 conditions resistance in this species (Kamoun *et al*. 1998a). In contrast, INF1 deficient strains remained unable to infect other *Nicotiana* species, such as tobacco. In this case, tobacco was hypothesized to react to additional elicitors, perhaps other elicitin-like proteins (Kamoun 2001; Kamoun *et al*. 1998a; Kamoun *et al*. 1999a). Indeed, in *P. infestans*, a complex set of elicitin-like genes was isolated using PCR amplification with degenerate primers, low stringency hybridizations, and random sequencing of cDNAs (Fabritius *et al*. 2002; Kamoun *et al*. 1997a; Kamoun *et al*. 1997b; Kamoun *et al*. 1999b). In total, eight elicitin and elicitin-like genes (termed *inf* genes) have so far been reported in *P. infestans*. All these genes encode putative extracellular proteins that share the
98 amino-acid elicitin domain corresponding to the mature class I elicitins, such as INF1. This domain is defined as the elicitin domain in many protein motif databases, such as pfam (PF00964) (Bateman et al. 2002) and InterPro (IPR002200) (Mulder et al. 2003). Six inf genes (inf2A, inf2B, inf5, inf6, inf7, and M-25) encode predicted proteins with a C-terminal domain in addition to the N-terminal elicitin domain. Sequence analysis of these C-terminal domains revealed a high frequency of serine, threonine, alanine, and proline. The amino-acid composition and the distribution of these four residues indicated the likely occurrence of clusters of O-linked glycosylation sites (Kamoun et al. 1997a). These proteins are likely to form a ‘lollipop on a stick’ structure in which the O-glycosylated domain forms an extended rod that anchors the protein to the cell wall leaving the extracellular N-terminal domain exposed on the cell surface (Jentoft 1990). Therefore, these INF proteins may be surface or cell wall associated glycoproteins that could interact with plant cells during infection.

The intrinsic biological function of elicitins in Phytophthora has long remained a mystery. Conclusive evidence finally emerged when it was demonstrated that class I elicitins bind sterols, such as ergosterol, and function as sterol-carrier proteins (Boissy et al. 1999; Mikes et al. 1997; Mikes et al. 1998; Vauthrin et al. 1999). Consequently, elicitins were hypothesized as having a biological function of essential importance to Phytophthora spp. since they cannot synthesize sterols and must assimilate them from external sources (Hendrix 1970). In addition, phospholipase activity was assigned to elicitin-like proteins from Phytophthora capsici with significant similarity to INF5 and INF6.
(Nespoulous et al. 1999) suggesting a general lipid binding/processing role for the various members of the elicitin family (Osman et al. 2001a). Other work by Osman et al. (2001b) using elicitin mutants altered in sterol binding revealed that sterol loading is important for specific-binding to a plasma membrane receptor and induction of the HR in tobacco. More recently, another gene with similarity to elicitins, M-25, was reported to be induced during mating in P. infestans (Fabritius et al. 2002) suggesting a wider role of elicitors in Phytophthora biology.

In this paper, we report the molecular and functional characterization of P. infestans genes encoding the class III elicitin-like INF2A and INF2B proteins (Kamoun et al. 1997a). We examined the occurrence of inf2 sequences in P. infestans and other Phytophthora spp., the full genomic sequence of the inf2A gene, and the expression of the inf2 genes in various isolates of P. infestans and during the P. infestans-tomato interaction. In addition, we compared INF2 proteins to the well characterized INF1 elicitin for their elicitor activity using both the binary potato virus X (PVX) expression system (agroinfection) and Agrobacterium tumefaciens transient transformation assays (agroinfiltration). Lastly, we characterized the defense responses induced by INF2 in N. tabacum, and showed that, similar to INF1, necrosis induction by INF2A in N. benthamiana requires the ubiquitin ligase-associated protein SGT1. These experiments revealed significant differences in intensity, specificity and consistency of HR induction between INF1, INF2A and INF2B. We found that N. tabacum, but not N. benthamiana, responded to INF2B. This could explain differences in resistance to P. infestans observed for these two species.
4.3 MATERIAL AND METHODS

Microbial strains and culture conditions

The various *P. infestans* isolates used in this study were described previously (Kamoun *et al.* 1998b). *P. infestans* isolates were routinely cultured on rye agar medium supplemented with 2% sucrose (Caten and Jinks 1968) or in still cultures in the synthetic medium described by Kamoun *et al.* (1994).

*E. coli* XL1-Blue and DH5α were used in most experiments and were routinely grown at 37°C in Luria-Bertani (LB) media (Sambrook *et al.* 1989). *A. tumefaciens* strain EHA105 (Hood *et al.* 1993) and GV3101 (Holsters *et al.* 1980) were used. All bacterial DNA transformations were conducted by electroporation.

DNA manipulations and plasmid constructions

DNA manipulations and screening of the λEMBL3 library were conducted essentially as described elsewhere (Ausubel *et al.* 1987; Sambrook *et al.* 1989). Total DNA of *P. infestans* was isolated from mycelium grown in liquid culture as previously described (Pieterse *et al.* 1991). Alkaline DNA transfer to Hybond N+ (Amersham, Arlington Heights, IL) and Southern hybridizations were performed at 65°C as described elsewhere (Ausubel *et al.* 1987; Sambrook *et al.* 1989). Filters were typically washed at 55°C in 0.5x SSC (75 mM NaCl and 7.5 mM sodium citrate) except for the blot shown in Figure 4.1, which was washed at low stringency (room temperature in 2x SSC). Dideoxy chain-termination sequencing was carried out using an AmpliCycle sequencing kit (Perkin Elmer, Foster City, CA).
Plasmid pFB60 was obtained by subcloning a gel purified 1.7 kb. *Hind*III fragment from an inf2A-containing λ.EMBL3 clone into pBluescript SK-(Stratagene, San Diego, CA). Sequencing of the full insert of pFB60 was conducted using vector primers as well as a series of internal sequencing primers.

Plasmids pGR106-INF2A and pGR106-INF2B were constructed by cloning polymerase chain reaction (PCR) amplified DNA fragments corresponding to a fusion between the signal sequence of the *PR-1a* gene of tobacco (Hammond-Kosack *et al.* 1995) and the sequence of the 98 amino-acid elicitin domain of INF2A and INF2B (Kamoun *et al.* 1997) into the *Cla*I site of pGR106 (Lu *et al.* 2004; http://www.jic.bbsrc.ac.uk/Sainsbury-Lab/david-aulcombe/Services/vigsprotocol.htm) using the overlap extension strategy described by Kamoun *et al.* (1999). The oligonucleotides used in the PCRs are PVX-F (5'- AATCAATCACAGTGTTGGCTTGC-3') and PR-INF2A (5'-GGCGAGCACGTCTCGGCACGGCAAGAGTGGGATATTAC-3'); PR-INF2B (5'-CTTGCCGTGCCGAGACGTGCCGCCCACG-3') and INF2-RSC (5'-GTGGAGCTCATCGATCAGGACGAGGAGCACTTCTTGGAG-3'). *Sac*I and *Cla*I restriction sites were introduced in INF2-RSC and are underlined. The resulting recombinant plasmids, pGR106-INF2A and pGR106-INF2B, were confirmed by DNA sequencing to have a *PR1a::inf2* fusion inserted in the sense orientation with regard to the duplicated PVX coat protein promoter. The pGR106-INF1 plasmid was constructed by cloning the *PR1a::inf1* fusion sequence (Kamoun *et al.* 1999) into the *Cla*I site of pGR106.
For agroinfiltration experiments, plasmids p35S-INF1 (previously named pInf1) was described earlier (Kamoun et al. 2003). Plasmids p35S-INF2A and p35S-INF2B were constructed by cloning PCR amplified DNA fragments corresponding to the PR-1a::inf fusions from the respective pGR106-INF constructs as Ncol and SacI fragments into pAvr9 (Van der Hoorn et al. 2000). The primers used for PCR were PR1-FNCO (5'-GCATCCATGGGATTTGTTCTTTTCACAA-3') and INF1-RSAC (5'-GGCGAGCTCTCATAGCGACGCACACGTAG-3') for PR1a::inf1 and INF2-RSC for PR1a::inf2. The introduced Ncol and SacI restriction sites are underlined. The resulting p35S-INF plasmids were confirmed by DNA sequencing to contain intact PR1a::inf ORFs flanked by the Cauliflower mosaic virus (CaMV) 35S promoter and the omega Tobacco mosaic virus (TMV) leader on the 5' side, and the potato proteinase-II terminator region on the 3' end.

**RNA manipulations, Northern blot hybridizations and RT-PCR analyses**

Total RNA was isolated from *P. infestans* mycelium using the guanidine hydrochloride extraction method (Logemann et al. 1987), and from *N. tabacum* using the Trizol RNA extraction protocol following the manufacturer recommendations (Gibco-BRL, Bethesda, MD). For Northern blot analyses, 10-15 µg of total RNA was denatured at 50°C in 1 M glyoxal, DMSO, and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N+ membranes (Amersham, Arlington Heights, IL) (Ausubel et al. 1987; Sambrook et al. 1989). Hybridizations were conducted at 65°C in 0.5 M sodium phosphate buffer, 7%
SDS and 1 mM EDTA. Filters were washed at 55°C in 0.5x SSC (75 mM NaCl and 7.5 mM sodium citrate) for the Phytophthora blots or at 65°C in 0.5x SSC for the plant blots. For the RT-PCR experiments, cDNA derived from a P. infestans-tomato time-course experiment was generated as previously described (Tian et al. 2004). Equal amounts of cDNA were subjected to PCR amplification using the following primers:

INF1TEV-F (5'-GGGAAATCGATACCACGTGCACCACCTCGCA-3'), INF1TEV-R (5'-GGGAAATCGATTAGCGACGCACACGTAGACG-3')
INF2TEV-F (5'-GGGAAATCGATGAGACGTGCTCGCCCACGGAC-3') INF2A-Rnew (5'-CGCATAGCACCTAAACAAGCCGCGGCGG-3'). Primers described by Torto et al. (2002) were used for amplification of elongation factor 2 alpha (ef2α).

**Hybridization probes**

Probes from the inf2 genes were obtained as gel purified DNA fragments containing essentially the signal peptide and elicitin domain (amino acids 1-126) of the inf2A and inf2B cDNA inserts, generated by digestions of the original cDNA plasmids (Kamoun et al. 1997a, 1997b) using appropriate restriction enzymes. A probe from the actA gene from pSTA31 (Unkles et al. 1991) was used as a loading control. The PR1a gene probe was generated through PCR amplification of tobacco genomic DNA using the gene specific primers PR1-tob-F (5'-ATGGGATTTGTTCTTTTTCACAA-3') and PR1-tob-R (5'-GTATGGACTTTGCCCTCTATAATTAC-3'). A probe from the alpha-tubulin gene was amplified from a Nicotiana otophora cDNA clone using vector primers.
Probes were radiolabeled with either $\alpha$-32P-dATP or $\alpha$-32P-dCTP using a random primer labeling kit (Gibco-BRL, Bethesda, MD).

In order to obtain probes specific to the various genes, we used the primer extension strategy described by Kamoun et al. (1997b). Single stranded, radio labeled probe complementary to the 3’ end untranslated region of the inf1 mRNA was generated by extending primer INF2-F1 (Kamoun et al. 1997b) from the gel purified inf1 insert from pFB7. Single stranded, radiolabeled probes complementary to the 3’ end untranslated region of the inf2A, and inf2B mRNAs were generated by extending primer INF2-F2 (5’-CCACCGCGGCTTGTAA-3’) from XhoI digested pFB5 and pFB24, respectively. The sequence corresponding to the TAA stop codon of the inf2A and inf2B open reading frames is underlined. The labeling reactions were performed as previously described (Kamoun et al. 1997b).

**PVX agroinfection assays**

*N. tabacum* (cv. Xanthi) and *N. benthamiana* plants with fully expanded leaves were used for the agroinfection assays. Plants were cultured and maintained in a greenhouse with an ambient temperature of 22-25°C and high light intensity. Inoculations were performed by dipping a wooden sterile toothpick in a recombinant *A. tumefaciens* GV3101 (pGR106-INF) colony grown on solid agar medium and wounding each leaf twice around the main vein. An excess of bacteria was used for the inoculations. Local necrotic symptoms were scored daily and typically started developing within 5-7 days after inoculation. In
addition, lesion sizes were measured and recorded. Frequencies of necrotic lesions were calculated and used to obtain percentage of necrosis induction.

All constructs were re-evaluated on young *N. benthamiana* plants at approximately the 3-4-leaf stage (ca. 3-week-old). Inoculations were then performed on two lower leaves by wounding each leaf twice around the main vein and near the base of the leaf with the *A. tumefaciens* strain. Mosaic, local and systemic necrotic symptoms were scored daily and typically started developing within 5-7 days after inoculation.

**Agroinfiltration assays**

Recombinant *A. tumefaciens* strains containing the various binary plasmids were prepared for agroinfiltration as previously described (Kapila *et al.* 1997; Van der Hoorn *et al.* 2000). Cultures were infiltrated into young and fully expanded leaves. Most p35S-INF1 and p35S-INF2 infiltrations were conducted side by side and repeated at least three times. Plants were visually evaluated 2-3 days after infiltration.

**GUS assays**

We used a transgenic tobacco line (cv. Samsun NN) carrying a *Bgl2::GUS* reporter construct. The selected line (gglb-1233-3), generated by Livne *et al.* (1997) to express a chimeric gglb50 promoter (basic beta-1,3-glucanase. GenBank X53600) fused to the GUS reporter gene, contains the gglb50 promoter region between positions -1233 to +19. Histochemical GUS staining was
performed using 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid (X-Gluc) (Rose Scientific, Edmonton Alberta, Canada) as previously described by Huijtema et al. (2003).

TRV-silencing experiments

*Agrobacterium* strains carrying the pBINTRA6 (RNA 1 vector), pTV00 (RNA 2 vector), pTV00:SGT1 (Ratcliff et al. 2001; Peart et al. 2002) were prepared for agroinfiltration as described above, and mixed in a 2:1 RNA 1:RNA 2 ratio. The SGT1 insert corresponds to the *NbSGT1.1* gene (GenBank accession no. AF516180). Mixed cultures were incubated for at least 2 hours before infiltration. The youngest fully expanded leaves of *N. benthamiana* plants (5-leaf stage) were infiltrated with the *Agrobacterium* suspensions using needleless syringes. Challenge inoculations using agroinfiltration or PVX agroinfection assays were started three weeks after TRV inoculation and performed as described above.

### 4.4 RESULTS

**Occurrence of inf2 sequences in Phytophthora species** (contributed by Sophien Kamoun and Vivianne Vleeshouwers)

The products of the *inf2A* and *inf2B* genes form a distinct class of elicitors previously designated class III (Kamoun et al. 1997a). In order to assess the occurrence and distribution of sequences similar to *inf2* across a range of *Phytophthora* species, total DNA from 13 isolates representing nine
Phytophthora species was hybridized at low stringency with a probe from the elicitin domain of inf2 (Figure 4.1). This inf2 probe hybridized to both inf2A and inf2B, but under similar hybridization conditions, no cross-hybridization between this probe and other P. infestans inf elicitin genes was observed. All tested isolates of the examined Phytophthora species appeared to contain from two to eight HindIII bands homologous to the inf2 elicitin domain (Figure 4.1). Similar hybridization experiments on total DNA from four additional oomycete species (Pythium aphanidermatum (isolate 28), Pythium sylvaticum (933), Aphanomyces leavis (465.64), and Saprolegnia ferax (G-1295) did not yield any detectable signals (data not shown). It therefore appears that inf2-like elicitin genes may occur as a small genus-specific gene family and are conserved in all tested species of Phytophthora.

Isolation and characterization of inf2A genomic region (contributed by Sophien Kamoun and Vivianne Vleeshouwers)

To determine the genomic structure of the inf2A gene, a λ.EMBL3 genomic library of P. infestans 88069 (Pieterse et al. 1993) was hybridized with the inf2 probe. A total of five hybridizing clones were identified. DNA from these clones was digested with HindIII, blotted, and hybridized with the inf2 probe (data not shown). Three of the clones contained a 1.7 kb. HindIII hybridizing band that co-migrated with one of the bands revealed on the total DNA blot (Figure 4.1, lane 1). The other two positive clones were not reconfirmed in subsequent hybridizations. The 1.7 kb. HindIII fragment was subcloned into pBluescript SK-
(Stratagene, San Diego CA) and fully sequenced using a primer walking approach. The nucleotide sequence revealed a 1,654 bp. *HindIII* fragment (GenBank accession AY693804) was found to contain a 558 bp. open reading frame (ORF) that perfectly matched the ORF in the *inf2A* cDNA sequence, suggesting that, similar to other elicitin genes from *Phytophthora*, the *inf2A* gene does not contain introns. Examination of the nucleotide sequence upstream of the ORF revealed at position -50 relative to the ATG start codon a sequence "TCTCATTCTACAATTT" similar to the oomycete transcriptional start site motif (Kamoun 2003; Pieterse et al. 1993; McLeod et al. 2004). Downstream of the ORF, the 51 bp. sequence that correspond to the 3' untranslated region contained a potential polyadenylation signal ATTAAA, located 18 bp. downstream of the TAA stop codon. No significant similarities between the noncoding sequences of the *inf2A* gene and the noncoding sequences of other elicitin genes were noted. In this screening, no genomic clone corresponding to *inf2B* was recovered from the genomic library.

**Occurrence of inf2A and inf2B in P. infestans** (contributed by Sophien Kamoun and Vivianne Vleeshouwers)

To determine whether the *inf2A* and *inf2B* genes are conserved in *P. infestans*, *BamH*I digested total DNA from a collection of 16 isolates of *P. infestans* (Kamoun et al. 1998b) was sequentially hybridized with gene-specific probes containing 3'end portions of the *inf2A* and *inf2B* cDNAs as well as a specific 3' end probe of the *inf1* cDNA (Kamoun et al. 1997a) (Figure 4.2).
probes lack a BamHI site. One to two genomic copies for each of the inf2A and inf2B genes could be detected in all 16 P. infestans isolates examined, whereas a single inf1 band was revealed. In some isolates, both the inf2A and inf2B probes revealed bands with lower intensity. Since, no cross-hybridization was noted between the inf2 probes and other inf elicitin genes under the hybridization conditions used, we expect these bands to contain inf2-like sequences. However, we cannot conclude at this stage whether the faint bands correspond to additional alleles/gene copies of inf2 or to pseudogene sequences. GE900083, an isolate from Germany, lacked the strongly hybridizing inf2A band observed in all other isolates.

*inf2A and inf2B mRNAs are produced by all tested P. infestans isolates*  
(contributed by Sophien Kamoun and Vivianne Vleeshouwers)

A small number of field isolates of P. infestans are deficient in mRNA of the elicitin gene inf1 and in INF1 protein (Kamoun *et al.* 1998b). To determine whether these and other isolates show altered levels of inf2A and inf2B mRNA, total RNA from the 16 isolates of P. infestans examined in Figure 4.2, as well as P. infestans 88069, was sequentially hybridized with the inf2A, inf2B, and inf1 gene-specific probes (Figure 4.3). All tested isolates showed detectable levels of inf2A and inf2B mRNA, suggesting that inf2 mRNAs are produced by all tested P. infestans isolates including the two isolates, DDR7602 and DDR7702, that were previously shown to lack inf1 mRNA (Kamoun *et al.* 1998b). However, in this experiment, levels of inf2 mRNA were variable between the examined isolates.
GE900083, the isolate that lacked the major inf2A band in the Southern blot analyses also produced a signal for inf2A mRNA.

**inf2A and inf2B are expressed during the P. infestans- tomato interaction**

We determined the expression profiles of inf2A and inf2B genes during a time course infection of tomato by *P. infestans* using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Gene specific primers for inf2A, inf2B, inf1 and the constitutive elongation factor 2-alpha (ef2α) gene were used. Expression of inf2A was detected 3 days after inoculation, whereas expression of inf1 and inf2B was observed as early as 1 day after inoculation (Figure 4.4). In contrast to inf1, which reached the highest levels of expression at the latest time point (day 4), inf2A expression peaked at day 3 and inf2B at days 3 and 4. These results show that both inf2 genes are expressed during *P. infestans* colonization of tomato.

**Heterologous expression of inf2A and inf2B in plants using agroinfection of Potato virus X**

To express the inf2 genes in plant cells and examine their elicitor activity, we first used the *Potato virus X* (PVX) system, which proved effective in assaying the HR-inducing activity of the inf1 gene (Kamoun *et al.* 1999c; Qutob *et al.* 2002; Torto *et al.* 2003). A fusion between the signal sequence of the PR-1α gene of tobacco (Hammond-Kosack *et al.* 1995) and the sequence of the 98 amino-acid elicitin domain of INF2A and INF2B (Kamoun *et al.* 1997a) was cloned into the binary PVX vector pGR106 (Lu *et al.* 2004). Two recombinant plasmids pGR106-
INF2A and pGR106-INF2B were confirmed to contain the correct inserts by DNA sequencing and were subsequently introduced into Agrobacterium tumefaciens to allow delivery of PVX in plants via agroinfection. *A. tumefaciens* strains carrying the pGR106-INF2 constructs were inoculated side-by-side on mature leaves of *N. tabacum* (tobacco, cv. Xanthi) and *N. benthamiana*, and compared to a strain carrying a pGR106 derivative expressing the *PR1a::inf1* construct (pGR106-INF1) (Figure 4.5). In tobacco, all three strains induced rapid symptoms consisting of localized HR-like necrotic lesions. In contrast, *N. benthamiana* leaves challenged with the pGR106-INF1 and pGR106-INF2A strains exhibited localized necrotic lesions, whereas the pGR106-INF2B strain failed to cause visible symptoms (Figure 4.5).

In addition to these qualitative differences, there was significant variation in the efficiency and extent of elicitation of necrotic symptoms between the elicitors (Table 4.1). On responding plants, the pGR106-INF1 and pGR106-INF2B strains were very consistent, resulting in necrotic symptoms in at least 93% of the inoculation sites (Table 4.1). In contrast, the pGR106-INF2A strain was poorly efficient resulting in necrotic symptoms in 36 and 70% of the inoculation sites on tobacco and *N. benthamiana*, respectively. Also, the pGR106-INF2A strain induced smaller necrotic lesions averaging 0.9 and 1.9 mm, in tobacco and *N. benthamiana*, respectively, whereas, the pGR106-INF1 and pGR106-INF2B strains induced lesions ranging from 3.2 to 5.8 mm in size on responding plants. Inoculations of all plants with mock and vector controls never resulted in necrotic symptoms.
To determine the significance of our observations, statistical analysis was performed on necrotic lesion size data. ANOVA analysis using the generalized linear models (GLM) procedure were applied using the model; \( Y = \mu + \text{Sp} + \text{Trt} + \text{Leaf} + (\text{Sp} \times \text{Trt}) + \epsilon \) where Sp denotes species and Trt equals elicitor treatment. Subsequent T-test comparisons indicated significant differences between the elicitors tested on both *N. benthamiana* and *N. tabacum* (Table 4.1).

To confirm the lack of response of *N. benthamiana* to INF2B, we repeated the agroinfection inoculations on individual young seedlings, an assay that is more sensitive than mature leaf inoculations since it allows systemic spread of the recombinant PVX and enhanced accumulation of the *inf* transcripts (Torto *et al.*, 2003). Inoculation of *N. benthamiana* seedlings with the pGR106-INF2B strain consistently failed to result in any necrotic symptoms and always resulted in mosaic virus symptoms similar to those obtained with the empty vector strain. In contrast, both the pGR106-INF1 and pGR106-INF2A strains induced necrotic lesions starting 6 days after inoculation (data not shown).

Altogether, these results show that, unlike *N. tabacum*, *N. benthamiana* does not respond to INF2B and suggest that INF2A may constitute a weaker HR elicitor than INF1 and INF2B.

**Heterologous expression of inf2A and inf2B in plants using agroinfiltration**

To validate the elicitor activity of INF2A and INF2B and further compare it to INF1, we used agroinfiltration to express the three *inf* genes in plant cells. The *PR1a::inf* gene fusions were transferred to a Cauliflower mosaic virus (CaMV)
35S promoter and a potato proteinase-II terminator cassette in a T-DNA binary vector as described in the methods. *A. tumefaciens* strains carrying the various p35S-INF constructs were infiltrated into young and fully expanded leaves of *N. tabacum* (cv. Xanthi) and *N. benthamiana* (Figure 4.6). As negative controls, *A. tumefaciens* carrying pGUSi, that contains a β-glucuronidase gene interrupted by an intron (Hood *et al.* 1993), as well as buffer solutions were used. In *N. tabacum*, confluent necrosis in the entire infiltrated areas appeared two days following infiltration of the p35S-INF2A, p35S-INF2B, and p35S-INF1 carrying strains. In contrast, in *N. benthamiana*, only the *A. tumefaciens* strain carrying p35S-INF1 consistently induced necrosis, generally starting at three to four days after infiltration. In repeated side by side infiltrations of *N. benthamiana* leaves with the p35S-INF1 and p35S-INF2 strains, the p35S-INF2B strain did not induce necrotic symptoms. However, occasionally, the p35S-INF2A construct caused necrosis in *N. benthamiana*. The negative control strain carrying pGUSi and the buffer solutions did not induce necrosis in both tobacco and *N. benthamiana*. These results confirm that *N. benthamiana* does not respond to INF2B and that INF2A may act as a weaker HR elicitor on this plant species.

**INF2B induces PR1a and Bgl2 expression in tobacco**

To assess whether the necrotic response elicited by the INF2 proteins is associated with the induction of plant defense response genes, we wound inoculated leaves of a transgenic tobacco line carrying the GUS reporter gene driven by the promoter of the pathogenesis related genes *Bgl2 (PR2)* (Livne *et al.*
1997) with *A. tumefaciens* strains carrying the pGR106-INF1, pGR106-INF2A and pGR106-INF2B (Figure 4.7A). Negative controls consisted of the *A. tumefaciens* strain carrying the vector pGR106 and mock inoculations. GUS histochemical staining of inoculated leaves showed some blue staining in the pGR106 treatment suggesting that the vector strain induces low levels of *Bgl2* expression. However, the pGR106-INF1 strain and particularly the pGR106-INF2B strain consistently induced stronger and larger areas of GUS staining than the vector control. The pGR106-INF2A strain did not consistently induce different GUS staining than the controls.

We also performed Northern blot analyses using RNA isolated from leaf discs surrounding inoculation sites of a non-transgenic tobacco line using the same treatments as in the *Bgl2::GUS* experiment (Figure 4.7B). Hybridization of the blots with probes of the pathogenesis related gene *PR1a* and the constitutive gene alpha-tubulin revealed moderate induction of *PR1a* by the vector construct. Nevertheless, both pGR106-INF1 and pGR106-INF2B elicited increased levels of *PR1a* expression. These experiments suggest that, similar to INF1, INF2B induces the expression of the pathogenesis related genes *PR1a* and *Bgl2* in tobacco. However, no significant induction by INF2A could be demonstrated under these experimental conditions.

**SGT1 is required for HR elicitation by INF2A**

Peart *et al.* (2002) demonstrated that the ubiquitin ligase associated protein SGT1 is required for HR induction by INF1 in *N. benthamiana*. To test
whether response to INF2A also requires SGT1, we used Tobacco rattle virus (TRV) to silence SGT1 in N. benthamiana (Ratcliff et al. 2001; Peart et al. 2002; Huitema et al. 2004). For this purpose, we infiltrated young N. benthamiana plants (5-leaf stage) with A. tumefaciens strains containing the binary vector pBintra6 (TRV RNA1) mixed with strains carrying the empty pTV00 vector (TRV RNA2) or pTV00:SGT1 (Peart et al. 2002). Three weeks after infiltration, we performed challenge inoculations using agroinfiltration (Figure 4.8A) or agroinfection (Figure 4.8B) as described above. In both experiments, INF1 consistently induced the HR on plants inoculated with the TRV vector but not on the TRV:SGT1 plants. Similarly, INF2A induced the HR in 20-50% of the inoculations in plants treated with the TRV vector but not on the TRV:SGT1 plants. As noted earlier, INF2B did not induce necrosis in N. benthamiana. These results suggest that, similar to INF1, the HR induced by INF2A in N. benthamiana is SGT1-dependent.

4.5 DISCUSSION

Elicitins form a ubiquitous family of structurally related proteins in Phytophthora. In P. infestans, eight elicitin and elicitin-like genes (inf genes) corresponding to distinct classes have been reported (Fabritius et al. 2002; Kamoun et al. 1997a; Kamoun et al. 1997b; Kamoun et al. 1999b). So far, most studies on P. infestans elicitins have focused on the 98 amino-acid canonical elicitin, INF1 (class Ia) (Kamoun et al. 1997a; Kamoun et al. 1998a; Kamoun et al. 1998b; Kamoun et al. 1999b; Kanzaki et al. 2003; Sasabe et al. 2000; Sharma
et al. 2003). In this paper, we report the molecular and functional characterization of the inf2 class (class III) of elicitin-like genes from *P. infestans* (Kamoun et al. 1997a). Our main finding is that variation in the resistance of *Nicotiana* to *P. infestans* is correlated with variation in the response to INF elicits. The ability of *N. tabacum*, but not *N. benthamiana*, to respond to INF2B could explain differences in resistance to *P. infestans* observed for these two species.

Despite the rapid accumulation of sequence data from numerous organisms, elicitin-like genes and proteins have only been identified in the oomycete genera, *Phytophthora* and *Pythium*. In *Phytophthora*, production of the 10 kDa class I elicits is quasi-ubiquitous and has been attributed to more than 30 species so far (Kamoun et al. 1994; Ponchet et al. 1999). The Southern blot hybridizations illustrated in Figure 4.1 suggest that the inf2 class of elicitin-like genes is similarly widespread since inf2-like sequences were detected in all nine *Phytophthora* species examined. In addition, we also identified sequences highly similar to inf2 by searching the expressed sequence tag (EST) database of *Phytophthora sojae* (Qutob et al. 2000; Qutob et al. 2003). These results indicate that the inf2 class of elicitin-like genes occurs as a small conserved family in *Phytophthora*.

Using Southern blot analyses with gene-specific probes, one to two genomic copies of the inf2A and inf2B genes could be detected in sixteen different *P. infestans* isolates. In addition, inf2 mRNA was present in all tested *P. infestans* isolates, although at variable levels. The biological basis and significance of this variation remains unclear. The two *P. infestans* isolates
previously described as naturally deficient in INF1 production (Kamoun et al. 1998b) were found to produce inf2 mRNA. In addition, inf1 sense and antisense transformants that showed no detectable levels of inf1 mRNA (Kamoun et al. 1998a) were found to be unaltered in inf2 mRNA using northern blot hybridizations (van West et al. 1999). These results suggest that down-regulation of inf1 mRNA does not correlate with altered levels of inf2 mRNA.

We monitored gene expression levels of both inf2A and inf2B during P. infestans tomato interactions. Semi-quantitative RT-PCR experiments revealed that both inf2A and inf2B are expressed during infection of tomato indicating that these proteins may be functionally relevant to P. infestans pathogenesis. Expression patterns of the inf2 genes in planta were slightly different from those of inf1, which tends to peak late in the infection cycle (Kamoun et al. 1997b). However, more precise methods for measuring gene expression need to be applied to confirm these results prior to speculating on their biological implications.

Comparative analyses of elicitor activity of INF1, INF2A, and INF2B using PVX agroinfection and agroinfiltration revealed that similar to INF1 and other elicitins, INF2A and INF2B induced HR-like symptoms on N. tabacum. However, using these assays, differences in HR induction were noted between the three elicitors in both N. tabacum and N. benthamiana. A significant difference in specificity of HR induction was obtained for INF2B, which, unlike INF1 and INF2A, failed to induce necrosis on N. benthamiana in both assays (Figures 4.4 and 4.5, Table 4.1). INF2A only occasionally induced the HR in N. benthamiana
using agroinfiltration and induced small and inconsistent necrotic lesions using agroinfection (Table 4.1). These results suggest that expression of \textit{inf2A} in \textit{N. benthamiana} via these transient assays may not be efficient enough to consistently result in necrosis. Alternatively, unknown environmental or host factors may affect the level of expression or response to INF2A in \textit{N. benthamiana}, resulting in the inconsistent responses. In any case, these results support the view that INF2A is an overall weaker HR elicitor than INF1 and INF2B. This conclusion is also supported by the experiments described in Figure 4.7 that show that INF2B and INF1 but not INF2A induced the expression of the defense genes \textit{PR1a} and \textit{Bgl2} in \textit{N. tabacum}. However, the extent to which these differences are significant to natural \textit{P. infestans}-plant interactions remains to be determined.

We determined that the ubiquitin ligase associated protein SGT1 is required for HR induction by INF2A as previously shown for INF1 (Peart \textit{et al.} 2002). SGT1 has emerged as a central player in \textit{R} gene mediated HR signaling in plants as diverse as barley, \textit{Arabidopsis}, and \textit{N. benthamiana} (Peart \textit{et al.} 2002; Shirasu and Schulze-Lefert 2003; Tör \textit{et al.} 2003). Using TRV-mediated gene silencing, Peart \textit{et al.} (2002) found that unlike abiotic inducers of cell death, all examined pathogen-derived elicitors required SGT1 for HR induction. Therefore, the result that INF2A induced necrosis is SGT1-dependent suggests that this protein is likely to induce a typical HR similar to the one induced by the better characterized INF1 protein and other HR elicitors. However, considering
the differences stated above, the extent to which INF2 and INF1 induce similar cell death pathways in *Nicotiana* remains to be determined.

The ability of *N. tabacum*, but not *N. benthamiana*, to respond to INF2B could explain differences in resistance to *P. infestans* observed for these two species (Kamoun 2001; Kamoun *et al.* 1998a). *P. infestans* strains engineered for INF1-deficiency by antisense gene silencing were found to reach significant levels of biomass and colonization in *N. benthamiana* but not in a number of other *Nicotiana* species, including *N. tabacum* (Kamoun *et al.* 1998a). This led to the hypothesis that resistance to *P. infestans* in *N. benthamiana* is mainly triggered by INF1, whereas the resistance reaction observed in *N. tabacum* may involve additional elicitor/avirulence factors (Kamoun 2001; Kamoun *et al.* 1998a). An attractive hypothesis is that the inability of *N. benthamiana* to respond to INF2B, contributes to the difference in response to INF1-deficient strains between these two *Nicotiana* species. Future experiments using *P. infestans* strains silenced for a combination of *inf* genes should help assess the contribution of the *inf2* genes to avirulence on *Nicotiana*.

The three-dimensional structure of cryptogein, the major basic elicitin (class Ib) of *P. cryptogea*, was determined both as a native protein and complexed with ergosterol (Boissy *et al.* 1996; Boissy *et al.* 1999; Fefeu *et al.* 1997; Gooley *et al.* 1998). The main features of the structure of cryptogein, three disulfide bridges, a beak-like motif formed by two antiparallel beta sheets, and an Ω-loop, are likely to be conserved among the *P. infestans* elicitins examined in this study (Figure 4.9). Ergosterol binding to cryptogein occurs in a hydrophobic
pocket and involves 15 amino-acid residues in cryptogein (Boissy et al. 1999). All these residues are fully conserved between cryptogein, INF1, and other class I elicitins (Figure 4.9) (Boissy et al. 1999). However, 6 of these 15 amino-acids are replaced in INF2A and INF2B, including Tyr87 (replaced by Leu) which was shown experimentally to be important in sterol binding and HR-induction in cryptogein (Osman et al. 2001b). This marked difference in amino-acid composition of the hydrophobic pocket suggests that INF2 may bind different substrates from class I elicitins, perhaps lipid molecules other than sterols. Variation in substrate binding could also explain the difference in elicitor activity between INF2 and class I elicitins, since sterol loading is important for the ability to specifically bind a plasma membrane receptor and induce the HR in tobacco (Osman et al. 2001b).

The differences in HR-inducing activity observed for INF elicitins in *N. benthamiana* and *N. tabacum* and the availability of facile functional assays suggest that these genes are ideal for probing structure-function relationships in elicitor proteins. Differences in activity of INF2A and INF2B were observed even though their elicitin domains differ only by three amino-acids (Figure 4.9). INF2A appeared weaker than INF1 and INF2B in inducing the HR on tobacco resulting in lower percentages of necrosis induction and smaller necrotic lesions when delivered through PVX (Figure 4.1, Table 4.1). On the other hand, INF2B consistently failed to induce the HR on *N. benthamiana* even though it functioned as a potent elicitor in tobacco (Figures 4.4 and 4.5, Table 4.1). In INF2B, Ser65 is replaced by Gly and Glu93 is replaced by Lys. Both of these residues are located
in the alpha helices and are not implicated in sterol binding. However, they are predicted to be surface exposed and are variable among class I elicitins (Figure 4.9). Our results suggest that these residues are important for specific HR activity in *N. benthamiana* and the overall elicitor activity in tobacco. Future domain swapping and amino acid exchange experiments should help determine the role of these residues in elicitin activity.

The *Nicotiana* genes involved in recognition of elicitins have not yet been identified and consequently one can only speculate about the molecular basis of the differences between tobacco and *N. benthamiana* with respect to their response to INF2 elicitins. Elicitin recognition genes in *Nicotiana* could be members of a variable *R* gene family, similar to those described in numerous plants to mediate HR induction by pathogen elicitors (Staskawicz *et al.* 1995; Bent 1996; Michelmore and Meyers 1998; Meyers *et al.* 1999). The difference observed between tobacco and *N. benthamiana* also indicates that similar to the phenotypic expression of resistance (Kamoun *et al.* 1998a), the genetic basis of *Nicotiana* resistance to *P. infestans* could be diverse. Perhaps, recognition of species-specific elicitors, such as INF elicitins, by an arsenal of *R* genes forms the basis of resistance of *Nicotiana* to *P. infestans* (Kamoun 2001). Considering this diversity, the *P. infestans-Nicotiana* pathosystem appears ideal for the dissection and comparative analyses of the molecular basis of nonhost recognition in closely related species.

3.6 ACKNOWLEDGMENTS
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A modified version of this chapter has been published as:

3.7 REFERENCES


Figure 4.1. Occurrence of inf2 sequences in Phytophthora species. DNA blot containing 20 microgram of HindIII digested total DNA from 13 isolates representing nine Phytophthora species, i.e., P. infestans (isolates 88069, 90128, and IPO-0), P. mirabilis (CBS 678.85, CBS136.86, and CBS150.88), P. phaseoli (CBS 556.88), P. parasitica (18), P. cactorum (436), P. palmivora (10), P. porri (HH), P. cinnamomi (2), and P. vignae (20853) was hybridized with a probe from the elicitin domain of inf2 which hybridizes to both inf2A and inf2B. Molecular marker sizes are shown on the left in kb.
Figure 4.2. Occurrence of the inf2A, inf2B, and inf1 sequences in isolates of Phytophthora infestans. DNA blot containing BamHI digested total DNA from P. infestans isolates (1) DDR7601, (2) DDR7602, (3) DDR7702, (4) GER7401, (5) GER8451, (6) GER8501, (7) GER8601, (8) 46210, (9) 66006, (10) 68308, (11) 70001, (12) UK7225, (13) UK7818, (14) IT8001, (15) GE900083, and (16) GE900089 (Kamoun et al. 1998) was hybridized with specific probes from the inf2A (A), inf2B (B), and inf1 (C) genes. The approximate sizes of the hybridizing bands are shown on the right in kb.
**Figure 4.3.** Occurrence of the inf2A, inf2B, and inf1 mRNA in isolates of *Phytophthora infestans*. RNA blots containing total RNA from (1) DDR7601, (2) DDR7602, (3) DDR7702, (4) GER7401, (5) GER8451, (6) GER8501, (7) GER8601, (8) 46210, (9) 66006, (10) 68308, (11) 70001, (12) UK7225, (13) UK7818, (14) IT8001, (15) GE900083, (16) GE900089, and (17) 88069 were hybridized with specific probes from the inf2A, inf2B, inf1, and actA genes. The approximate sizes of the inf and actA transcripts are shown on the right.
Figure 4.4. Expression of *P. infestans* inf2A, inf2B and inf1 during infection of tomato. Total RNA from *P. infestans* -infected leaves of tomato 0, 1, 2, 3, and 4 days after inoculation, and *P. infestans* mycelium (My) grown in a synthetic medium were used in RT-PCR amplifications as described in the text. Amplification of the *P. infestans* elongation factor 2 (ef2α) was used as a control to determine the integrity of the RNA.
Figure 4.5. Agroinfection assays. Symptoms observed on *Nicotiana tabacum* (tobacco) (A) and *Nicotiana benthamiana* (B) leaves after inoculation with *Agrobacterium tumefaciens* containing the binary potato virus X (PVX) vector expressing *Phytophthora infestans* *inf* genes. Inoculated leaves were photographed 10 days after inoculation with *A. tumefaciens* containing, from left to right, the binary PVX vector pGR106, pGR106-INF1, pGR106-INF2A, and pGR106-INF2B.
Figure 4.6. Agroinfiltration assays. Symptoms observed on *Nicotiana tabacum* (tobacco) (left panel) and *Nicotiana benthamiana* (right panel) leaves after infiltration with *Agrobacterium tumefaciens* containing binary vectors expressing *Phytophthora infestans* inf genes. Inoculated leaves were photographed 6 days after inoculation with *A. tumefaciens* containing the binary vector p35S-INF2A (top left section of the leaves), p35S-INF2B (bottom left), p35S-INF1 (top right), and the negative control pGUSi (bottom right).
Figure 4.7. Induction of defense response genes in tobacco by INF1 and INF2. (A) Histochemical GUS assay of a leaf from transgenic tobacco line carrying a Bgl2::GUS construct. The leaf was wound-inoculated with a toothpick only (Wo) as well as with Agrobacterium tumefaciens strains carrying pGR106 (Wt), pGR106-INF1, pGR106-INF2A, and pGR106-INF2B. The picture illustrates a representative leaf stained 8 days after inoculation. (B) Northern Blot hybridization of RNA isolated from tobacco leaves (cv. Xanthi) that were wound-inoculated with a toothpick only (Wo) or toothpick inoculated with A. tumefaciens strains carrying pGR106 (Wt), pGR106-INF1, pGR106-INF2A, and pGR106-INF2B. The blot was hybridized with probes from the defense gene PR1a and the constitutive gene alpha-tubulin (tub). Total RNA was harvested from leaf discs surrounding the inoculation sites immediately after the onset of necrosis. Different leaves were used for the different treatments.
Figure 4.8. Symptoms observed in *Nicotiana benthamiana* silenced for the ubiquitin ligase-associated gene SGT1 following agroinfiltration and *Potato virus X* (PVX) agroinfection with *inf1* and *inf2* constructs. *N. benthamiana* plants were first inoculated with *Agrobacterium tumefaciens* carrying the *Tobacco rattle virus* vector (TRV) or a TRV:SGT1 construct (TRV:SGT1), and then challenged after 3 weeks with (A) *A. tumefaciens* carrying vector (top left), p35S-INF1 (top right), p35S-INF2A (bottom left), and p35S-INF2B (bottom right) constructs, or (B) *A. tumefaciens* carrying the binary PVX vector pGR106, pGR106-INF1, and pGR106-INF2A. Leaves in panel A were photographed 5 days after the secondary agroinfiltration. The bars in panel B correspond to the percentage of *A. tumefaciens* binary PVX inoculation sites showing the HR over time (N = 40).
Figure 4.9. Multiple alignment of the elicitin domain of selected *Phytophthora* elicitin and elicitin-like protein highlighting the major structural features. Multiple alignment of elicitin sequences from *Phytophthora cryptogea* cryptogein (CRY-B) and *P. infestans* (INF1, INF2A, and INF2B) was conducted using the program CLUSTAL-W (J.D. Thompson *et al.*, EMBL, Heidelberg, Germany). Identical amino acids are shaded in dark gray and similar amino acids shaded in light gray. Residue numbers are indicated above the sequences. The secondary structure elements indicated above the sequences (six alpha helices, omega loop, and two antiparallel beta-sheets) correspond to CRY-B as described in Boissy *et al.* (1996). Residues in blue were shown by Boissy *et al* (1999) to interact with an ergosterol substrate. Residues in red differ between INF2A and INF2B. Residue numbers flank the sequences.
Table 4.1. Recombinant potato virus X expressing *inf* elicitin genes induce variable levels of necrosis on *Nicotiana tabacum* and *Nicotiana benthamiana*. Results were obtained from one representative experiment. A total of 14 inoculation sites were analyzed for *N. tabacum* and 20 for *N. benthamiana*.

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Table 4.1. Recombinant potato virus X expressing *inf* elicitin genes induce variable levels of necrosis on *Nicotiana tabacum* and *Nicotiana benthamiana*. Results were obtained from one representative experiment. A total of 14 inoculation sites were analyzed for *N. tabacum* and 20 for *N. benthamiana*. 
CHAPTER 5

THE PHYTOPHTHORA INFESTANS NEP1-LIKE PROTEIN PINPP1.1
INITIATES A PLANT CELL DEATH PATHWAY THAT IS DISTINCT FROM
INF1 INDUCED HR

5.1 ABSTRACT

Cell death plays a ubiquitous role in plant-microbe interactions and can be
associated with both susceptible and resistant responses. In recent years, a
novel class of cell death-inducing proteins, known as Nep1-like proteins (NLPs),
have been identified in bacteria, fungi and oomycetes. These proteins induce
hypersensitive-like necrosis in a variety of dicotyledonous plants. In this study,
we describe four members of the NLP family from the oomycete plant pathogen
Phytophthora infestans (PiNPP1.1-1.4) and compared their activity to the well-
characterized hypersensitive response (HR) elicitor INF1. Using binary potato
virus X (PVX) agroinfection assays, we showed that PiNPP1.1 induce HR-like
cell death in the host plant tomato and in Nicotiana benthamiana.
Expression analyses indicated that *PiNPP1.1* is differentially expressed throughout infection of tomato and is up-regulated during late stages of infection. We also characterized the defense responses induced by *PiNPP1.1*, and two other NLPs *PsojNIP* and *Nep1* in *N. benthamiana* and showed that necrosis induction by these genes is dependent of the ubiquitin ligase-associated protein SGT1 and the heat-shock protein HSP90. In addition, NLP but not INF1 induced cell death was found to depend on COI1, MEK2, NPR1 and TGA2.2, suggesting distinct signaling requirements for NLPs. Finally, we unveil cross-talk between NLP and INF1 induced pathways. Co-expression of elicitor combinations resulted in accelerated and enhanced cell death. These results point to two distinct cell death pathways that act synergistically when induced simultaneously.

### 5.2 INTRODUCTION

Cell death plays a ubiquitous role in plant-microbe interactions and can be associated with both susceptible and resistant responses. Indeed, the effect of cell death on the outcome of a particular plant-pathogen interaction varies depending on the mode of infection of the pathogen and the pathosystem (Dickman *et al.*, 2001; Bonas and Lahaye, 2002). For example, *R* gene mediated disease resistance and many types of nonhost resistance involve the hypersensitive response (HR), a form of programmed cell death in plants that is particularly effective against pathogens that require living host cells (so called biotrophs) (Staskawicz *et al.*, 1995; Dangl *et al.*, 1996; Kamoun *et al.*, 1999a; Heath, 2000; Dangl and Jones, 2001; Kamoun, 2001; McDowell and Woffenden,
In contrast, necrotrophic pathogens that obtain nutrients from dead plant tissue, trigger programmed cell death in host plants to facilitate colonization (Wang et al., 1996; Navarre and Wolpert, 1999; Asai et al., 2000; Govrin and Levine, 2000; Dickman et al., 2001; McDowell and Woffenden, 2003). The ambiguous role of cell death is particularly evident in hemibiotrophic pathogens, such as the oomycetes Phytophthora infestans and Phytophthora sojae. These pathogens adopt a two-step infection style. During the phase of infection that follows penetration of host tissue, they require living cells much like biotrophic pathogens. In contrast, in a later phase of the disease, they cause extensive necrosis of host tissue resulting in profuse colonization and sporulation. This infection cycle suggests that host cell death may differently impact the disease depending on timing of occurrence. The extent to which different cell death pathways regulate the HR and disease associated necrosis remains unclear.

In recent years, a novel class of necrosis-inducing proteins, known as Nep1-like proteins (NLPs) (Pemberton and Salmond, 2004), have been identified in bacteria, fungi and oomycetes. These proteins named after the canonical 24-KDa necrosis and ethylene-inducing protein (Nep1) that was originally purified from culture filtrates of Fusarium oxysporum f. sp. erythroxyli (Bailey et al., 1997), have been described from species as diverse as Bacillus (Takami and Horikoshi, 2000), Erwinia (Pemberton et al., 2005; Bell et al., 2004) Verticillium (Wang et al., 2004), Pythium (Veit et al., 2001), and Phytophthora (Fellbrich et al., 2002; Qutob et al., 2002). Despite their diverse phylogenetic distribution, NLPs share a high degree of sequence similarity and several members of the family have the
remarkable ability to induce cell death in as many as 20 dicotyledonous plants (Bailey, 1995; Bailey et al., 1997; Pemberton and Salmond, 2004). The contribution of NLPs to disease remains unclear at this stage. NLPs induce defense responses in both susceptible and resistant plants and, therefore, are thought of as general or non-specific elicitors. Based on similarity to self and non-self recognition models of the animal innate immune system, general elicitors, such as NLPs, have been likened to pathogen associated molecular patterns (PAMPs) (Nurnberger and Brunner, 2002). NLPs fulfill many criteria for functioning as PAMP triggers immunity in plants, including the occurrence of functional orthologues in a diversity of pathogenic oomycetes, fungi and bacteria, and the absence of similar sequences in plant genomes (Veit et al., 2001; Fellbrich et al., 2002; Qutob et al., 2002). Nevertheless, in P. sojae, the PsojNIP gene was expressed late during the colonization of soybean and thus may function as a toxin that facilitates colonization of host tissue during the necrotrophic phase of infection (Qutob et al., 2002). The hypothesis that NLPs have virulence functions is supported by a recent report in which disruptions of the NIP_{ecc} and NIP_{eca} genes in E. carotovora subsp carotovora and E. carotovora subsp atroseptica respectively, resulted in reduced virulence phenotypes on potato tubers (Pemberton et al., 2005).

The host factors involved in cell death signaling triggered by NLPs remain poorly understood. Plants accomplish recognition of pathogen molecules through a large set of endogenous receptor like proteins (R proteins) that can either bind effectors or monitor their activity resulting in the HR (Dangl and Jones, 2001).
Characterization of the genetic requirements for the HR has culminated in models that explain effector recognition and subsequent resistance signaling through tightly controlled multi-step cascades. Despite the large number of identified $R$ genes, there are only a small number of known signaling components downstream of protein recognition, pointing to a set of shared signaling steps in plants. Among such signaling components, the ubiquitin ligase-associated protein SGT1 is required for many $R$ gene mediated HR pathways (Peart et al., 2002; Austin et al., 2002; Azevedo et al., 2002). SGT1 interacts with the COP9 protein complex and was recently found to interact with RAR1 and the molecular chaperone HSP90, suggesting the presence of multiprotein complexes involved in resistance signaling (signalosomes) (Liu et al., 2002; 2004; Takahashi et al., 2003). In this model, SGT1 as well as RAR1 function as co-chaperones, folding and stabilizing signalosome protein complexes (Liu et al., 2004).

Localized resistant interactions or localized wounding can result in systemic induction of elevated resistance. Salicylic acid (SA) and jasmonic acid (JA) are important secondary metabolites that participate in induction of systemic acquired resistance (SAR) and induced systemic resistance (ISR) respectively. Recently, COI1, an important regulator of Jasmonic Acid (JA) mediated responses was found to interact with ASK1 as well as ASK2 in the formation of at least two separate SCF$^{COI}$ ubiquitin-ligase complexes, presumably regulating JA signaling via ubiquination \textit{in planta} (Feng et al., 2003; Xu et al., 2002). These results provide a possible direct connection between signalosome complexes and regulation of defense pathways mediated through systemic secondary
signals. Most R-gene mediated HR responses results in SA accumulation and lead to SAR (Feys and Parker, 2000) but whether similar mechanisms are involved in regulation however, is unknown.

Whether or not there are cell death or HR pathways independent of signalosome protein complexes remains unclear. In particular, we do not know the extent to which the biochemical components involved in NLP response overlap with those mediated by R proteins.

The Nep1 family is unusual in many respects. Unlike other inducers of cell death, it is remarkably conserved in different phyla. Many questions about the role of these proteins during plant-microbe interactions remain unanswered. Do NLPs function as PAMPs or toxins? Do they trigger resistance or do they facilitate colonization in the late stages of infection? These questions prompted us to characterize Nep1-like proteins in the well-established *P. infestans*–solanaceae pathosystem. In this study, we describe four members of the NLP family in *P. infestans* (PiNPP1.1-1.4) and compared their activity to the well-characterized HR elicitor INF1. Using the binary potato virus X (PVX) agroinfection assays, we showed that PiNPP1.1 induce HR-like necrosis in the host plant tomato and in *N. benthamiana*. Expression analyses indicated that *PiNPP1.1* is differentially expressed throughout infection of tomato and is up-regulated during late stages of infection. We used virus induced gene silencing (VIGS) to identify signaling and regulatory components that contribute to NLP-induced cell death. These analyses showed that cell death induction depends on the ubiquitin ligase-associated protein SGT1 and the heat-shock protein HSP90.
In addition, we showed that COI1, MEK2, NPR1 and TGA2.2 contribute to PINPP1.1 but not INF1-induced cell death, suggesting the presence of distinct signaling pathways. Finally, we demonstrated synergistic interplay between INF1 and NLP-induced pathways through transient co-expression assays in N. benthamamiana.

5.3 MATERIALS AND METHODS

Microbial strains and growth conditions

*P. infestans* isolate 90128 (A2 mating type) was used for the time course infection assay. Isolates were grown routinely on rye agar medium supplemented with 2% sucrose (Caten and Jinks, 1968). For RNA extraction, plugs of mycelium were transferred to modified Plich medium (Kamoun *et al.*, 1993) and grown for 2 to 3 weeks before harvesting.

*A. tumefaciens* strain GV3101 (Holsters *et al.*, 1980) was used in molecular cloning experiments and was routinely cultured at 28°C in Luria-Bertani (LB) media using appropriate antibiotics (Sambrook and Russell, 2001). All bacterial DNA transformations were conducted by electroporation using standard protocols (Sambrook and Russell, 2001).
PVX-agroinfection and agroinfiltration constructs

The cDNA inserts of PiNPP1.1, PiNPP1.2 and PiNPP1.3 were PCR-amplified from their respective cDNA clones using PSPORT-Ascl ('5'-GGCCGGCGCGCCCTCCCCGGGTGCACCCACCGGTCC-3') and SP6 ('5'-TACGATTAGGTGACACTATAG-3') primers and high-fidelity Pfu DNA polymerase (Stratagene). The fragments were cloned in the binary PVX vector pGR106 (Lu et al., 2003) using the Clai and Not1 restriction enzymes, and transformed into A. tumefaciens strain GV3101 to generate agroinfection ready clones. Recombinant clones were tested for the presence of PiNPP inserts by PCR-amplification with PVX primers, PVX-F (5'-AATCAATACAGTGTTGGCTTGC-3') and PVX-R (5'-AGTTGACCCTATGGGCTGTGTTG-3'), and the inserts were checked by DNA sequencing. pGR106-PSOJNI, pGR106-NEP1, and pGR106-INF1 were described elsewhere (Qutob et al., 2002; Torto et al., 2003). PiNPP1.1 was cloned into pCB302-3 to obtain p35S-PiNPP1.1. For this purpose, PCR amplification was performed from the original cDNA clone using the primers PiNPP1.1-F: 5'-GCGACTAGTATGAACATCCTTCAACTCTGC-3' and PiNPP1.1-R: 5'-GCGACTAGTCTAGGCGTAGGATTG-3', adding SpeI restriction sites flanking both termini. Fragments were ligated into pre-digested pCB302-3 vector and transformed into Agrobacterium. Recombinant colonies were tested directly for insertion of the gene in the right orientation using a primer binding to the 35S promoter, 35S-F: 5'-ATGACGCACAATCCACTCCTTCC-3' and PiNPP1.1-R. Amplified insert was used for sequence verification.
Virus-induced gene silencing constructs

TRV:SGT1 that carries a fragment of the NbSGT1.1 gene (GenBank accession no. AF516180) was described elsewhere (Peart et al., 2002). We amplified LeHSP90-1 from tomato cDNA using HSP90-F: 5'-GGAGGATCCCGAGGGTCTGTGCAAGGATTAAGG-3' and HSP90-R: 5'-TCAGGATCCTCAATGGTGCCAT-3’. coi1, mek2, tga2.2 and npr1 were amplified from N. benthamiana cDNA using primers modified from Ekengren et al. (2003) as follows: COI1-F: 5’-GAGCCCGGGCAGATCTGCGGACTTCAATGGTG-3’ and COI1-R: 5’-GCGGATCCTGAGCCCTTCATCGGATTCC-3’; NtMek2-F: 5’-GAGCCCGGGCTACCTCGGCCCCACCAGGAAGACT-3’ and NtMek2-R: 5’-GCGGATCCCCATCGTACTGCATCTCGATCGATCTGTG-3’; TGA2.2-F: 5’-GAGCCCGGGGACATATTCAGGATAAGGGGA-3’ and TGA2.2-R: 5’-GCGGATCCTATTCGCGGGGCAGGAAGCCA-3’; NPR1-F: 5’-GAGCCCGGGGAAAGCCTAAAATTGTAGTGTC-3’ and NPR1-R: 5’-GCGGATATCCCTATTCTCAAAATTGTAGTGTC-3’. For LeHSP90 and mek2, amplified fragments were digested with BamHI, whereas for coi1, tga2.2 and npr1, BamHI and XmaI were used. Digested fragments were ligated into the pTV00 vector to yield pTV-COI1, pTV-MEK2, pTV-NPR1, pTV-TGA2.2 and pTV-HSP90 (http://www.jic.bbsrc.ac.uk/Sainsbury-Lab/davidaulcombe/Services/vigsprotocol.htm) and were transformed into electro-competent A. tumefaciens cells (strain GV3101). Recombinant strains were tested for having the correct insert
through colony PCR. For all constructs, amplified inserts were sequenced to confirm cloning of the correct gene fragment.

**DNA sequencing and sequence analyses**

The cDNAs as well as the recombinant clones were sequenced by primer walking using Applied Biosystems 3730 DNA Analyzer. Sequences were assembled and annotated using SequencherTM 4.1 (Gene Codes Corp, Ann Arbor, MI, USA). Similarity searches were performed locally on an Apple Macintosh OSX workstation using BLAST (Altschul et al., 1997). Multiple alignments of protein sequences were conducted using the program CLUSTAL-X (Thompson et al., 1997). The sequences described in this paper were deposited in GenBank (accession No. AAK25828, ---and --).

**Transient gene expression assays**

PVX agroinfection assays were performed as described by Torto et al., (2003). For routine screens, *N. benthamiana* plants at approximately the 3 to 4-leaf stage (3-week-old) were used for the PVX agroinfection assays. Plants were grown and maintained in a greenhouse with an ambient temperature of 22°-25°C and high light intensity. Recombinant *A. tumefaciens* GV3101 strains carrying pGR106 derivatives were streaked onto LB solid media plates containing 50 mg ml⁻¹ kanamycin and grown for 2 to 3 days at 28°C. Inoculations were performed on two lower leaves by dipping a wooden sterile toothpick in a culture grown on solid agar medium and then wounding each leaf twice around the main vein and
near the base of the leaf. An excess of bacteria was used for the inoculations. Multiple experiments were performed and each experiment consisted of at least four plants per construct. For comparative analyses, side-by-side inoculations on mature *N. benthamiana* (6-8 leaf stage) and tomato plants (4-6 weeks old) were performed. For each construct, two inoculations per leaf and at least 3 leaves per plant were used. For agroinfiltration, recombinant *Agrobacterium* strains (GV3101) were grown as described elsewhere (Van der Hoorn et al., 2000) except that all culturing steps were performed in LB media. For infiltrations of silenced plants, suspensions were spun down and re-suspended in MMA infiltration medium to a final OD$_{600}$ of 0.8 (1 liter MMA: 5 g of MS salts, 1.95 g of MES, 20 g of sucrose, 200 µM acetosyringone, pH=5.6). For the co-expression assays, exact same procedures were followed except that for the effector combinations, recombinant strains were mixed in a 1:1 ratio. In addition, a final OD$_{600}$ of 0.2 was used for infiltration of all treatments. All suspensions were incubated 1-3 hours in MMA prior to infiltration. For both silencing and co-infiltration assays, at least 4 plants were infiltrated for each treatment and 3-4 leaves were used per plant.

**RT-PCR analyses**

Total RNA from *P. infestans* and from infected tomato leaves was isolated using the Trizol reagent (Gibson-BRL, Bethesda, Md.) according to the manufacturer instructions. First-strand cDNAs were synthesized from 4.5 µg of total RNA with a universal polyT primer and the ThermoScript reverse
transcriptase from the ThermoScript RT-PCR system (Gibson-BRL, Bethesda, Md.). Reactions were performed according to the manufacturer’s instructions and were incubated at 50°C for 1 h. PCR amplifications were carried out with equal amounts of cDNAs using the primer pairs PiNPP1.1-F (5’-GCGAAGCTTGATGTGATTTCACACGATGCAGT-3’) and PiNPP1.1-R (5’-GCGTCTAGACTAGCCAGCTGGACGAATAGTCCAC-3’); PiNPP1.2-F (5’-GCGAAGCTTTAAGTCAGGGAATGCATCCTCGAGT-3’) and PiNPP1.2-R (5’-GCGTCTAGATTTAAGGGAATGCATCCTCGAGT-3’); PiNPP1.3-F (5’-GCGGAATTCCGAAGTCACGTACATCAACCACGA-3’) and PiNPP1.3-R (5’-GCGTCTAGATACCAACCGGATAAGCATCTTTTCAG-3’). Integrity of the mRNA and cDNA was controlled with primers EF2-F1 (5’-TGACGCTATCGCCAAGGAATC-3’) and EF2-R1 (5’-TAACGCTGACCGTATGAGGGG-3’), which are specific for the constitutive elongation factor 2 (ef2) gene of P. infestans (W.R. Morgan and S. Kamoun, unpublished data).

**TRV-induced gene silencing**

*A. tumefaciens* strain GV3101 carrying the binary TRV RNA 1 construct, pBINTRA6, and the TRV RNA2 vector, pTV00 (Ratcliff *et al.*, 2001), or pTV00 derivatives were prepared for agroinfiltration assays as described above. The bacterial suspensions were mixed in 2:1 ratio (RNA1: RNA2) in induction buffer (final OD600= 0.6), MES (10 mm MgCl2, 10 mm MES, pH 5.6 and 150 µm acetosyringone) and maintained at room temperature for 2-3 h. Infiltrations were
conducted by gently pressing a 1-ml disposable syringe to the abaxial surface of fully expanded leaves of 3-week old *N. benthamiana* plants slowly depressing the plunger. A sufficient amount of bacterial suspension was used to infiltrate the leaves to give a water-soaked appearance. This typically required 1-4 infiltrations per leaf. Following agroinfiltration, plants were maintained in a greenhouse at 22°C with a 16 h photoperiod. Secondary challenges were performed about three weeks after agroinfiltration with the TRV strains.

### 5.4 RESULTS

**PiNPPs are members of NLP family** (contributed by Thirumala Kanneganti)

We mined expressed sequence tag (EST) data sets from *P. infestans* using PexFinder to identify cDNAs encoding extracellular proteins (Torto *et al.*, 2003). ESTs corresponding to putative extracellular proteins were annotated by similarity and motif searches against public databases. Sequences derived from four cDNAs, named *PiNPP1.1*, *PiNPP1.2*, *PiNPP1.3* and *PiNPP1.4* showed significant similarity to members of the NLP family. Three out of four cDNAs contained full-length open reading frames (ORFs). Sequence analysis revealed ORFs of 717, 738 and 738-bp corresponding to a predicted translated product of 238, 245 and 245 amino acids for *PiNPP1.1*, *PiNPP1.2* and *PiNPP1.3*, respectively (Figure 5.1). SignalP (Bendtsen *et al.*, 2004) analysis of the predicted proteins identified N-terminal signal peptides of 19 amino acid residues with significant mean S values of 0.750, 0.855, 0.910 and hidden Markov model scores of 1.0, 0.999, 0.998 for PiNPP1.1, PiNPP1.2 and PiNPP1.3 respectively.
PINPP1.1 induces necrosis in N. benthamiana and tomato (contributed by Thirumala Kanneganti)

To express the PiNPP cDNAs in planta, we performed PVX agroinfection assays using the binary vector pGR106 (Lu et al., 2003). Agroinfection of N. benthamiana plants (4 leaf stage) with Agrobacterium tumefaciens strain GV3101 carrying the constructs pGR106-PiNPP1.1 and pGR106-INF1 resulted in necrosis at the inoculated sites 4 to 5 days post infection (dpi) (Figure 5.2a). Inoculation with the empty vector pGR106, pGR106-PiNPP1.2 and pGR106-PiNPP1.3 did not produce any symptoms. We also tested the host plant tomato. Inoculation of 3 to 5 week old tomato plants with A. tumefaciens carrying PGR106-PiNPP1.1 resulted in necrosis 5 dpi (Figure 5.2b). At 8 to 9 dpi, necrotic lesions started to spread throughout the entire plant leading to death (Figure 5.2c).

Comparative analyses of necrosis-inducing activity

We compared the timing and appearance of PINPP1.1 induced cell death to those of known defense-response elicitors, such as the P. infestans elicitin INF1 (Kamoun et al., 1998; Kamoun et al., 1999b) and NLP family members PSOJNIP of P. sojae (Qutob et al., 2002) and NEP1 of F. oxysporum (Bailey, 1995; Nielsen and Krogh, 1998). A. tumefaciens carrying pGR106 derived constructs were inoculated side-by-side onto fully expanded leaves of N. benthamiana and tomato. Dark necrotic lesions were observed at the site of...
inoculation in *N. benthamiana* with pGR106-PINPP1.1, pGR106-PSOJNIP and pGR106-NEP1 within 5 dpi (Figure 5.3a) but necrosis was only observed starting at 7 dpi with the pGR106-INF1 construct (Figure 5.3b). On tomato, necrotic symptoms developed at PINPP1.1, PSOJNIP AND NEP challenged sites 7 dpi (Figure 5.3c). These results suggest that the examined NLPs are potent cell death inducers.

**PiNPP1.1, PiNPP1.2 and PiNPP1.3 are temporally regulated during infection**

(contributed by Thirumala Kanneganti)

We used semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) to determine the expression patterns of *PiNPP1.1, PiNPP1.2*, and *PiNPP1.3* during a time course infection of tomato by *P. infestans*. *In vitro* grown mycelium was used as a positive control for constitutive gene expression. cDNA obtained from healthy tomato leaves served as a negative control. RT-PCR analysis with gene specific primers revealed that *PiNPP1.1* transcripts accumulated during late stages of infection and were up-regulated compared to *in vitro* grown mycelium. *PiNPP1.1* transcripts were first detected at 3 days post infection (dpi) but dramatically increased at 4 dpi. Both *PiNPP1.2* and *PiNPP1.3* appeared constitutively expressed and showed amplification patterns similar to the constitutive *P. infestans* elongation factor 2 (*ef2*) gene (Figure 5.4). These results indicate that *PiNPP1.1* is differentially expressed throughout various stages of infection and is up-regulated during late infection of tomato similar to the *P. sojae* NLP *PsojNIP* (Qutob *et al.*, 2002).
**PINPP1.1 induced cell death is SGT1 and HSP90 dependent**

Previous studies have shown that the ubiquitin ligase associated protein SGT1 (Peart *et al.*, 2002) and heat-shock protein HSP90 (Kanzaki *et al.*, 2003) are required for HR induction by the *P. infestans* elicitin INF1 in *N. benthamiana*. We tested whether SGT1 and HSP90 are required for *PINPP1.1* induced cell death using virus induced gene silencing (VIGS) and agroinfiltration. For this purpose, young *N. benthamiana* plants (5 leaf stage) were infiltrated with mixtures of *A. tumefaciens* strains carrying combinations of pBINTRA6 (TRV:RNA1) and either pTV:00, pTV-SGT1 or pTV-HSP90 (TRV:RNA2). Three weeks after TRV infection, silenced plants were infiltrated with *Agrobacterium* strains containing p35S-INF1 and p35S-PINPP1.1. Recombinant *A. tumefaciens* containing the empty pCB302-3 vector was used as a negative control. Infiltration sites were evaluated 2 and 3 days after infiltration and frequencies of necrosis development were calculated as the frequency of necrotic panels x 100%. Silencing of SGT1 and HSP90 resulted in reductions of necrosis induced by all tested effectors. INF1 induced cell death was reduced 80 and 75% in SGT1 and HSP90 silenced plants respectively when compared to control plants. Similarly, SGT1 and HSP90 were found to be required for NLP induced cell death since SGT1 and HSP90 silenced plants exhibited significant reductions (>80%) in PINPP1.1 and PSOJNIIP induced cell death (Fig. 5.5). These experiment were performed several times and similar results were obtained.
COI1, MEK2, NPR1 and TGA2.2 contribute to PINPP1.1 but not INF1 induced cell death

To further compare INF1 and PINPP1.1 induced cell death, we elected to silence additional disease resistance signaling components and test their importance in effector-induced cell death. We selected and cloned gene fragments representing Coi1, Mek2, Tga2.2 and Npr1 from N. benthamiana in pTV00 and tested their involvement in PINPP induced cell death. Plants at the 4-5 leaf stage were infiltrated with mixtures of recombinant A. tumefaciens strains carrying the binary pBINTRA6 and either pTV00, pTV-COI1, pTV-NPR1, pTV-TGA2.2 and pTV-MEK2 (RNA2). Three weeks after TRV infection, silenced plants were infiltrated with A. tumefaciens strains containing p35S-INF1 and p35S-PINPP1.1. A recombinant strain containing the empty pCB302-3 vector was used as a negative control. Infiltration sites were scored 2 and 3 days after infiltration for development of necrosis. Knock down of coi1, npr1 gene expression resulted in a 35% reduction of necrosis frequencies for PINPP1.1 whereas MEK2 and TGA2.2 silenced plants showed 41 and 29 % reduction respectively when compared to control plants. In contrast to PINPP1.1, no reduction of INF1 induced necrosis was observed (Figure 5.6). These results indicate differences of signal requirements between INF1 and PINPP1.1 induced cell death, pointing to the existence of separate cell death signaling pathways.
Co-expression of *inf1* and *PiNPP1.1* or *PsojNIP* leads to synergistic enhancement of cell death.

We performed *Agrobacterium* co-infiltration assays to test the relationships between INF1, PINPP1.1 and PSOJNIP induced signaling pathways. To allow detection of possible antagonistic or synergistic effects between pathways, we determined the concentration threshold that resulted in significant reductions of necrosis frequencies (data not shown). *N. benthamiana* leaves were infiltrated with various concentrations of recombinant *A. tumefaciens* containing either p35S-INF1, p35S-PINPP1.1 and p35S-PSOJNIP. We experimentally determined that INF1 had the lowest sub-lethal OD$_{600}$ (~0.2) and used this value for further experiments (data not shown). To test for antagonistic or synergistic relationships, mixtures of recombinant *A. tumefaciens* strains carrying p35S-INF1 and p35S-PINPP1.1 or p35S-PSOJNIP (1:1 ratio) were infiltrated side-by-side with individual treatments on *N. benthamiana* leaves (Final OD$_{600}$ = 0.2). Infiltrated panels were evaluated and scored for necrosis development 1, 2 and 3 days after infiltration. Percentage of necrosis development was calculated (number of panels showing necrosis/total number of panels infiltrated x100%) per treatment for every comparison. Pair wise comparisons revealed distinct differences in timing and intensity of necrosis between treatments. 33% and 44% of the leaf panels challenged with INF1-PINPP1.1 and 79% and 100% of INF1-PsojNIP co-expressing sites already showed necrosis after 2 days. In contrast, infiltrations with individual recombinant strains only resulted in a 0% and 8% necrosis induction rate for INF1, 6% for
PiNPP1.1 and 13% for PsojNIP (Figure 5.7). Differences were sustained 3 days after infiltration since 83 and 94% of INF1-PiNPP1.1 and 100% of INF1-PsojNIP infiltrated panels developed necrosis compared to 19 and 29% for INF1, 17% for PINPP1.1 and 42% for PSOJNIP challenged panels respectively (Figure 5.7). These results confirm the presence of two distinct cell death pathways and in addition, showcase a synergistic interaction between INF1 and NLP induced cell death signaling.

5.5 DISCUSSION

Plant pathogenic microbes have the remarkable ability to manipulate cellular and biochemical processes in their host plants. These manipulations are achieved through a diverse array of effectors that can either promote infection (virulence factors) or trigger defense responses (elicitors or avirulence factors). As part of ongoing functional screens for *P. infestans* effectors, particularly those that induce plant cell death, we identified four members of the NLP family from *P. infestans*. One of these, PINPP1.1, induces necrosis in both the host plant tomato and *N. benthamiana*. Expression analyses of the PiNPP1.1 gene showed that it is up-regulated during late stages of colonization of tomato. PINPP1.1 induces a cell death pathway that is distinct from INF1 induced cell death. We found in parallel to INF1, SGT1 and HSP90 are required for PINPP1.1 and POSJNIP induced cell death. COI1, MEK2, NPR1 and TGA2.2 however, were identified as components that contribute to PINPP1.1 induced necrosis whereas INF1 induced cell death was not affected. Co-expression of INF1 and either
PINPP1.1 or PSOJNIP led to intensified and accelerated cell death induction reinforcing this observation and in addition suggesting some extent of interplay between pathways.

NLPs are widely distributed in plant pathogenic oomycetes and fungi, and occur as well in several plant-associated bacteria. Nonetheless, no NLP-like sequences could be detected in plants. This suggests that NLPs are a common feature of microbial organisms and are likely to be of central importance to their lifestyle. In *P. infestans* and *P. sojae*, NLPs form gene families with only a single member capable of triggering cell death. The prevalence of NLPs in *Phytophthora* species and the presence of orthologous genes in other plant pathogenic oomycetes, such as *Pythium* spp., suggests that these proteins may contribute to their parasitic mode of life. *Phytophthora* NLPs evoke complex defense responses including cell death in a large number of dicotyledonous plant species, as diverse as tomato, parsley, tobacco, *Arabidopsis*, and soybean (our findings; Fellbrich et al., 2002; Qutob et al., 2002). Taken together, NLPs may therefore be considered PAMPs or pathogen-specific molecular patterns that upon recognition trigger innate defense mechanisms in dicotyledonous plants. At present, however, it cannot be ruled out that NLPs trigger plant defense responses indirectly, for example, through enzymatic or toxic activity. Fellbrich et al., (2002) showed that NPP1 induces defense responses in parsley protoplasts. Thus, NLPs may facilitate the release of an endogenous elicitor from the plasma membrane of a variety of dicotyledonous plants, which in turn triggers defense responses.
We monitored expression levels of the three *PiNPP1* genes during *P. infestans*-tomato interaction. Semi-quantitative RT-PCR experiments revealed that all the genes are expressed during infection suggesting that they are functionally relevant to pathogenesis. However, *PiNPP1.1*, the only gene that triggers cell death, exhibited a markedly different expression profile from the other two genes. *PiNPP1.1* was significantly up-regulated during late stages of infection particularly during the transition from biotrophy to necrotrophy, when *P. infestans* moves to a destructive phase that results in collapse and necrosis of host tissue. This expression pattern is not expected for a PAMP and would suggest that *PINPP1.1* may be a toxin that facilitates colonization of host tissue during the late phase of infection. These results are consistent with transcript analyses of another NLP family member, PSOJNIP, during infection of soybean by *P. sojae* (Qutob *et al.*, 2002). This indicates that down-regulation of NLP genes during the early phase of infection followed by up-regulation during the necrotrophic phase could be a common feature in hemibiotrophic *Phytophthora* spp. A role for *PINPP1.1* in virulence is consistent with recent findings in *Erwinia* where deletion of NLP-like genes resulted in a reduced virulence phenotype (Pemberton *et al.*, 2005).

The NLP family is represented by an ever-growing number of proteins. The wide distribution of NLPs across several classes of plant pathogenic microbes, and their activity on a wide variety of plants, raises questions about the function and significance of these proteins in plant-microbe interactions.

Biochemical analysis of the activity of *P. parasitica* NPP1 in cultured
parsley cells and protoplasts showed convergent signaling with PEP13, a known and well-characterized PAMP from *Phytophthora*. To obtain more insights about NLP induced defense responses, we used VIGS to test the genetic requirements of NLP-induced cell death. We showed that in addition to the previously characterized elicitin INF1, PINPP1.1 as well as PSOJNIP require both SGT1 and HSP90 for cell death induction. Recently, SGT1 was proposed to act, together with RAR1, as a co-chaperone of HSP90, stabilizing and regulating signalosome complexes (Takahashi *et al.*, 2003; Liu *et al.*, 2004). Silencing of either component would reduce signalosome stability and or its activity. It is therefore tempting to speculate that both SGT1 and HSP90 are present in signalosome complexes, involved in NLP-induced responses. In this model, HSP90 would act as a molecular chaperone to increase the stability of protein complexes that include receptor-like proteins and other regulatory proteins (e.g. kinases). HSP90 is thought to have multiple targets, some of which could be involved in alternative cellular processes contributing to cell death. Therefore we cannot exclude the possibility that HSP90 silencing affected unknown cellular processes thereby inhibiting cell death. Genetic analyses of the role of additional signalosome components and HSP90 partners will help clarify this issue.

COI1, MEK2, NPR1 and TGA2.2 were identified as signaling components that are important in NLP-induced cell death. Silencing of COI1, MEK2, NPR1 and TGA2.2 resulted in reduced levels of necrosis induction in p35S-PINPP1.1 but not p35S-INF1 infiltrated panels. COI1 is required for jasmonic acid (JA) mediated signaling presumably via SCF$^{COI}$ mediated ubiquination *in planta* (Feng

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et al., 2003; Xu et al., 2002). It is possible that SCF^{COI} complexes play a part in NLP induced signaling and that during this process, they interact or require either HSP90, SGT1 or both. Expression of PiNPP1.1 in ASK1 and ASK2 silenced *N. benthamiana* plants should enable us to investigate whether this is the case. In this study, MEK2, a MAPK kinase, was found to contribute to NLP-induced cell death. MAPK signaling pathways constitute modules of protein kinases that regulate cellular processes and act as molecular switches between pathways. For example, upon elicitor treatment, NtMEK2 activates Wounding Induced Protein Kinase (WIPK) and Salicylic acid Induced Protein Kinase (SIPK) before onset of HR (Zhang and Liu, 2001). Although the upstream MAPKK kinase has not been identified as of yet, a three-component signaling module is proposed to modulate defense responses. These findings combined with our results, suggest that NLP expression in planta, induces cell death through discrete signaling cascades that include MEK2. Whether WIPK, or SIPK are part of a module that regulates NLP induced cell death, and which MAPKK kinase lies upstream within such a module, remains to be determined. It was shown recently that StMEK1, the closest *Solanum tuberosum* homolog of MEK2, does not require either HSP90 or HSP70 for necrosis induction. This may indicate that HSP90 acts either upstream or independent of a MAPK cascade (Kanzaki et al., 2003). The identification of a MEK2 dependent inducer of cell death could be useful towards elucidating the mechanisms of signal transduction and chaperone activity. Here, we showed that NPR1 and TGA2.2 silencing results in reduced levels of necrosis induction. Both NPR1 and TGA2.2 are transcriptional regulators of defense
responses dependent on SA. NPR1 physically interacts with TGA2.2, a requirement for activation of PR-gene expression and onset of defense responses (Despres et al., 2000; Despres et al., 2003; Niggeweg et al., 2000). It is therefore possible that the NLP-induced cascade is either aided by SA signaling or that it overlaps with some of its signaling steps. The contribution of SA (NPR1 and TGA2.2) and JA (COI-1) signaling genes to NLP induced cell death point to an intriguing possible role of NLP-like genes in pathogenesis. It is possible, that NLP-like genes are functionally analogous to coronatine, aiding in pathogen virulence through modulation of defense pathways (Bender et al., 1999; Feys et al., 1994; Zhao et al., 2003). This hypothesis would fit findings of a recent study of the Arabidopsis-Pseudomonas syringae pv tomato (Pst) association, where mutants deficient in coronatine production exhibit reduced virulence (Block et al., 2005). Silencing additional SA, JA as well as ethylene signaling genes will determine the level of interplay that exists between defense signaling and NLP-induced cell death and provide a more comprehensive overview of cellular reprogramming and virulence.

In our silencing experiments, the requirements for NLP-induced cell death was investigated and compared with that of INF1. In contrast to NLP-induced necrosis, silencing of COI1, MEK2, NPR1 as well as TGA2.2 did not affect INF1 induced HR. These observations point to the existence of separate signaling pathways leading to cell death. Plants have the ability to trigger intricate webs of multiple signaling pathways, enabling appropriate and dosed responses to a multitude of signals. For instance, RPP4, RPP7 and RPP8, R-genes active
against a defined set of *Pe. parasitica* isolates, have different but overlapping signaling requirements that mediate defense induction (Eulgem *et al.*, 2004; Aarts *et al.*, 1998). Although our knowledge of signaling pathways and defense induction is growing, understanding the complexity of their interactions remains a challenge. To investigate the relationship between signaling pathways, we co-expressed both *PiNPP1.1* and *PsojNIP* with *inf1* and assayed the effect on cell death induction. Co-expression of INF1 and PINPP1.1 or INF1 and PSOJNIP resulted in accelerated and intensified necrosis development compared to individual treatments. These results confirm the existence of two separate signaling cascades and in addition, showcase synergistic interplay between pathways. Pathogen recognition invariably results in cellular reprogramming. Signaling downstream of pathogen perception can induce transcriptional activation of multiple classes of defense related genes initially, followed by up-regulation of genes that are involved in signal transduction (Eulgem *et al.*, 2004). Similarly, perception of *flg22*, a PAMP molecule that induces innate immunity in *Arabidopsis*, results in up-regulation of signaling genes predominantly (Navarro *et al.*, 2004). It is possible, that recognition of PAMPs or other pathogen molecules leads to sensitization or priming of host cells to other biotic stimuli. It is therefore sensible to propose that either INF1 or NLP recognition leads to increased expression of signal transduction components, thereby sensitizing plant cells to either or both molecules. To further understand such regulatory networks and perhaps feedback loop mechanisms, future efforts could be
directed at those genes that are induced by either INF1 or NLPs and investigate their role in synergism or cell sensitization.

In summary, NLPs and INF1 represent an example of pathogen molecules that induce cell death through distinct signaling pathways. Although many questions remain unanswered, this contribution to our current knowledge has raised some intriguing possibilities with respect to NLP-gene function. Future studies will help to further identify and characterize NLP-specific signaling components and provide more insight in the mechanics that underlie host cell death during pathogenesis and virulence.

5.6 ACKNOWLEDGEMENTS

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5.7 REFERENCES


Figure 5.1. Multiple alignment of *P. infestans* PINPP proteins. Identical amino acids are shaded in dark grey and similar amino acids are shaded in light gray. Residue numbers are indicated above the sequences. Signal peptides are underlined.
Figure 5.2. Symptoms observed on *N. benthamiana* and *L. esculentum* plants following inoculation of lower leaf with recombinant PVX constructs. Photographs were taken 14 days post-inoculation. Panel 1 with the entire leaves, panel 2 under a dissecting microscope to visualize the inoculated sites and panel three under UV light using GFP filter. (a) Symptoms observed on *N. benthamiana*. Left section, lack of plant response at the inoculated site of the empty vector pGR106, middle section, necrosis at the site of inoculation in pGR106:INF1 and on the right section, necrosis at the site of inoculation in pGR106:PiNPP1. (b) Symptoms observed at the inoculated sites on *L. esculentum*. Left panel with lack of plant response at the inoculated site of the empty vector pGR106 and on the right necrosis at the site of inoculation in pGR106:PiNPP1 (c) Spreading necrosis and plant death induced by pGR106:PiNPP1.1 on three plants shown on the right section. Same age plant inoculated with pGR106 is shown on the right section.
Figure 5.3. Comparative analysis of *P. infestans* elicitin (*inf1*); *P. infestans* necrosis-inducing *Phytophthora* protein 1 (*PiNPP1.1*, *PiNPP1.2*, *PiNPP1.3*; *P. sojae* necrosis-inducing protein, *PsojNIP*; *F. oxysporum* necrosis- and ethylene-inducing peptide, *NEP1*, inoculated on the same leaf on *N. benthamiana* and *L. esculentum*. The leaf base flanking the main vein was inoculated, as described in the experimental procedures. Test plants were inoculated with *A. tumefaciens* harboring the PVX expression vector containing a DNA construct encoding an open reading frame, as follows: 1. pGR106-INF1 2. pGR106-PiNPP1.1 3. pGR106-PiNPP1.2, 4. pGR106-PiNPP1.3; 5. pGR106-PsojNIP; 6. pGR106-NEP1 and 7. empty vector pGR106.
Figure 5.4. RT-PCR analysis of the PiNPPs. Time course expression of PiNPPs and ef2 of *P. infestans* isolate 90128 during colonization of tomato. Total RNA isolated from infected leaves of tomato 0, 1, 2, 3, 4 days after inoculation, from non-infected leaves (To), and from *P. infestans* mycelium grown in synthetic medium (My). Amplification of *P. infestans* elongation factor 2 gene (ef2) was used as a control to determine the integrity of the RNA. Appropriate sizes of the transcripts are 492bp for PiNPP1.1, 693 for PiNPP1.2, 693 for PiNPP1.3 and 700bp for ef2.
Figure 5.5. PiNPP1 induces SGT1 and HSP90-dependent cell death. *N. benthamiana* plants were infiltrated with mixtures of combinations containing *A. tumefaciens* pBINTRA6 (TRV RNA1) and either pTV00 (TRV), pTV:NbSGT1 (TRV:NbSGT1) or pTV:LeHSP90 (TRV:LeHSP90). Silenced plants were challenged with *A. tumefaciens* carrying binary pp35S-INF1, p35S::PiNPP1.1 and empty vector. Leaves were photographed 3 days after the secondary PVX challenge (panel A). Frequency of necrotic lesion occurrence after agroinfiltration was determined on control as well as SGT1 and HSP90 silenced plants. Occurrence of necrosis induction was recorded 2 and 3 days after infiltration and subsequently, percentages of necrotic panels were calculated for each elicitor within every silencing treatment (Figure 5B). Black bars represent the levels of necrosis induction on TRV infected plants, white bars represent observed frequencies on SGT1 silenced plants and red bars represent observations on HSP90 silenced plants.
Figure 5.6. COI1, MEK2, TGA2.2 and NPR1 contribute to PINPP1.1 but not INF1 induced cell death. *N. benthamiana* plants at the 4-5 leaf stage were infiltrated with mixtures of recombinant *A. tumefaciens* strains carrying the binary pBintra6 (TRV:RNA1) and either pTV00, pTV::coi-1, pTV::mek2, pTV::npr1 and pTV::tga2.2 (TRV:RNA 2). Three weeks after TRV infection, silenced leaves were infiltrated with recombinant *A. tumefaciens* strains containing p35S-INF1, 35S::PINPP1.1 and PCB302-3 (negative control). Infiltrated leaf panels were scored 3 days after infiltration. Top panel, control (TRV), COI-1, MEK2, NPR1 and TGA2.2 silenced leaves 3 days after infiltration. Bottom panel, percentage of necrosis (Y-axis) occurring on p35S::inf1 and p35S::PINPP1.1 infiltrated leaf panels after 3 days.
Figure 5.7. Co-expression INF1 and PiNPP1.1 or PsojNIP leads to synergistic induction of cell death. Mixtures of recombinant *A. tumefaciens* strains carrying *p35S-INF1* and *35S::PiNPP1.1* or *p35S-INF1* and *35S::PsojNIP* were infiltrated side-by-side with individual treatments on *N. benthamiana* leaves (Final OD$_{600}$ = 0.2). Infiltrated panels were evaluated and scored for necrosis development 1, 2 and 3 days after infiltration. Left panel: symptom development 3 days after infiltration. Percentages of necrotic panels were calculated (number of infiltrated sites showing necrosis/total number of infiltrations*100%) for each treatment and per comparison (right panel).
CHAPTER 6

THE CRN GENES OF PHYTOPHTHORA INFESTANS ENCODE A COMPLEX
AND DIVERSE FAMILY OF EFFECTORS THAT TRIGGER CELL DEATH IN
HOST AND NONHOST PLANTS

6.1 ABSTRACT

Plant-pathogen associations are established through multiple and complex
exchanges of plant and microbe molecules. In plants, receptor-mediated
perception of pathogen molecules or their activity, initiates discrete signaling
steps leading to defense induction and resistance. The challenge for plant
pathogens therefore is to circumvent, suppress, or evade defense responses.
Consequently, plant pathogens employ effector molecules that perturb cell
signaling, metabolism and transcription. Pathogenic microbes and their host are
continuously co-evolving while competing. The conceptual result is described as
an arms race, in which continuous invention and modification of pathogen
virulence-and host target genes occurs.
Here we characterized the *P. infestans* CRN protein family. Sixteen full-length CRN sequences were obtained and used for sequence and phylogenetic analyses. Two gene clusters were identified in this family and patterns of reticulate evolution were discovered within cluster II. *In planta* expression of 13 crn paralogs revealed that most but not all genes induce cell death in *Nicotiana benthamiana* and *Solanum spp*. We expanded our analysis of CRN induced cell death. Expression of CRN2 and its effector domain in A. thaliana resulted in cell death. Gene expression profiling of CRN1 and CRN2 induced responses revealed that genes from multiple functional classes are induced. We silenced known signaling components and assayed their importance in CRN2 induced cell death. The ubiquitin ligase-associated SGT1b protein and the molecular chaperone HSP90 were found to be required for cell death. We identified four additional signal and regulatory genes involved in cell death. COI1, MEK2, NPR1 and TGA2.2 contribute to CRN2 but not INF1 induced cell death, pointing to two separate cell death pathways. Finally, we confirmed these results via co-infiltration assays. Co-expression of INF1 and CRN2 resulted in accelerated cell death whereas CRN2 and PINPP1.1 co-expression had no detectable effect. Altogether these results implicate the crn gene family as a group of effector molecules that are relevant to *P. infestans* host interactions.
6.2 INTRODUCTION

Plant-pathogen associations are established through multiple and complex interactions between microbe and plant molecules that result in either plant susceptibility or resistance (Kamoun et al., 2001; Huitema et al., 2004). In plants, receptor-mediated perception of pathogen molecules or their activity, initiates defense induction and resistance (Dangl and Jones, 2001; Staskawicz et al., 1995). Resistance genes ($R$) encode receptor like proteins that continuously survey extracellular as well as intracellular spaces for foreign molecules or perturbations of host cellular processes (Martin et al., 2003). Perception of biotic abnormalities initiates a set of discrete signaling steps that lead to transcriptional changes, onset of defense responses and in some cases the hypersensitive response (HR) a form of programmed cell death (Dangl and Jones, 2001). Multiple $R$ genes have now been identified and genetic analyses have culminated in models that explain effector recognition and subsequent resistance signaling through tightly controlled multi-step cascades. Despite the large number of identified $R$ genes, only a small number of downstream signaling components are known, pointing to a set of shared signaling proteins in plants. Among such signaling components, the ubiquitin ligase-associated protein SGT1 is required for a multitude of $R$ gene mediated HR reactions (Peart et al., 2001; Austin et al., 2002; Azevedo et al., 2002). SGT1 interacts with the COP9 signalosome and was recently found to interact with RAR1 and the molecular chaperone HSP90, suggesting the presence of multiprotein complexes involved in resistance signaling (signalosomes) (Liu et al., 2002; 2004; Takahashi et al.,
In this model, SGT1 as well as RAR1 function as co-chaperones, folding and stabilizing signalosome protein complexes (Liu et al., 2004).

Plants are exposed to a multitude of potential pathogens and consequently, R gene mediated recognition only explains the minority of associations where resistance is the outcome. Both salicylic acid (SA) and jasmonic acid/ethylene (JA/E) dependent defense pathways have been identified and linked to systemic acquired resistance (SAR) and induced systemic resistance (ISR) respectively (Ryals et al., 1996; Reymond and Farmer, 1998; Feys and Parker, 2003). Local induction of either or both pathways results in elevated levels of systemic resistance to most pathogens (Glazebrook, 2001; Ellis et al., 2002; van Wees et al., 2000). Based on genetic analysis, important players in both SAR and ISR pathways have been identified and in addition, extensive cross-talk was found to occur (Shah et al., 1999; Felton et al., 1999; van Wees et al., 2000). Among these genes, Npr1 and Coi1 were identified as regulatory components for SA and JA/E mediated defenses respectively (Cao et al., 1997; Ryals et al., 1997; Xie et al. 1998). Taken together, these and other components integrate multiple biotic signals and tightly regulate subsequent responses.

The challenge for the pathogen is to circumvent, suppress, or evade defense responses. Plant pathogens have the remarkable ability to affect host metabolism and signaling (Kamoun et al., 2001). Suppression of host defenses is thought to occur through delivery of effector molecules that perturb cellular processes including cell signaling, metabolism and transcription (Kjemtrup et al., 2003).
In a quest for survival, pathogenic microbes are continuously competing and consequently co-evolving with their host. The result is often times described as an arms race, in which continuous invention and modification of pathogen virulence genes, is countered by the plant through alteration of recognition capabilities or modification of effector targets (Stahl and Bishop, 2000). Consequently, birth-and-death evolution, reticulate evolution as well as diversifying selection contribute to co-evolution, in particular to formation of gene families in pathogenic microbes (Gotesson et al., 2002; Bos et al., 2003; Liu et al., 2005).

The oomycete pathogen, *Phytophthora infestans* is the causal agent of late blight of potato and tomato causing billions of dollars in economic losses annually (Erwin and Ribeiro, 1996; Kamoun, 2001; Kamoun, 2003). *P. infestans* infection is marked by an early biotrophic stage followed by a distinct necrotrophic stage in which the infected tissue turns necrotic (Kamoun, 2003). Despite its economic importance, relatively little is known about the molecular mechanisms of host specificity and pathogenicity. During infection, *P. infestans* secretes a vast array of effector molecules that are aimed at reprogramming host cellular processes, enhancing virulence (Kamoun, 2003; Huitema et al., 2004; Tian et al., 2004). Targets of these effector molecules not only reside in the apoplast but may also be present in the cell cytoplasm, suggesting a mechanism of protein translocation into plant cells (Armstrong et al., 2005). With the advent of sequencing technologies, computational tools and robust functional assays, putative effector molecules have now been identified successfully (Torto et al., 2000).
2003; Qutob et al., 2002; this thesis). Consequently, there is a precedent for using pre-determined criteria towards selecting candidate gene families for further analyses. Computational and functional characterizations of effector gene families are expected to significantly impact our understanding of virulence mechanisms and evolution in Phytophthora.

Here, we present the characterization of the CRN gene family in P. infestans. Previously, using a functional genomics approach, crn1 and crn2 were identified as necrosis-inducing genes in N. benthamiana and tomato and subsequent data mining revealed a large gene family (Torto et al., 2003). We obtained full-length sequences and performed comparative analyses. Sequence alignment of all 16 crn gene family members pointed to a conserved motif of seven N-terminal amino acids. Across all CRN protein sequences, C-terminal portions exhibit a mosaic structure, suggesting exchanges of gene segments (reticulate evolution). We therefore investigated the evolutionary mechanisms underlying the diversification of the crn family. Sawyer’s test and the Phylogenetic Profile method revealed evidence of gene conversion and gene recombination events, the main genetic mechanisms of reticulate evolution.

We expanded our functional analysis of the crn gene family and identified a subset of paralogs that exhibit necrosis-inducing activity in both N. benthamiana and Solanum species. We expressed crn2 in multiple Solanum as well as a non-solanaceous species and found induction of cell death in most cases. These results suggest that the crinklers form a diverse family of protein molecules that exhibit activity in a wide range of plant species.
We used microarray experiments to investigate crn1 and crn2 induced transcriptional changes in tomato. CRN1 as well as CRN2 affected expression of genes that represent multiple functional classes. To enhance our understanding of CRN induced cell death, we applied virus induced gene silencing (VIGS) to silence known signaling components in plants. Subsequent challenges of SGT1, HSP90, MEK2, COI1, TGA2.2 and NPR1 silenced plants with CRN2 showed involvement of these genes in CRN2 induced cell death and distinct differences with INF1 induced signaling. These differences were confirmed in co-infiltration assays. Co-expression of INF1 and CRN2 led to intensified and accelerated cell death induction. Altogether, these findings implicate the crinklers as a diverse family of effector genes in Phytophthora. Their complex evolutionary history suggests mechanisms of co-evolution with either its present or ancient host. Future studies into the mechanisms of crn gene function and the basis of cell death induction will shed more light on their role and importance in Phytophthora pathogenesis.

6.3 MATERIAL AND METHODS

Data sets

A total of 16 full-length cDNA sequences of the crn gene family members were used in this study (Torto et al. 2003, Torto and Kamoun, unpublished). cDNA coding sequences were translated into corresponding amino acid sequences using the computer program DNA strider™1.3f.
Multiple sequence alignment and phylogenetic tree

The amino acid sequences of CRN proteins were aligned using the program Clustal X 1.64. The phylogenetic tree was constructed using the neighbor-joining method implemented in Clustal X 1.64. Alternative topologies were viewed with the program TreeView PPC 1.6.6.

Sawyer’s test

The aligned cDNA coding regions of the crn genes were used to detect gene conversion using Sawyer’s test implemented in the software GENECONV version 1.81 (Sawyer, 1999). We performed Sawyer’s test on the two major clusters (cluster I and II) of the crn gene family. Sawyer’s test is based on the analysis of the distribution of maximal length aligned fragments shared by a pair of homologous DNA sequences, which is assumed to occur as a result of gene conversion (Sawyer 1989). The significance of the distribution is estimated by a Monte Carlo test involving permutation of sites. In detail, Sawyer’s test searches for identical or similar fragments from the alignment of pairs of sequences, which are members of a gene family consisting of at least three members. Because mismatches could occur as a result of mutations following a gene conversion event or incomplete mismatch repair during the gene conversion event, mismatches within fragments were allowed in our study. The parameter option gscale, indicating the mismatch penalty for fragment scores, was set to 1. Higher positive values of gscale indicate more severe penalties. The original data were randomly permutated 10,000 times. Then, the statistics were calculated for each
permutation. Comparisons of the observed shared fragment with the data sets obtained from the random 10,000 times permutations of the original data were used to estimate the significance of the observed shared fragment. Global and pairwise probability $P$-values can be estimated by two methods. The first method is based on the 10,000 permutations and the second is based on the method of Karlin and Altschul (1990; 1993), which is similar to the method used by the BLAST sequence similarity search program. However, we only used the first method (10,000 permutations) since it was reported to be more accurate and conservative (Sawyer 1989). The pairwise probability $P$-value for an original fragment is the proportion of permuted alignments having a fragment with a higher score than the observed fragment in the same sequence pair. Whereas, the global probability $P$-value for an original fragment is the proportion of permuted alignments having a fragment with a higher score than the observed fragment in any possible pairs of sequences. In this study, we only used global probability $P$-value because global $P$-values are multiple sequence comparisons corrected for all possible sequence pairs, while pairwise $P$-values are not. Only global permutation $P$-values smaller than 0.05 ($P < 0.05$) were considered as significant, and, therefore, providing evidence of gene conversion. These fragments are inner fragments, which arose through gene conversion event between ancestors of two sequences in the alignment. Sawyer’s test can also detect outer fragments, which are evidence of ancient gene conversion events. This ancient gene conversion event may either come from outside of the alignment or within the alignment. However, later mutation or gene conversion
may have damaged evidence for this ancient gene conversion event. The theory for detecting outer fragments is the same as one used for detecting inner fragments.

**Phylpro program**

The amino acid sequence alignments of the *crn* gene family were analyzed for gene recombination using the Phylogenetic Profile method implemented in the computer program Phylpro (Weiller 1998). The Phylogenetic Profile method is based on the theory that phylogenetic relationships between two different segments of a multiple sequence alignment will be similar unless they have been affected by gene recombination events. The method will define two sequence windows for each test position in a given sequence of the multiple sequence alignment. One window is called the upstream window located immediately before the test position, and the other is called the downstream window located directly after the test position. In order to determine the differences between the test sequence and all other sequences in the window, two vectors of distance data are estimated by pairwise comparison from the upstream and downstream windows of a given test position. The phylogenetic correlation of a given test sequence at a given test position is calculated as the linear correlation coefficient of the upstream and downstream distance vectors of a given test position. If a gene recombination event occurred in the test position of the test sequence, then the two distance vectors will correlate poorly and the phylogenetic correlation of the given test position will exhibit a low value. All
phylogenetic correlations for every position of every sequence, named a “phylogenetic profile”, can be plotted and superimposed in a single graph using the program Phylpro. If the phylogenetic correlation for a given test position of a given test sequence is very low, and therefore appears as a downward peak appeared in the phylogenetic profile graph, then this position is a likely site for gene recombination in that test sequence. In our analysis, we used the default value (10 differences) of window limits. We separately applied Phylogenetic Profile method on the two clusters of the *crn* gene family because sequences from different clusters could not be unambiguously aligned.

**Functional assays using PVX-based expression**

For functional assays of *crn* paralogs on *N. benthamiana* and *Solanum* species, plants were maintained under greenhouse conditions with a temperature of 22-25°C and high light intensity throughout the experiments. To determine the activity of 13 *crn* paralogs in *N. benthamiana*, we performed three independent experiments using the PVX-based expression vector, pGR106. Cloning of the 13 *crn* paralogs into this PVX-vector was performed as previously described by Torto *et al* (2003). In these experiments, side-by-side wound-inoculations with recombinant *Agrobacterium* GV3101 (carrying pGR106-cDNA) as previously described (Torto *et al.*, 2003) were performed. In each experiment we inoculated at least two plants. Finally, the activity of CRN2 was assayed on a selection of 34 *Solanum* species, represented by 65 clones using a similar PVX-based
approach. In all functional assays the empty vector pGR106 was used as a control. Symptoms were observed during time ranges of 5-12 or 4-17dpi.

**Plant growth conditions.**

*Nicotiana benthamiana* seeds were seeded into soil and germinated in large pots under a cheesecloth mesh. Young seedlings were transferred into soil in individual styro-foam pots. Plants were grown under greenhouse conditions at 22-25°C high light intensity before and throughout the experiments.

**CRN2 functional analysis in Arabidopsis thaliana.**

We used the floral dip method for *Arabidopsis* transformation as reported (Clough and Bent, 1998). Plants were grown to the flowering stage in growth chambers using a 16 hr light, 8 hr dark regime. Flowering plant parts were submerged in recombinant *Agrobacterium* suspensions (OD600 ~0.8) containing 5% sucrose and 0.02% Silwet L-77. Dipped plants were incubated over night in the dark at 22-24 °C. This procedure was repeated on the same plants after 1 week. Seeds collected from the treated plants were surface sterilized and plated onto MS-sucrose plates containing 30 µg hygromycin/mL (company). Plates were incubated at 4°C for 3-4 days after which they were transferred to the growth chamber. Healthy root forming seedlings were transferred to soil after approximately 2 weeks and grown to maturity. T1 seeds were collected from transformants after PCR confirmation.
**DEX induction experiments:**

Transgenic *Arabidopsis* plants were grown from T1 seeds as mentioned previously. Mature, 4-5 week old plants were sprayed with 30 μM DEX in 0.1% EtOH solution and covered with a plastic dome. Applications of 0.1% EtOH were used as negative controls. Symptom development was monitored and recorded between 1 and 5 days after treatment.

**Plant material**

For gene expression analysis of the tomato response to CRN1 and CRN2, cotyledons of 2-week old tomato plants were wound-inoculated with recombinant *Agrobacterium* strain GV3101 containing pGR106-CRN1, pGR106-CRN2 pGR106-EPI and the empty vector control (pGR106). Tomato plants were grown and maintained throughout the experiments under greenhouse conditions with a temperature of 22-25°C and high light intensity. Leaf material was harvested when plants showed symptoms of systemic infection (12-14 dpi), frozen in liquid nitrogen and stored at -80°C. Two independent biological replications were conducted, each including two plants for the treatment (pGR106-CRN2, pGR106-CRN1 or pGR106-EPI1) and two plants for the reference samples (pGR106-empty).
Plasmid construction

pGR106 constructs containing CRN1 and CRN2 (pGR106-CRN1 and pGR106-CRN2) were described previously (Torto et al., 2003). cDNAs of all other tested paralogs were cloned using an identical approach described by Torto et al. (2003). Deletion constructs were prepared using gene specific primers amplifying regions of varying lengths using the following primers: 1F: 5'-GGAAATCGATGGCTTTCCCTGGACACCCGACGC-3'
2F: 5'-GGAAATCGATGGCTGCTCTGAAGCTGGTG-3'
3F: 5'-GGAAATCGATGGGAATCTGATGACTACATCCATCG-3'
4F: 5'-GGAAATCGATGCACAGGAATTCAAGTG-3'
7F: 5'-GGAAATCGATGGTATTGATCTTGGATATCATC-3'
8F: 5'-GGAAATCGATGTACACGAAGAACAATGGAG-3'
9F: 5'-GGAAATCGATGGGTTCAGCGAGTGACACTTCC-3'
10F: 5'-GGAAATCGATGTCCAAGCGTACGCGATATGTC-3'
11F: 5'-GGAAATCGATGGGATTTCGCCTGTGGACACA-3'
5R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'
6R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'; 7R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'; 8R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'; 9R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'; 10R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'; 11R: 5'-GGAAATCGATGGGTCAGCGAGTGACACTTCC-3'. Amplified gene regions were ligated into pGR106 predigested with ClaI and NotI. For binary
vector constructs, we amplified both the CRN2 full-length gene as well as its effector domain using the following primer combinations: CRN2-Xho: 5'GGGCTCGAGATGGTCAAGCTTGTGGCGATCGTTG-3' and CRN2-Spe: 5'-GGGactagtTCAGCTGAAATAACTCAAAGTAGGAATAG-3'. CRN2-Spe/Xho: 5'-GGAAACTAGTCTCGAGATGTCCAAGCGTACGATATGTCACCAC-3' and CRN2-Spe. Xhol and SpeI digested CRN2 fragments were ligated into the inducible expression cassette pTA7002 (Aoyama and Chua, 1997) to yield pTA-CRN2 and pTA-CRN2del10 respectively. For agroinfiltration assays, a pCB302-3 derivative (Xiang et al., 1999) was used. CRN2 PCR fragments were digested with SpeI and ligated into pCB302-3 to obtain p35S-CRN2. Obtained constructs were sequence verified using either primers used for cloning (pTA and pGR constructs) or 35S-F: 5'-ATGACGCACAATCCCACTATCCTTC-3' (p35S-CRN2).

**RNA manipulations and microarray analysis**

RNA was extracted using Trizol reagent (Gibco-BRL, Bethesda, MD, USA) according to the manufacturer’s recommendations with slight modifications. To increase the quality of the RNA, an additional chloroform extraction step was included in the protocol. Finally, the quality and amount of RNA was assessed using a spectrophotometer and gel electrophoresis. Probe preparation and hybridization with cDNA microarrays (composed of 10,000 cDNA clones/genes from potato) was conducted as described in protocols available on the TIGR website ([http://www.tigr.org/tdb/potato/microarray_SOPs.shtml](http://www.tigr.org/tdb/potato/microarray_SOPs.shtml)). For hybridization purposes, the treatment cDNA was labeled with CY3 and the reference cDNA
with CY5 fluorescent dye. For each treatment and corresponding reference two slides were used for hybridization, one for each biological replication. After hybridization, the slides were scanned using a GenePix 4000B scanner and data was acquired using the GenePix Pro 4.0 software (Axon Instruments, Molecular Devices Corporation, Union City, Ca, USA). Two data points per slide were obtained for each gene and these were evaluated in further analyses as technical replicates. Data normalization was performed using the LOWESS algorithm in MIDAS (http://www.tigr.org/software/tm4/midas.html).

**Data analysis**

Regression analysis and outlyer detection was performed using the SAS software package version 8 (SAS institute, Cary, NC, USA). The four replications (two biological replications and two technical replications/array) were analyzed separately by using the log transformed ‘raw’ expression values as input data. For regression analysis, expression data generated in the effector treatment (Y-axis) was plotted against the reference (X-axis). A 99% confidence interval was used to select genes that fell out of this interval were identified as being differentially expressed. A series of grep commands in Bbedit were used to identify genes that were differentially expressed in all four replications and to extract the corresponding expression data across all treatments. For clustering purposes, two data sets were generated. First, the average ratios from two technical replications were calculated for each biological replication. Then, for each gene, the total average ratio was calculated using all replications. Genes
with missing data points for one of the treatments were removed before clustering. Cluster analyses were performed using the Cluster software package (Eisen et al., 1998). First, the genes list was organized using the SOM (self organizing map) algorithm on the log transformed data. The generated output file was used as input file for average linkage hierarchical clustering in both dimensions. Data was visualized and evaluated in TreeView 1.0.8. (Eisen et al., 1998) Finally, putative functions of significantly up-or down-regulated genes provided by TIGR were checked using the BLAST algorithm against the GenBank nr database. The complete microarray data set is publicly available at the TIGR Solanaceae Gene Expression Data Base website (http://www.tigr.org/tigrscripts/sgedb/studies_SGED.pl).

**TRV induced gene silencing.**

Constructs and procedures for TRV induced gene silencing were performed as described previously (This thesis, Chapter 5). In short, A. *tumefaciens* strain GV3101 carrying the binary TRV RNA 1 construct, pBINTRA6, and the TRV RNA2 vector, pTV00 (Ratcliff et al., 2001), or pTV00 derivatives were prepared for agroinfiltration assays and mixed in 2:1 ratio (RNA1: RNA2; final OD$_{600}$ = 0.6). Infiltrations were conducted by gently pressing a 1-ml disposable syringe to the abaxial surface of fully expanded leaves of 3-week old *N. benthamiana* plants slowly depressing the plunger. A sufficient amount of bacterial suspension was used to infiltrate the leaves to give a water-soaked appearance. This typically required 1-4 infiltrations per leaf. Following
Agroinfiltration, plants were maintained in a greenhouse at 22°C with a 16 h photoperiod. Secondary challenges were performed about three weeks after agroinfiltration with the TRV strains.

**Agroinfiltration assays**

Recombinant *Agrobacterium* strains (GV3101) were grown as described elsewhere (Van der Hoorn *et al.*, 2000) except that all culturing steps were performed in LB media. For infiltrations of silenced plants, suspensions were spun down and re-suspended in MMA infiltration medium to a final OD$_{600}$ of 0.8 (1 liter MMA: 5 g of MS salts, 1.95 g of MES, 20 g of sucrose, 200 µM acetosyringone, pH=5.6). For the co-expression assays, exact same procedures were followed except that for the effector combinations, recombinant strains were mixed in a 1:1 ratio. In addition, a final OD$_{600}$ of 0.2 was used for infiltration of all treatments. All suspensions were incubated 1-3 hours in MMA prior to infiltration. For both silencing and co-infiltration assays, at least 4 plants were infiltrated for each treatment and 3-4 leaves were used per plant.

### 5.4 RESULTS

**CRN sequence analysis** (contributed by Trudy Torto-Alalibo and Zhenyu Liu)

We identified 14 additional CRN-like genes from available EST sequence collections (Table 6.1). For each gene, the original cDNA clone was retrieved and used for further sequencing. We applied a primer walking approach from both 5' and 3' prime ends to obtain full-length cDNA sequences. For this purpose,
sequences derived from each individual cDNA were generated using the Phred base calling software (Ewing et al., 1998) compiled and assembled to obtain contigs with high quality sequences. Putative open reading frames were identified from each consensus sequence and were used to generate predicted amino acid sequences (for Genbank accession numbers, see table 1). The CRN gene family encodes a diverse family of proteins ranging in size from 190 (CRN12) to 847 (CRN9) amino acids (Table 6.1). We applied the SignalP V. 2.0 (Nielsen et al., 1997) program to identify putative signal peptide sequences (Data not shown). Our analyses show that all CRN proteins are putatively extracellular, indicating that they can function as effector molecules. We complied all CRN-like amino acid sequences and used ClustalX (Thompson et al., 1997) to generate alignments (Figure 6.1). ClustalX analysis identified both sequence conservation and divergence between gene family members (Figure 6.1). Sequence alignment of CRN proteins sequences revealed a conserved motif in the CRN N-terminus consisting of a positively charged residue (D, K, N, E or Q) followed by LQ/HFLAK. Conservation of this motif implies a generalized role in processes such as post-translational processing, protein targeting or perhaps translocation.

**Phylogenetic analysis** (contributed by Zhenyu Liu)

To investigate the relationship among members of the *crn* gene family, we constructed the phylogeny of 16 *crn* genes using the neighbor-joining method (Figure 6.2). A total of 1,000 bootstrap replications were conducted to determine the statistical significance of the obtained branches. When considering bootstrap
values higher than 850, the *P. infestans crn* genes fell into two major clusters, cluster I and cluster II (Figure 6.2). Cluster I consists of *crn1, crn8, crn9, crn12* and *crn14*. Cluster II includes *crn2, crn3, crn7, crn10, crn11, crn15* and *crn16* (Figure 6.2).

**Evidence for gene conversion in the crn genes** (contributed by Zhenyu Liu)

In order to detect whether gene conversion has occurred in the examined *crn* genes, we applied Sawyer’s test implemented in the software GENECONV 1.81 on *crn* genes from cluster I and cluster II. A total of 10 gene conversion events were detected in genes from cluster I (Table 6.2), whereas 20 gene conversions were detected in genes from cluster II (Table 6.3). Eight out of 10 (80%) of inner polymorphic fragments shared by members of cluster I had global permutation *P*-values equal to 0.0000, whereas 2/10 (20%) had global permutation *P*-values lower than 0.0006 (Table 6.2). In cluster II genes, 18/20 (90%) of inner fragments had global permutation *P*-values equal to 0.0000, whereas 2/20 (10%) had global permutation *P*-values lower than 0.003 (Table 6.3). All the inner fragments mentioned above are significant with global permutation *P*-values lower than 0.05. For example, there is significant evidence for an 84-bp inner polymorphic fragment (with global permutation *P*-values equal to 0.0000) between *crn8* and *crn1* in the region between nucleotide 7 and 90, with only 5 mismatches (Table 6.2). Examples of gene conversion within cluster II genes include two large fragments (positions 198 to 1901 in *crn2* and *crn10*,...
positions 277 to 1136 in \textit{crn15} and \textit{crn16}) with global permutation \( P \)-values lower than 0.003 (Table 6.3).

Seven outer fragments were detected in cluster II of the \textit{crn} gene family (Table 6.4). Two fragments in \textit{crn3} (position 305 to 312 and position 425 to 411), two fragments in \textit{crn7} (positions 169 to 274 and positions 285 to 306), one fragment in \textit{crn10} (positions 1057 to 1060), one fragment in \textit{crn11} (positions 1057 to 1060), and one fragment in \textit{crn16} (positions 1158 to 1169) had global permutation \( P \)-values lower than 0.04, indicating the presence of ancient gene conversions events (Table 6.4). These ancient gene conversion events are thought to come from outside the sequences examined in the alignment. This prompted us to study which sequences outside cluster II may have shared these seven outer polymorphic fragments. Therefore, the coding sequences of \textit{crn3}, \textit{crn7}, \textit{crn10}, \textit{crn11} and \textit{crn16} were aligned with sequences of all non-cluster II permutation \( P \)-values lower than 0.05 (Table 6.5). \textit{crn3} was shown to share polymorphic fragments with \textit{crn4}, \textit{crn5}, \textit{crn9} and \textit{crn13}. \textit{crn7} shared inner polymorphism fragments with \textit{crn1}, \textit{crn4}, \textit{crn5}, \textit{crn8}, \textit{crn9}, \textit{crn12} and \textit{crn14}. Only one inner fragments was shared by \textit{crn10} and \textit{crn4}. Three inner polymorphic fragments shared by \textit{crn11} and \textit{crn4}, \textit{crn9} and \textit{crn12} were also detected. Finally, \textit{crn16} was found to share three inner fragments with \textit{crn4}, \textit{crn9}, and \textit{crn13}, respectively. Overall, these results provide strong evidence for gene conversion in the \textit{crn} gene family.
Gene recombination in cluster II of crn gene family (contributed by Zhenyu Liu)

To further investigate reticulate evolution in the crn gene family, we analyzed cluster I and cluster II sequences for gene recombination using PhylPro (Weiller, 1998). We did not find evidence for gene recombination in the cluster I genes. However, strong recombination signals, visualized by single sharp downward peaks were identified in the phylogenetic profiles of cluster II genes (Figure 6.3). Two recombination sites were observed for all genes of cluster II, suggesting that all seven genes (crn2, crn3, crn7, crn10, crn11, crn15 and crn16) are recombinants (Figure 6.3). We mapped all the recombination sites derived from the phylogenetic profiles of the multiple sequence alignments of cluster II genes (Figure 6.4). Mosaic structures were clearly observed, suggesting that rearrangements of sequences appeared through multiple gene recombination events during the evolution of the crn gene family (Figure 6.4).

CRN paralogs induce cell death in N. benthamiana (contributed by Trudy Torto-Alalibo)

Since CRN1 and CRN2 were previously identified as elicitors of cell death in N. benthamiana (Torto et al., 2003), and because of the interesting evolutionary history of the CRN gene family, we tested additional CRN genes for effector activity in planta. Eleven additional crn paralogs were cloned into the binary PVX vector (pGR106), transformed into Agrobacterium (GV3101) and toothpick inoculated side-by-side on fully expanded N. benthamiana leaves.
Besides CRN1 and CRN2, six additional CRN coding genes, \textit{crn6}, \textit{crn7}, \textit{crn8}, \textit{crn9}, \textit{crn10} and \textit{crn11}, induced necrosis in \textit{N. benthamiana} (Figure 6.5, Table 6.6). Interestingly, levels of cell death induction varied markedly between the \textit{cm} genes. CRN2, CRN7 and CRN8 induced necrosis on 100\% of inoculated sites across two independent experiments. Timing of cell death appearance between effectors however, varied between 5 and 9 days after inoculation (Figure 6.5). CRN6, CRN9, CRN10 and CRN11 showed lower levels of cell death inducing capacity. Development of necrosis for constructs carrying these genes occurred in lower frequencies and varied between experiments (Table 6.6). In addition, cell death occurred between six and twelve days after inoculation, indicating that these proteins are less potent cell death inducers. Amongst all the \textit{cm} family members tested, expression of \textit{cm4}, \textit{cm12}, \textit{cm13}, \textit{cm14} and \textit{cm16} did not result in cell death. These results indicate that most but not all \textit{cm} family members induce necrosis in \textit{N. benthamiana}.

**CRN paralogs induce necrotic symptoms in \textit{Solanum tuberosum}**

(contributed by Vivianne Vleeshouwer)

Torto \textit{et al.} (2003) showed that CRN1 and CRN2 induce cell death in \textit{Nicotiana} and tomato. We expanded our analysis of the \textit{cm} family in the host species \textit{Solanum tuberosum}. For this purpose, a subset of \textit{cm} genes, present in recombinant \textit{Agrobacterium} strains (table 6.7) were inoculated side-by-side on \textit{S. tuberosum} leaves. With each recombinant strain, a total of eight inoculations were performed on two plants (four inoculations per plant) after which symptom
development was monitored between 4 and 17 dpi. CNR2, CNR6, CRN7 as well as CRN8 and CRN9 induced necrosis on *S. tuberosum* (Table 6.7). In contrast, CRN4, CRN13, CRN14 and CRN16 did not induce any visible symptoms. The levels of necrosis induction were markedly lower when compared to our results in *N. benthamiana*. CRN2, CRN6 and CRN7 induced necrosis in 75%, 88% and 63% of the inoculation sites respectively, whereas CRN8 and CRN9 inoculations resulted only in 13% necrosis induction. Our results however, suggest that either conserved CRN targets or recognition specificities are conserved within the *Solanaceae*.

**CRN2 induces cell death in a diverse collection of Solanum species**

(contributed by Vivianne Vleeshouwers)

To further determine the spectrum of CRN2 activity in *Solanum*, we expanded our functional analysis using PVX agroinfection assays. 65 clones representing 34 *Solanum* species were wound-inoculated with recombinant *Agrobacterium* GV3101 containing either pGR106-CRN2 or the empty vector (pGR106) control. Development of necrotic lesions around the inoculation site was monitored and recorded. Thirty-four clones representing 22 different *Solanum* species exhibited necrosis around pGR106-CRN2 wound-inoculated sites whereas none was observed with the respective negative controls. Frequencies of inoculation sites with symptoms of local necrosis ranged from 13-100% between different *Solanum* species as well as between clones (Table 6.8). An additional 34 clones representing 24 species were found to have either
background responses to the pGR106 empty vector or no response to crn2. Clones corresponding to thirteen species fell in both categories. These results indicate that CRN2 is able to induce necrosis in a wide range of species within Solanum but with varying degrees of activity.

**CRN2 contains a C-terminal Effector domain** (contributed by Trudy Torto-Alalibo)

We performed deletion analysis to identify the CRN2 effector domain. For this purpose, gene specific primers were designed to allow amplification of gene fragments with desired length, introduce proper start and stop codons when necessary and generate appropriate flanking restriction sites. The deleted gene constructs were cloned into pGR106 and tested in N. benthamiana. For deletion analysis, recombinant A. tumefaciens colonies were toothpick inoculated side by side on mature N. benthamiana leaves. Inoculations were repeated for each construct and development of local necrosis was monitored and recorded. Deletion of the signal peptide did not result in abolishment of necrosis (Figure 6.6). Additional deletion analysis revealed that up to 172 N-terminal amino acids could be removed before loss of effector function occurred (Figure 6.6). We extended our analysis and removed C-terminal portions of CRN2. Up to 49 amino acids were deleted without an effect on cell death induction (Figure 6.6). Removal of 22 additional amino acids however, resulted in loss of effector function (Figure 6.6). These results show that a C-terminal domain spanning 283 amino acids (173-456) is required for effector function. However, considering
deletion analysis from both protein termini, we suggest that a 234 amino acid region (173-407) is sufficient for cell death induction.

**The CRN2 effector domain induces cell death in Arabidopsis thaliana.**

We tested whether CRN2 and its effector domain exhibits necrosis-inducing activity in the nonhost Arabidopsis thaliana (Huitema et al., 2003). For this purpose, we cloned crn2 and its C-terminal effector domain (173-456) into the dexamethasone (DEX) inducible expression cassette pTA7002 (Aoyama and Chua, 1997). Empty vector, pTA-CRN2 and pTA-CRN2del10 constructs were used to generate transgenic Arabidopsis plants using the floral dip method (Clough and Bent, 1998). Transgenic crn2, crn2del10 and two transgenic control lines containing the empty expression cassette, were identified in seedling selection assays. Presence of the crn2 gene was confirmed using PCR on genomic DNA of putative pTA-CRN2 and pTA-CRN2del10 derived transformants (data not shown). T1 seeds were collected and used to obtain mature plants for DEX induction experiments. Mature transgenic crn2, control and wildtype (Col-3) plants were sprayed with a 30 µM DEX, 0.1% EtOH solution. A 0.1% EtOH solution was used as the respective negative control for the induction treatment. 48-72 hrs after DEX application, discrete necrotic lesions appeared on the younger leaves of crn2 transgenic plants (Figure 6.7). Three to five days after DEX application, necrotic lesions became apparent on older leaves of these plants. None of the control induction treatments on crn2 transgenic plants (Figure 6.7) or DEX treatments on the control plants (not shown) resulted in lesion
formation. The DEX induction experiment was repeated several times and similar results were obtained. These results show that CRN2 expression induces cell death in *Arabidopsis*, a nonhost species that is phylogenetically distinct from the *Solanaceae* and indicates that CRN2 either has a conserved plant target or is recognized through an ancient perception mechanism.

**Gene expression profiling of the tomato response to CRN1 and CRN2**
(contributed by Jorunn Bos)

We used potato microarrays to examine the transcription profiles of genes corresponding to approximately 10,000 cDNAs, upon expression of *crn1* and *crn2* in planta. For this purpose, we inoculated young tomato plants with recombinant *A. tumefaciens* strains containing either pGR106 (Lu *et al.*, 2003), pGR106-CRN1 and pGR106-CRN2. We included a PVX construct containing the *P. infestans* kazal-like protease inhibitor EPI1 (pGR106-EPI1) (Tian *et al.*, 2004) as an external control. For all tested constructs, we allowed systemic PVX infection and symptom development to occur before tissue harvest. Symptomatic leaf tissue was harvested and used for RNA extractions. This experiment was repeated in order to obtain two biological replications for each treatment. Extracted RNA samples were assessed for purity and quality before cDNA synthesis, labeling and subsequent array hybridization. Each treatment was compared to its reference (PVX only), within one microarray hybridization. Raw gene expression values were retrieved and processed before statistical analysis. First, only cDNAs that did not have missing data points within each comparison.
were considered for analysis. Second, expression values for the remaining cDNAs were log transformed (\(\ln(x)\)) to improve normal distributions. We then performed regression analysis and outlier detection using the SAS software package, to compose lists of genes that fell out of a 99% confidence interval. We classified genes as significantly up or down regulated when significant differences between the treatment and reference samples were found in both biological and technical replicates. CRN1, CRN2 and EPI1 all induced transcriptional changes. 70, 143 and 32 genes were found to be either up-or down regulated by CRN1, CRN2 and EPI1 respectively. The number of genes that were up-or down regulated by CRN2 exceeded CRN1 and induction levels for the majority of genes were significantly higher (Table 9). This indicates that CRN2 elicits similar but stronger transcriptional changes than CRN1.

We further characterized and compared transcriptional changes induced by CRN1, CRN2 and EPI through cluster analysis (Figure 6.8). For this purpose, sets of differentially expressed genes from all treatments were combined and corresponding data points across all treatments were extracted. Genes with data missing for one or more of the treatments were removed after which fold changes of gene expression (treatment/reference) were calculated for the remaining set. We performed hierarchical cluster analysis of the 219 genes using the ratios (treatment/reference) across all treatments and including biological replicates. As expected, the biological replications within each treatment clustered together (data not shown) indicating that highest level of similarity occurred between replications. Furthermore, cluster analysis showed that CRN1 and CRN2 elicit
similar responses in tomato, while EPI1 induced a different expression profile (Figure 6.8). In our analysis, four distinct groups of genes emerged based on their expression patterns across treatments. Cluster I mainly contains genes that are up regulated by CRN2 and are affected by CRN1. Cluster II constitutes genes that are specifically up regulated by EPI1. Genes down-regulated by both CRN1 and CRN2 or by all three of the treatments are defined by cluster III. Finally, cluster IV contains a small set of genes specifically down regulated by EPI1.

Finally, we checked the putative gene functions provided by TIGR using Blast searches against GenBank. Genes were categorized based on predicted function. A large number of CRN2-induced genes encode proteins involved in stress and defense related responses (Table 6.9). Amongst these sets, a substantial number of CRN2 induced genes encode defense response related enzymes, some of which were also induced in CRN1 challenged plants. These results confirm previously reported Northern analysis of CRN2 induced genes (Torto et al., 2003). Other genes that were up-regulated by CRN2 include ones with putative functions in gene regulation, such as WRKY DNA binding protein and sigma-like transcription factors (Data not shown). Finally, a large number of genes up-regulated by CRN2 were predicted to encode proteins involved in primary as well as secondary metabolism, including phytoalexin synthesis (Data not shown). In contrast to CRN2, the majority of genes affected by CRN1 were down regulated and show similarity to 40S and 60S ribosomal proteins. CRN2 affects these genes in a similar way since they group together in cluster III of the
hierarchical cluster analysis. These results indicate that CRN1 as well as CRN2 induce transcriptional changes in tomato that result in defense activation, alterations of metabolic pathways and perhaps modulate signal transduction capacities.

**CRN2 induced cell death requires the ubiquitin ligase SGT1 and the molecular chaperone HSP90**

To further understand CRN2 induced cell death, we investigated the signaling requirements for CRN2 induced necrosis in *N. benthamiana*. We elected to silence SGT1 and HSP90, two components required for R-gene mediated as well as INF1 induced HR (Peart et al., 2002; Kanzaki et al., 2003) and tested their requirement for CRN2 induced cell death. For this purpose, young *N. benthamiana* plants (5 leaf stage) were infiltrated with mixtures of *A. tumefaciens* strains carrying combinations of pBINTRA6 (TRV:RNA1) and either pTV:00, pTV-SGT1 or pTV-HSP90 (TRV:RNA2). Three weeks after TRV infection, silenced plants were infiltrated with *Agrobacterium* strains containing p35S-INF1 and p35S-CRN2. Recombinant *A. tumefaciens* containing the empty pCB302-3 vector (Xiang et al., 1999) was used as a negative control. Infiltration sites were evaluated 3 and 4 days after infiltration and frequencies of necrosis development were recorded. Silencing of SGT1 and HSP90 resulted in reductions of necrosis induced by our treatments. When compared to control plants, INF1 induced necrosis was reduced by at least 60% and 90% on SGT1 and HSP90 silenced plants whereas 100% and 50% reductions were observed in
CRN2 induced cell death (Figure 6.9). This experiment was performed two times and similar results were obtained.

**COI1, MEK2, NPR1 and TGA2.2 contribute to CRN2 but not INF1 induced cell death**

To further characterize and compare INF1 and CRN2-induced signaling, we elected to silence additional disease resistance signaling components and test their requirement for cell death. We selected a set of four genes, *coi1*, *mek2*, *tga2.2* and *npr1*, and cloned corresponding fragments in pTV00 (Peart *et al.*, 2002). Plants at the 4-5 leaf stage were infiltrated with mixtures of recombinant *A. tumefaciens* strains carrying the binary pBINTRA6 and either pTV00, pTV-COI1, pTV-NPR1, pTV-TGA2.2 and pTV-MEK2 (RNA2). Three weeks after TRV infection, silenced plants were infiltrated with *A. tumefaciens* strains containing p35S-INF1 and p35S-CRN2. A recombinant strain containing the empty pCB302-3 vector was used as a negative control. Infiltration sites were scored 3 and 4 days after infiltration for development of necrosis. Knock down of *coi1*, *npr1*, *mek2* and *tga2.2* gene expression resulted in reductions of necrosis frequencies for CRN2 whereas no reduction of INF1 induced necrosis was observed (Figure 6.10). These results indicate differences of signal requirements between INF1 and CRN2 or PINPP1.1 induced necrosis, pointing to the existence of two separate cell death signal transduction pathways.
Inf1 and crn2 co-expression leads to enhancement of cell death.

We performed Agrobacterium co-infiltration assays to test the relationships between INF1, CRN2 and PINPP1.1 induced signaling pathways. In order to allow detection of possible antagonistic or synergistic effects between pathways, we determined the concentration threshold that resulted in significant reductions of necrosis frequencies (data not shown). N. benthamiana leaves were infiltrated with various concentrations of recombinant A. tumefaciens containing either p35S-INF1, p35S-CRN2 and pCB302-3 (negative control). We experimentally determined that INF1 had the lowest sub-lethal OD$_{600}$ (~0.2) and used this value for further experiments. To test for antagonistic or synergistic relationships, mixtures of recombinant A. tumefaciens strains carrying p35S-INF1 and p35S-CRN2 (1:1 ratio) were infiltrated side-by-side with individual treatments on N. benthamiana leaves (Final OD$_{600}$ = 0.2). Infiltrated panels were evaluated and scored for necrosis development 2 and 3 days after infiltration. Percentage of necrosis development was calculated (number of panels showing necrosis/total number of panels infiltrated x100%) per treatment for every comparison. Pair wise comparisons revealed distinct differences in timing and intensity of necrosis between treatments (Figure 6.11). 53% and 44% of the leaf panels challenged with INF1-CRN2 showed necrosis after 2 days. In contrast, infiltrations with individual recombinant strains only resulted in a 0% and 6% necrosis induction rate for INF1 and 0% necrotic panels for CRN2 (Figure 6.11). Differences were sustained 3 days after infiltration since 97 and 100% of INF1-CRN2 infiltrated panels developed necrosis compared to 50% for INF1 and 0%
for CRN2 respectively (Figure 6.11). These results confirm the presence of two distinct cell death pathways and in addition, showcase a synergistic interaction between INF1 and CRN2 induced cell death signaling.

6.5 DISCUSSION

Pathogens have the ability to manipulate host cellular processes and increase susceptibility. Generally, secreted effectors are thought to govern perturbations of cellular processes in the host although in most cases the exact mechanisms are not known (Kjemtrup et al., 2000). Through a combination of functional and computational methods, a diverse set of putative effector molecules has been identified from *P. infestans* (Armstrong et al., 2005; Bos et al., 2004; Tian et al., 2004; Kanneganti et al., in preparation, Liu et al., 2005; Torto et al., 2003; Huitema et al., 2004). Some of these effectors promote infection (virulence factors) whereas others induce defense responses (avirulence/elicitor genes). Because of their supposed roles in virulence or pathogenicity and the selective pressures that accompany host-pathogen interactions, these effectors could be subject to diversification. Arguably, effector genes can therefore become members of diverse multi-gene families with complex phylogenies (Bos et al., 2004; Liu et al., 2005). Understanding both the phylogeny as well as functions of such gene families will address questions about their roles in virulence and the relevance of gene family expansion and diversification to pathogenesis.
Here, we investigated the role of the CRN family in pathogenesis. Previously, CRN1 and CRN2 were identified as inducers of cell death in *N. benthamiana* and tomato (Torto *et al*., 2003). We extended our analysis and reconstructed the phylogeny of the CRN gene family. Sequence similarity searches identified a diverse family of 16 gene paralogs. SignalP analysis predicted that most but not all paralogs have a signal peptide, strongly suggesting that these genes are extracellular and indicating that they contribute to pathogenesis. Comparative analysis identified a motif of seven amino acid residues that was conserved across all CRNs. This motif (LQ/HLFLAK) was present within the first 60 amino acids of all CRN protein sequences suggesting a more generalized role in gene function such as post-translational processing, localization or translocation. Members of this gene family varied in length as well as sequence composition, showcasing both sequence conservation, divergence at the amino acid level and recombination. These results imply that extensive evolutionary changes occurred. These results prompted us to expand our analysis of the CRN family.

We investigated the evolutionary history of the *crn* gene family and unveiled a pattern of reticulate evolution that resulted in a complex gene family. Our discovery of reticulate evolution in gene families of co-evolving organisms is not unprecedented. For instance, unequal or equal recombination within the leucine rich repeat region of plant disease resistance (*R*) genes is thought to alter recognition specificity and generate novel *R* genes (Michelmore and Meyers 1998, Ellis *et al*. 2000). Site recombination events within particular regions of *R*
genes might promote gene diversification and generate new resistance specificities, and therefore could be selectively advantageous (Bennetzen and Hulbert 1992, Michelmore and Meyers 1998). Reticulate evolution was also demonstrated for the large and diverse \textit{pg} gene family of \textit{P. cinnamomi} (Götesson \textit{et al.} 2002). Götesson \textit{et al.} (2002) proposed that reticulate evolution in these \textit{pg} genes may have contributed to functional diversity and improved pathogen fitness, perhaps by evading inhibition by polygalacturonase inhibiting proteins (PGIPs) of plants (Götesson \textit{et al.} 2002). Our observation of reticulate evolution therefore is consistent with a proposed function of \textit{crn} genes as disease effectors that may have coevolved with target molecules in host plants (Torto \textit{et al.} 2003). In this model, reticulate evolution caused by gene conversion or gene recombination contributed to functional diversification of \textit{crn} genes, creating novel combinations of structural features.

\textit{In planta} expression of effector genes oftentimes results in phenotypic changes. For example, expression of \textit{Xanthomonas AvrBs3} or the \textit{Pseudomonas syringae AvrB, AvrPto, and AvrPphE/B} genes results in distinct phenotypic changes that include chlorosis, necrosis and tissue browning (Kjemtrup \textit{et al.}, 2000). This phenomenon is useful in effector gene characterizations where phenotypic alterations indicate perturbations of cellular processes (Kjemtrup \textit{et al.} 2000, Huitema \textit{et al.}, 2004). Thus, \textit{in planta} expression of candidate effector genes is a viable diagnostic approach towards identifying effector functions.

We utilized PVX based transient assays to express \textit{crn} paralogs in plants. Agroinfection assays revealed that in addition to \textit{crn1} and \textit{crn2}, two additional
cluster I genes (crn8 and crn9) and three additional cluster II genes (crn7, crn10, crn11) induce cell death. These results indicate that these family members are either recognized or have effector function in planta, suggesting that these genes are relevant to P. infestans-host interactions. We found differences in cell death inducing activity between cluster II genes and in addition, crn12 and crn14 from cluster I as well as crn15 and crn16 in cluster II did not induce necrosis. These observed differences could be due to subtle variations between these genes, affecting protein stability, function or binding affinity to its target. Alternatively, recombination and diversification in these genes may have helped generate novel gene function or structural features.

We expanded our functional analysis of the crn2 gene and used PVX to challenge a large collection of Solanum species. In some species, the wild type virus induced necrosis, indicating resistance to PVX. A majority of species however, displayed necrosis that could only be attributed to crn2 expression. In those cases, none of the control treatments induced cell death. We therefore conclude that CRN2 has effector activity across a wide range of Solanum species. To further understand CRN gene function, we performed deletion analysis. A CRN2 domain spanning 283 amino acid (residues 173 to 456) was identified using PVX-based functional expression of deletion constructs. These results showcase the presence of a C-terminal effector domain.

We tested the activity of CRN2 as well as its minimal effector domain in Arabidopsis through a stable transformation approach. Induced expression of crn2 as well as its effector domain in Arabidopsis resulted in the formation of
necrotic lesions whereas none of the control treatments and plants showed any responses. This result indicates that the CRN2 effector domain has cell death inducing activity in a wide range of species that includes non-solanaceous plants. It is therefore sensible to speculate that plants have conserved factors that are either virulence targets or mediate perception of CRN2.

Expression of CRN2 without a signal peptide is sufficient to induce cell death in both *N. benthamiana* and *Arabidopsis*. These results suggest that CRN2 has either an intracellular receptor or plant target. Consequently, we hypothesize that during *P. infestans* infection, effector molecules are translocated into the host cell where they exhort virulence function. In a major recent advance, a novel eukaryotic translocation mechanism was identified (Hiller *et al.*, 2004; Marti *et al.*, 2004). A specific RXL motif was found amongst a set of diverse proteins from *Plasmodium* species and in addition, requirement of this motif for translocation across host plasma membranes was shown (Hiller *et al.*, 2004; Marti *et al.*, 2004). More recently, a similar motif (RxLR) was found to be common in a diverse set of secreted oomycete proteins including AVR3A, a *P. infestans* avirulence protein that is recognized by the intracellular R3a resistance gene (Armstrong *et al.*, 2005) and *atr13* a novel avirulence gene from *Pe. parasitica* (Rehmany *et al.*, in press). These results altogether indicate the presence of a protein translocation machinery in oomycete plant pathogens. In this light, it is tempting to speculate that because of its striking similarities, the LQ/HLFLAK motif has a role in translocation. The presence of a motif across all CRN proteins suggests that is not required for effector function but that it has a more basic role.
Subsequent deletion analysis of CRN2 confirmed this assertion since function was retained in the C-terminal domain. Because of these results, we speculate that the CRNs form a family of intracellular effectors that are delivered through a novel translocation machinery. Future experiments will perhaps provide more support or confirm this hypothesis.

Previously, CRN2 was found to induce expression of *hsr201* and *PR-1a*, two defense related genes commonly upregulated upon treatment with elicitor preparations (Czernic *et al*., 1996; Torto *et al*., 2003). In order to further characterize plant responses to members of the *crn* gene family, we used microarray analysis to identify CRN1 and CRN2-induced transcriptional changes in tomato. PVX-mediated expression of CRN1 and CRN2 resulted in differential expression of genes that fell into various functional categories. Importantly, expression of *crn1* and *crn2 in planta*, resulted in the induction of a broad range of stress and defense-related genes in both cases. These results confirm that CRN1 and CRN2 induce cell death and that recognition or activity of either gene is sufficient to induce subsequent defense responses. CRN2 appeared to induce more dramatic gene expression changes. This observation is consistent with previous results where CRN2 was found to induce stronger defense gene responses then CRN1 (Torto *et al*., 2003). Whether CRN1 and CRN2 are perceived in plants, or whether these results are representative for all family members, remains to be determined. Additional gene expression profiling experiments are needed to assess the nature of responses induced by other members of this family.
Cell death signaling upon perception of biotic stimuli often occurs in
discrete and tightly regulated multi cascade steps (Dangl and Jones, 2001). The
identification of numerous $R$ genes has allowed the identification of their
respective downstream signaling components that regulate cellular
reprogramming. Despite the vast number of $R$ genes, a relatively few signal
components have been identified. Amongst those signaling proteins, the
ubiquitin-ligase associated gene SGT1 and the molecular chaperone HSP90
have emerged as conserved and required components of disease signaling
pathways (Liu et al., 2004; Takahashi et al., 2003; Lu et al., 2003). In addition,
genetic analyses of resistance to multiple pathogens have identified key genes
amongst other functional classes that contribute to resistance (Egendren et al.,
2003; Liu et al., 2002). These findings have culminated in models that propose
larger multi-protein complexes that mediate perception and regulate downstream
signal transduction (Bech-Otschir et al., 2002). To understand the role of
resistance signaling genes in CRN2 induced responses, we used Virus Induced
Gene Silencing (VIGS) to identify the genetic requirements of cell death. First, we
silenced SGT1 and HSP90 and found abolishment of cell death in both cases.
We extended our analysis and tested four candidate genes (COI1, MEK2, NPR1
and TGA2.2) for their involvement in CRN2 induced cell death. We cloned
representative gene fragments in the pTV00 vector and silenced these genes in
$N. benthamiana$. Silencing of all these genes resulted in transient reductions in
cell death. These observations indicate the involvement of COI1, MEK2, NPR1
and TGA2.2 in CRN2 induced signaling. The interpretation of our findings
remains difficult. CRN2 may be perceived after which a subsequent set of signaling steps is initiated. HSP90 and SGT1 could act as chaperone and co-chaperone in signalosomes, stabilizing multi protein complexes that include receptor like components as well as signaling factors. These complexes would participate in cell death signaling and regulate other signaling components such as COI1, MEK2, NPR1 and TGA2.2. In another scenario, CRN2 effector function perturbs host cellular processes strictly as an effector, ultimately killing the cell through activation of programmed cell death. The requirement of COI1 for CRN2 induced cell death would point to a mechanism that is similar to that of coronatine (COR), a phytotoxin and structural analog of Methyl Jasmonate (MeJA) (Bender et al., 1999; Feys et al., 1994; Zhao et al., 2003). CRN2 activity in plant cells would alter plant responses and render plants more susceptible to infection. This hypothesis would fit with recent observations in Arabidopsis and Pseudomonas syringae pv tomato (Pst) associations, where mutants deficient in coronatine production exhibit reduced virulence (Block et al., 2005). More extended analyses would be required however to provide firm answers to either option.

Co-expression of INF1 and CRN2 led to intensified and accelerated cell death induction. Plants have the ability to trigger intricate webs of multiple signaling pathways, enabling appropriate and dosed responses to a multitude of signals. For instance, RPP4, RPP7 and RPP8, R-genes active against a defined set of Pe. parasitica isolates, have different but overlapping signaling requirements that mediate defense induction (Eulgem et al., 2004; Aarts et al., 1998). Co-expression of INF1 and PiNPP1.1 results in accelerated cell death
Although our knowledge of signaling pathways and defense induction is growing, understanding the complexity of their interactions remains a challenge. Co-expression of INF1 and CRN2 resulted in accelerated and intensified necrosis development compared to individual treatments. These results confirm the existence of two separate signaling cascades but importantly, showcase synergistic interplay between pathways. Pathogen recognition invariably results in cellular reprogramming. Signaling downstream of pathogen perception can induce transcriptional activation of multiple classes of defense related genes initially, followed by up-regulation of genes that are involved in signal transduction (Eulgem *et al.*, 2004). Similarly, perception of *flg22*, a PAMP molecule that induces innate immunity in *Arabidopsis*, results in up-regulation of genes predominantly involved in signaling (Navarro *et al.*, 2004). It is possible, that recognition or activity of an effector leads to sensitization or priming of plant cells to other biotic stimuli. It is therefore sensible to propose that either INF1 or CRN2 recognition leads to increased expression of signal transduction components, thereby sensitizing plant cells to either or both molecules. To further understand such regulatory networks and perhaps feedback loop mechanisms, future efforts could be directed at those genes that are induced by either INF1 or CRN2 and investigate their role in synergism or cell sensitization.

### 6.6 ACKNOWLEDGMENTS

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center (ABRC) at The Ohio State University for Col-3 seeds. Computational analysis of crn evolution was performed by Zhenyu Liu and corresponding sections were extracted from her thesis. All functional analyses in Solanum were performed by Vivianne Vleeshouwers. Characterizations of CRN paralogs and CRN2 deletion analysis in N. benthamiana was performed by Trudy Torto-Alalibo. Jorunn Bos performed microarray experiments in tomato and subsequent data analyses. I thank Cahid Cakir for his contributions and expert assistance as well as Diane Kinney for her supporting role in my research. Research on functional genomics of Phytophthora-plant interactions is supported by NSF Plant Genome Research Program grant DBI-0211659. Salaries and research support were provided, in part, by State and Federal Funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

6.7 REFERENCES


Figure 6.1. Alignment of N-terminal region of 16 CRN proteins.
Figure 6.2. A phylogenetic tree of the crn gene family from *Phytophthora infestans*. The tree was constructed using the neighbor-joining method of Clustal X 1.64, based on the amino acid sequence alignments, and topology were shown using TreeviewPPC 1.6.6. Bootstrap
**Figure 6.3.** Phylogenetic profiles of the cluster II of the *crn* gene family from *Phytophthora infestans* using Phylpro based on amino acid sequence alignments. The x-axis indicates amino acid positions in the sequence alignment. The y-axis indicates the phylogenetic correlation value. The sequences analyzed were *crn*2, *crn*3, *crn*7, *crn*10, *crn*11, *crn*15, and *crn*16 indicated by blue, dark, pink, light blue, green, yellow and red, respectively. Recombination signals appear in areas of low phylogenetic correlation, visualized by single sharp downward peaks. All the sequences in the profiles are recombinant.
Figure 6.4: Mosaic structures for cluster II of the *crn* gene family using Phylpro. Only 357 variable amino acid sites were shown. a to f are the locations of the predicted recombination sites at variable amino acid 97, 98, 15, 14, 27 and 17, respectively. Open white box indicate alignment gaps.
Figure 6.5. Induction of necrotic symptoms by CRN paralogs in *N. benthamiana*. Symptoms of localized (A) and systemic (B) necrosis were observed during a time range of 6-12 dpi for the different pGR106::crn construct used for PVX-expression. For both experiments 2 plants were used for wound-inoculations with recombinant *Agrobacterium*. In A, each plant was inoculated 4 times and the average number of inoculation sites with local necrosis was calculated. In B, the average number of leaves with systemic necrosis was calculated. For A, only CRN paralogs inducing necrosis and the control (pGR106) are shown.
Figure 6.6. Deletion analysis of the \textit{crn2} gene. CRN2 amino acid regions are indicated (left) whereas necrosis-inducing activity is shown on the right.
Figure 6.7. Induction of cell death in Arabidopsis. Top panel: plants carrying the full length crn2 gene were treated with either Dexamethasone or ethanol. Bottom panel: the CRN2 effector domain induces cell death in Arabidopsis. Control (Col-3, Col-3:pTA) and plants carrying crn2del10 were treated with DEX. Only plants carrying the crn2 constructs displayed leaf necrosis.
Figure 6.8. CRN2 and CRN1 induce similar expression profiles in tomato. Hierarchical cluster analysis was performed in both gene and array dimensions of 219 genes that were found to be differentially expressed between the reference (empty vector) and the treatment samples in statistical analyses. Red color indicates that the genes is up-regulated compared to the reference whereas green color indicates down-regulation. We identified four clusters of genes among the different treatments, based on their expression profiles as described in the text.
Figure 6.9. CRN2 induced cell death is SGT1 and HSP90 dependent. SGT1 and HSP90 silenced plants showed reduced necrosis (panel A) upon infiltration with p35S-INF1 and p35S-CRN2 constructs. Percentage of necrosis induction was reduced in both INF1 (top graph panel B) and CRN2 (bottom graph panel B) challenged panels.
Figure 6.10. Silenced plants infiltrated with p35S-INF1 and p35S-CRN2. Infiltrated leafs showed phenotypic reduction in necrosis (panel A) as well as reductions in % of leaf panels showing necrosis.
Figure 6.11. Mixtures of recombinant *A. tumefaciens* strains carrying p35S-INF1 and p35S-CRN2 were infiltrated side-by-side with individual treatments on *N. benthamiana* leaves (Final OD$_{600} = 0.2$). Infiltrated panels were evaluated and scored for necrosis development 1, 2 and 3 days after infiltration. Top panel (A): symptom development 3 days after infiltration. Percentage of necrosis development was calculated (number of infiltrated sites showing necrosis/total number of infiltrations) for each treatment and per comparison (lower panel, B).
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**Table 6.1.** Description of CRN proteins including: Genbank accession numbers, number of amino acid residues, putative functional domains and score of hits to known domains.
Note: The analyses were performed on the protein-coding regions. Mismatches in the converted region were allowed (the parameter gscale was set to 1 in GENECONV in our analysis.

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Note: The analyses were performed on the protein-coding regions. Mismatches in the converted region were allowed (the parameter gscale was set to 1 in GENECONV in our analysis.

<sup>a</sup> Begin = first nucleotide of the potential converted regions.
<sup>b</sup> End = last nucleotide of the potential converted regions.
<sup>c</sup> Length = length of the potential converted regions with alignment gaps.
<sup>d</sup> Sim P-values = simulated P value of the global inner fragments based on 10,000 permutations.
<sup>e</sup> Dif. = the number of nucleotide mismatches within the potential converted regions of two sequences.
<sup>f</sup> Total dif. = the total number of nucleotide mismatches between two sequences.

Table 6.2: Evidence of gene conversion events between pairs of sequences in the alignment of cluster I of the crn gene family from Phytophthora infestans using Sawyer’s test implemented in GENECONV1.81.
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<td>465</td>
<td>0.0000</td>
<td>2</td>
<td>541</td>
</tr>
<tr>
<td>CRN15 vs. CRN16</td>
<td>277</td>
<td>1136</td>
<td>860</td>
<td>0.0001</td>
<td>5</td>
<td>82</td>
</tr>
</tbody>
</table>

Note: The analyses were performed on the protein-coding regions. Mismatches in the converted region were allowed (the parameter gscale was set to 1 in GENECONV in our analysis.

<sup>a</sup> Begin = first nucleotide of the potential converted regions.
<sup>b</sup> End = last nucleotide of the potential converted regions.
<sup>c</sup> Length = length of the potential converted regions with alignment gaps.
<sup>d</sup> Sim P-values = simulated P value of the global inner fragments based on 10,000 permutations.
<sup>e</sup> Dif. = the number of nucleotide mismatches within the potential converted regions of two sequences.
<sup>f</sup> Total dif. = the total number of nucleotide mismatches between two sequences.

Table 6.3. List of gene conversion events between pairs of sequences in the alignment of cluster II of the crn gene family from Phytophthora infestans using Sawyer’s test implemented in GENECONV1.81.
<table>
<thead>
<tr>
<th>Sequences</th>
<th>Sim P-value</th>
<th>Begin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>End&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Length&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Dif.&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Total Mats.&lt;sup&gt;f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRN3</td>
<td>0.0007</td>
<td>305</td>
<td>312</td>
<td>8</td>
<td>0</td>
<td>658</td>
</tr>
<tr>
<td>CRN3</td>
<td>0.0067</td>
<td>425</td>
<td>441</td>
<td>17</td>
<td>2</td>
<td>658</td>
</tr>
<tr>
<td>CRN7</td>
<td>0.0000</td>
<td>169</td>
<td>274</td>
<td>106</td>
<td>5</td>
<td>659</td>
</tr>
<tr>
<td>CRN7</td>
<td>0.0040</td>
<td>285</td>
<td>306</td>
<td>22</td>
<td>4</td>
<td>659</td>
</tr>
<tr>
<td>CRN10</td>
<td>0.0096</td>
<td>1057</td>
<td>1060</td>
<td>6</td>
<td>0</td>
<td>722</td>
</tr>
<tr>
<td>CRN11</td>
<td>0.0260</td>
<td>1057</td>
<td>1060</td>
<td>4</td>
<td>0</td>
<td>721</td>
</tr>
<tr>
<td>CRN16</td>
<td>0.0359</td>
<td>1158</td>
<td>1169</td>
<td>12</td>
<td>0</td>
<td>704</td>
</tr>
</tbody>
</table>

Note:  
<sup>a</sup> Sim P-values = simulated P value of the global inner fragments based on 10,000 permutations.  
<sup>b</sup> Begin = first nucleotide of the potential converted regions.  
<sup>c</sup> End = last nucleotide of the potential converted regions.  
<sup>d</sup> Length = length of the potential converted regions with alignment gaps.  
<sup>e</sup> Dif. = the number of nonunique nucleotide within the potential converted regions.  
<sup>f</sup> Total Mats. = the total number of nonunique nucleotide for that sequence.

**Table 6.4:** List of outer fragments of the cluster II of the *crn* gene family from *Phytophthora infestans* using Sawyer's test implemented in GENECONV1.81.
<table>
<thead>
<tr>
<th>Sequence comparisons</th>
<th>Sim P-value$^a$</th>
<th>Begin$^b$</th>
<th>End$^c$</th>
<th>Length$^d$</th>
<th>Dif.$^e$</th>
<th>Total Diff.$^f$</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRN3 vs. CRN4</td>
<td>0.0302</td>
<td>367</td>
<td>389</td>
<td>23</td>
<td>0</td>
<td>276</td>
</tr>
<tr>
<td>CRN3 vs. CRN5</td>
<td>0.0464</td>
<td>367</td>
<td>389</td>
<td>23</td>
<td>0</td>
<td>271</td>
</tr>
<tr>
<td>CRN3 vs. CRN9</td>
<td>0.0055</td>
<td>106</td>
<td>158</td>
<td>53</td>
<td>3</td>
<td>231</td>
</tr>
<tr>
<td>CRN3 vs. CRN13</td>
<td>0.0000</td>
<td>88</td>
<td>173</td>
<td>86</td>
<td>8</td>
<td>258</td>
</tr>
<tr>
<td>CRN7 vs. CRN1</td>
<td>0.0334</td>
<td>453</td>
<td>481</td>
<td>29</td>
<td>1</td>
<td>252</td>
</tr>
<tr>
<td>CRN7 vs. CRN4</td>
<td>0.0036</td>
<td>453</td>
<td>478</td>
<td>26</td>
<td>0</td>
<td>272</td>
</tr>
<tr>
<td>CRN7 vs. CRN5</td>
<td>0.0388</td>
<td>453</td>
<td>478</td>
<td>26</td>
<td>0</td>
<td>251</td>
</tr>
<tr>
<td>CRN7 vs. CRN8</td>
<td>0.0000</td>
<td>274</td>
<td>396</td>
<td>123</td>
<td>9</td>
<td>189</td>
</tr>
<tr>
<td>CRN7 vs. CRN9</td>
<td>0.0000</td>
<td>274</td>
<td>396</td>
<td>123</td>
<td>10</td>
<td>184</td>
</tr>
<tr>
<td>CRN7 vs. CRN12</td>
<td>0.0000</td>
<td>274</td>
<td>396</td>
<td>123</td>
<td>9</td>
<td>198</td>
</tr>
<tr>
<td>CRN7 vs. CRN14</td>
<td>0.0000</td>
<td>274</td>
<td>396</td>
<td>123</td>
<td>6</td>
<td>184</td>
</tr>
<tr>
<td>CRN10 vs. CRN4</td>
<td>0.0000</td>
<td>34</td>
<td>96</td>
<td>63</td>
<td>6</td>
<td>296</td>
</tr>
<tr>
<td>CRN11 vs. CRN4</td>
<td>0.0000</td>
<td>34</td>
<td>96</td>
<td>63</td>
<td>6</td>
<td>296</td>
</tr>
<tr>
<td>CRN11 vs. CRN9</td>
<td>0.0000</td>
<td>103</td>
<td>177</td>
<td>75</td>
<td>5</td>
<td>289</td>
</tr>
<tr>
<td>CRN11 vs. CRN13</td>
<td>0.0000</td>
<td>91</td>
<td>177</td>
<td>87</td>
<td>6</td>
<td>293</td>
</tr>
<tr>
<td>CRN16 vs. CRN4</td>
<td>0.0000</td>
<td>34</td>
<td>96</td>
<td>63</td>
<td>6</td>
<td>296</td>
</tr>
<tr>
<td>CRN16 vs. CRN9</td>
<td>0.0000</td>
<td>103</td>
<td>177</td>
<td>75</td>
<td>6</td>
<td>299</td>
</tr>
<tr>
<td>CRN16 vs. CRN13</td>
<td>0.0000</td>
<td>91</td>
<td>177</td>
<td>87</td>
<td>6</td>
<td>285</td>
</tr>
</tbody>
</table>

Note: The analyses were performed on the protein-coding regions. Mismatches in the converted region were allowed (the parameter gscale was set to 1 in GENECONV in our analysis.

$^a$ Begin = first nucleotide of the potential converted regions.

$^b$ End = last nucleotide of the potential converted regions.

$^c$ Length = length of the potential converted regions with alignment gaps.

$^d$ Sim P-values = simulated P value of the global inner fragments based on 10,000 permutations.

$^e$ Dif. = the number of nucleotide mismatches within the potential converted regions of two sequences.

$^f$ Total dif. = the total number of nucleotide mismatches between two sequences.

Table 6.5. List of gene conversion events between pairs of sequences of the *cm* gene family from *Phytophthora infestans* using Sawyer’s test implemented in GENECONV1.81.
<table>
<thead>
<tr>
<th>CRN paralog</th>
<th>loc. necrosis I (12 dpi)</th>
<th>loc. necrosis II (12 dpi)</th>
<th>loc. necrosis III (12 dpi)</th>
<th>loc. necrosis III (17 dpi)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRN1</td>
<td>0%</td>
<td>0%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CRN2</td>
<td>100%</td>
<td>100%</td>
<td>62.50%</td>
<td>100%</td>
</tr>
<tr>
<td>CRN4</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>CRN6</td>
<td>100%</td>
<td>75%</td>
<td>0%</td>
<td>62.5%</td>
</tr>
<tr>
<td>CRN7</td>
<td>100%</td>
<td>100%</td>
<td>62.50%</td>
<td>100%</td>
</tr>
<tr>
<td>CRN8</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>CRN9</td>
<td>12.5%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>CRN10</td>
<td>87.5%</td>
<td>25%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CRN11</td>
<td>75%</td>
<td>37.5%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CRN12</td>
<td>0%</td>
<td>0%</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>CRN13</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>CRN14</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>37.5%</td>
</tr>
<tr>
<td>CRN16</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Table 6.6. Necrosis inducing activity of CRN paralogs on *N. benthamiana* plants.
Table 6.7. Overview of *Solanum tuberosum* MaR7 differential to *Agrobacterium* carrying pGR106-CRN paralogs. Percentages are based on the number of inoculation sites showing localized necrosis out of a total number of eight. Symptoms were evaluated 17 dpi.
<table>
<thead>
<tr>
<th>Plant species</th>
<th>Clone</th>
<th>pGR106</th>
<th>CRN2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Species responding to CRN2 only</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. andreanum</td>
<td>18344-1</td>
<td>0 (16)</td>
<td>31.25 (16)</td>
</tr>
<tr>
<td>S. andreanum</td>
<td>18344-6</td>
<td>0 (8)</td>
<td>75 (8)</td>
</tr>
<tr>
<td>S. agrimonifolium</td>
<td>18285-1</td>
<td>0 (8)</td>
<td>0 (8)</td>
</tr>
<tr>
<td>S. brachycarpum</td>
<td>17721-2</td>
<td>0 (8)</td>
<td>0 (8)</td>
</tr>
<tr>
<td>S. brachycarpum</td>
<td>17721-3</td>
<td>0 (8)</td>
<td>37.5 (8)</td>
</tr>
<tr>
<td>S. bulbocastanum</td>
<td>17693-2</td>
<td>nd</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. bulbocastanum</td>
<td>17693-5</td>
<td>0 (18)</td>
<td>100 (18)</td>
</tr>
<tr>
<td>S. bulbocastanum</td>
<td>21306-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. cha??</td>
<td>18060-7</td>
<td>0 (8)</td>
<td>25 (8)</td>
</tr>
<tr>
<td>S. canasense/canense?</td>
<td>18062-1</td>
<td>0 (8)</td>
<td>25 (8)</td>
</tr>
<tr>
<td>S. cardiophyllum</td>
<td>18326-1</td>
<td>0 (48)</td>
<td>100 (48)</td>
</tr>
<tr>
<td>S. fendleri</td>
<td>17717-3</td>
<td>0 (6)</td>
<td>66.67 (6)</td>
</tr>
<tr>
<td>S. fendleri</td>
<td>17717-8</td>
<td>0 (48)</td>
<td>100 (48)</td>
</tr>
<tr>
<td>S. guerreroense</td>
<td>18290-1</td>
<td>0 (8)</td>
<td>12.5 (8)</td>
</tr>
<tr>
<td>S. guerreroense</td>
<td>18290-1</td>
<td>0 (8)</td>
<td>62.5 (8)</td>
</tr>
<tr>
<td>S. guerreroense</td>
<td>18290-2</td>
<td>0 (8)</td>
<td>0 (8)</td>
</tr>
<tr>
<td>S. huancabambense</td>
<td>17719-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. huancabambense</td>
<td>18306-6</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. hjertingii</td>
<td>17718-1</td>
<td>0 (8)</td>
<td>75 (8)</td>
</tr>
<tr>
<td>S. hjertingii</td>
<td>17718-2</td>
<td>0 (8)</td>
<td>75 (8)</td>
</tr>
<tr>
<td>S. jamesii</td>
<td>18349-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. leptophytes</td>
<td>18174-8</td>
<td>0 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>S. microdontum</td>
<td>17596-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. multinterruptum</td>
<td>17829-2</td>
<td>0 (8)</td>
<td>50 (8)</td>
</tr>
<tr>
<td>S. multinterruptum</td>
<td>17829-2</td>
<td>0 (40)</td>
<td>2.5 (40)</td>
</tr>
<tr>
<td>S. multinterruptum</td>
<td>17829-2</td>
<td>0 (8)</td>
<td>0 (8)</td>
</tr>
<tr>
<td>S. neocardenasii</td>
<td>18000-1</td>
<td>0 (8)</td>
<td>62.5 (8)</td>
</tr>
<tr>
<td>S. okadae</td>
<td>18109-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. polyadenium</td>
<td>17749-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. polyadenium</td>
<td>17749-4</td>
<td>0 (6)</td>
<td>100 (6)</td>
</tr>
<tr>
<td>S. polytrichon</td>
<td>17750-4</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. pinnatisectum</td>
<td>17743-1</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>S. pinnatisectum</td>
<td>17743-4</td>
<td>0 (8)</td>
<td>50 (8)</td>
</tr>
<tr>
<td>S. papita</td>
<td>17830-1</td>
<td>0 (4)</td>
<td>100 (4)</td>
</tr>
<tr>
<td>S. stoloniferum</td>
<td>17605-4</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
</tbody>
</table>

**Table 6.8 continued**

**Table 6.8.** Overview of results obtained upon agroinfection of *Solanum* species with pGR106 and pGR106-CRN2. Data is shown as percentage of inoculations showing localized necrosis. The number of examined inoculations per plant species shown between brackets. Species responding to inoculations with pGR106-CRN2 (upper), to pGR106-CRN2 and empty vector (middle) and plants not showing any visible symptoms (bottom) are shown. Absence of data is indicated by nd.
Table 6.8 continued

<table>
<thead>
<tr>
<th>Species</th>
<th>Variety</th>
<th>Response 1</th>
<th>Response 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{S. tuberosum}</td>
<td>Bintje</td>
<td>0 (4)</td>
<td>25 (4)</td>
</tr>
<tr>
<td>\textit{S. tuberosum}</td>
<td>Bintje</td>
<td>0 (8)</td>
<td>25 (8)</td>
</tr>
<tr>
<td>\textit{S. tuberosum}</td>
<td>Bintje</td>
<td>0 (40)</td>
<td>30 (40)</td>
</tr>
<tr>
<td>\textit{S. tuberosum}</td>
<td>Bintje</td>
<td>0 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>17810-01</td>
<td>0 (4)</td>
<td>50 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>17810-06</td>
<td>0 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>17810-14</td>
<td>0 (4)</td>
<td>50 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>17820-01</td>
<td>0 (4)</td>
<td>25 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>17820-12</td>
<td>0 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>17820-21</td>
<td>0 (4)</td>
<td>25 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>18313-05</td>
<td>0 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>20571-01</td>
<td>0 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>20571-02</td>
<td>0 (4)</td>
<td>25 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>20571-11</td>
<td>0 (4)</td>
<td>25 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>20571-12</td>
<td>0 (4)</td>
<td>50 (4)</td>
</tr>
</tbody>
</table>

Species showing non-specific responses

<table>
<thead>
<tr>
<th>Species</th>
<th>Variety</th>
<th>Response 1</th>
<th>Response 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{ABPT}</td>
<td>707-TG-11-1</td>
<td>100% (8)</td>
<td>75 (8)</td>
</tr>
<tr>
<td>\textit{S. agrimonifolium}</td>
<td>18285-2</td>
<td>37.5 (8)</td>
<td>75 (8)</td>
</tr>
<tr>
<td>\textit{S. bulbocastanum}</td>
<td>17693-2</td>
<td>100 (8)</td>
<td>nd</td>
</tr>
<tr>
<td>\textit{S. bulbocastanum}</td>
<td>17693-5</td>
<td>50 (4)</td>
<td>75 (4)</td>
</tr>
<tr>
<td>\textit{S. brachistotrichum? (bst)}</td>
<td>17681-1</td>
<td>75 (8)</td>
<td>75 (8)</td>
</tr>
<tr>
<td>\textit{S. brachistotrichum? (bst)}</td>
<td>17681-2</td>
<td>100 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>\textit{S. cardiophyllum}</td>
<td>18326-1</td>
<td>25 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>18313-02</td>
<td>50 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>18313-04</td>
<td>25 (4)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>\textit{S. demissum}</td>
<td>20571-18</td>
<td>50 (4)</td>
<td>75 (4)</td>
</tr>
<tr>
<td>\textit{S. fendleri}</td>
<td>17717-8</td>
<td>37.5 (8)</td>
<td>100 (8)</td>
</tr>
<tr>
<td>\textit{S. microdontum}</td>
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<td>50% (8)</td>
<td>37.5 (8)</td>
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<td>12.5 (8)</td>
<td>12.5 (8)</td>
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<tr>
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<td>75 (8)</td>
<td>100 (8)</td>
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<tr>
<td>\textit{S. okadae}</td>
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<td>75 (8)</td>
<td>nd</td>
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<tr>
<td>\textit{S. papita}</td>
<td>17830-1</td>
<td>100 (4)</td>
<td>100 (4)</td>
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<td>75 (8)</td>
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<tr>
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<tr>
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<td>62.5 (8)</td>
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<tr>
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<td>87.5 (8)</td>
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Non-responsive species

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<th>Variety</th>
<th>Response 1</th>
<th>Response 2</th>
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<tbody>
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<td>\textit{crc?}</td>
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<td>\textit{S. demissum}</td>
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<td>Bintje</td>
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<td>\textit{S. vernei}</td>
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Table 6.9 Genes showing significant up-regulation in tomato by pGR106::CRN1 and pGR106-CRN2 as defined by statistical analysis. We selected the stress/defense related genes displayed in this table based on the putative gene function as provided by TIGR. Ratios indicated, are based on the average of two biological replicates and two spots per gene on the arrays. The right column indicates for which treatment the genes are significantly up-regulated.
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