Structure and function of the (a)virulence protein NIP1 of *Rhynchosporium secalis*

Structuur en functie van het (a)virulentie eiwit NIP1 van *Rhynchosporium secalis*

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Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr. ir. L. Speelman
in het openbaar te verdedigen
op maandag 15 april 2002
des namiddags te vier uur in de Aula

Structure and function of the (a)virulence protein NIP1 of *Rhynchosporium secalis*.
Slot, Klaas A.E., van ‘t
Ph.D. Thesis Wageningen University. – with summary in Dutch. – with references
Subject headings: plant-pathogen interaction/Rhynchosporium secalis/elicitor
ISBN 90-5808-625-9

The research presented in the thesis was carried out at the Max-Planck-Institut für Züchtungsforschung in Cologne, Germany. The research was financed by the Deutsche Forschungsgemeinschaft.

"*Experiments are the only means of knowledge at our disposal.*
The rest is poetry, imagination."
Max Planck (1858-1947)

Contents

Chapter 1

Outline of this thesis

Plants have to cope with a large array of potential microbial pathogens. However, the majority of potential pathogens is unable to initiate infection on most plants. In these cases of non-host interactions, the plant does not support the nutritional requirements of the pathogen, or the microbe lacks the necessary "tools" to overcome the plants' barriers (Heath, 1991). Relatively few microbes possess the proper molecular equipment to infect a given plant species. In these cases, there are two possibilities for the outcome of the interaction. If the plant fails to arrest the development of the pathogenic microbe, the plant becomes colonized tissues by the microbe. The plant is susceptible; compatible interaction. Alternatively, cultivars (or lines) of the host plant are capable of inhibiting the pathogen's life cycle. The plant is resistant; incompatible interaction. In case the plant successfully defends itself against invasion by the pathogen. Resistance at the cultivar level is genotype-specific and reflects the struggle for existence of both the host and microbe, resulting in highly specialized interactions of plants carrying a large number between resistance genes and pathogens carrying a number of matching genes encoding potential elicitors of defense reactions.

Plant pathogen interactions

Genotype-specific resistance is mostly a dominant or co-dominant trait, which inherits in a monogenic fashion. Through products of resistance (*R*) genes host plants are capable to recognize specific, pathogen-derived molecules and launch a defense response against the invader. These elicitors of resistance reactions are direct or indirect products of a microbial avirulence (*Avr*) gene. Both *R* and *Avr* genes form the basis of the gene-for-gene hypothesis, stating that for each *R* gene that confers resistance of the plant, there is a corresponding *Avr* gene that confers avirulence of the pathogen (Flor, 1946). Lacking or disfunctioning of either *R* gene or *Avr* gene results in a compatible interaction (Keen 1982). Prerequisite for any active defense is recognition of the attacker. A biochemical model, the elicitor-receptor model, was subsequently proposed that explains the gene-for-gene relationship by a direct interaction between the products of both *R* and *Avr* genes (Keen, 1990; Gabriel and Rolfe, 1990). In its simplest form, this model predicts the product of the *R* gene to be a receptor that perceives the product of the *Avr* gene.

The barley leaf scald disease

Although very few microbes are plant-pathogenic, barley (*Hordeum vulgare* L.) is host to a large number of fungal pathogens. *Rhynchosporium secalis* (Oudem.) J. J. Davis belongs to the group of *Deuteromycetes*, also referred to as *Fungi Imperfecti*, for which a sexual stage has not been described. The fungus is known as the causal agent of leaf scald on barley, rye and other grasses since the end of the nineteenth

century (Caldwell, 1937). Typical symptoms of this disease are necrotic spots on barley leaves that increase until the total leaf surface is covered. Leaf scald can cause dramatic yield losses, particularly in cool, semi-humid barley-growing regions (Shipton *et al.,* 1974). Studies involving barley cultivars that carry the *R* gene, *Rrs1*, and fungal strains expressing the *Avr* gene, *AvrRrs1,* have shown that the interaction complies with the gene-for-gene concept. Like many other resistances, including resistance mediated by the *Cf-9* gene from tomato, *Rrs1*-mediated resistance appears to be a semi-dominant trait.

Fungal development in the plant deviates substantially from that described for most other phytopathogenic fungi as the major infection court are the subcuticular regions of host leaves. Penetration of the cuticle is followed by hyphal growth between the cuticle and the epidermal cell walls (Ayesu-Offei and Clare, 1970, Lehnackers and Knogge, 1990). This type of infection has been described so far only for *R. secalis* and the scab causing species belonging to the genus *Venturia* (Agrios, 1988). During early stages of the infection process, the epidermal cell walls are not damaged, indicating that physical contact between plasma membranes of plant cells and fungal hyphae does not occur. Three days after infection, in susceptible plants a few epidermal cells collapse, followed by the underlying mesophyll cells several days later. After ten days, a dense subcuticular stroma is formed. Fungal hyphae could be detected between dead mesophyll cells only in late stages of pathogenesis, when leaf tissue was already heavily degraded. In this stage, conidia are formed in the necrotic lesions but conidiophores breaking through the cuticle are also observed in regions of the leaf that looked healthy. In resistant plants, only the primary infected epidermal cells collapse, fungal development slows down and is subsequently inhibited completely (Lehnackers and Knogge, 1990).

Necrosis Inducing Proteins

A family of Necrosis Inducing Proteins (NIP1, NIP2, NIP3) was identified in fungal culture filtrates of *R. secalis* (Wevelsiep *et al.,* 1991). NIP1, a small (6440 Da), cysteine-rich protein was found to be toxic to leaves of all tested barley cultivars as well as to leaves of several other cereals and bean (*Phaseolus vulgari*s, Wevelsiep *et al.,* 1991; W. Knogge, unpublished data). This host-nonselective toxic activity may be based on the indirect stimulation by NIP1 of the plant plasma membrane H^+-ATPase (Wevelsiep *et al.,* 1993). Evidence for a role of NIP1 in virulence came from a fungal *NIP1* replacement mutant, which displayed a reduced level of virulence compared to the parental $NIP1^+$ strain on susceptible barley plants. However, NIP1 also specifically induced mRNA encoding the pathogenesis related protein PR5 in leaves of barley cultivars carrying the *R* gene *Rrs1* (Hahn *et al.,* 1993). All fungal strains secreting an elicitor-active NIP1 were unable to infect *Rrs1*-barley. In contrast,

virulent races do not carry the *NIP1* gene, or carry *NIP1* alleles that encode inactive forms of NIP1 (Rohe *et al.,* 1995). Final proof of NIP1 being the product of the *Avr* gene, *AvrRrs1*, came from *NIP1* complementation and replacement mutants that showed the expected phenotypes on *Rrs1* plants (Rohe *et al.,* 1995). So far, four naturally occurring types of NIP1 have been characterized. Types I and II NIP1 from fungal isolates UK7 and AU1, respectively, are active both as elicitors of PR protein synthesis and as stimulators of the plasma membrane H^+-ATPase. In contrast, types III and IV from fungal isolates AU2 and AU3, respectively, are inactive.

Scope of this thesis

This thesis covers studies on the activity of various forms of NIP1 from *R. secalis* on its host barley. An overview of proteins produced in other plant-microbe interactions that have a dual function in both virulence and *R* gene-mediated recognition is presented in chapter two of this thesis. A central issue in this chapter deals with the finding that many microbes appear to maintain elicitor genes despite their host-range restricting effects. It appears therefore likely that, like NIP1, these elicitors contribute to virulence.

The *NIP1*-gene encodes an 82 amino acid (a.a.) protein, including a 22 amino acid signal peptide, which is cleaved off upon secretion. The 60 amino acid mature NIP1 contains 10 cysteine residues. A heterologous expression system was established to produce sufficient quantities of NIP1 to allow structural characterization and identification of its receptor. In addition, an efficient expression system was required to produce ^{15}N-NIP1 required to determine its 3D structure. Chapter 3 describes the expression in *E. coli* of His-tagged NIP1 and the subsequent purification of milligram quantities of elicitor-active NIP1. An *in vitro* folding procedure was developed and applied to the purified NIP1 to increase the yield of protein with the correct disulfide bond pattern.

A central research question addressed in this thesis is to prove or disprove whether NIP1, both as virulence factor and as elicitor, is mediated through a single receptor in the plant or whether the elicitor receptor (triggering resistance) is distinct from the toxin receptor (conditioning disease). Chapter 4 describes the identification and characterization of a single class of high-affinity binding sites for NIP1, which was detected using ^{125}I-labeled NIP1 as ligand. Binding of ^{125}I-NIP1 to microsomes from resistant barley (*Rrs1*) was specific, reversible and saturable. A binding site with identical binding characteristics was detected in membranes from a near-isogenic susceptible (*rrs1*) barley cultivar as well as in microsomes from wheat, oat and rye. Surprisingly, in heterologous competition experiments the elicitor-inactive mutant proteins S23-P (type III*) and G45-R (type IV*) were shown to compete efficiently for the NIP1 binding site. In contrast, the elicitor-active NIP1 type II, which differs in

three amino acid positions from type I, showed a significantly lower affinity for the binding site in heterologous competition experiments when compared to type I.

In chapter 5 the NIP1 type I solution structure is presented, determined from a ^{15}N-labeled NIP1 sample. The NIP1 structure can be regarded as a two-domain structure that is dominated by β-sheet elements, which are generally stabilized by disulfide bridges. The structure shows no homology to other protein structures in the databases. With the high-resolution structural data being available on the NIP1 molecule, many of the biochemical data can be explained. In addition, the dynamic properties of NIP1 can possibly be connected to the transduction of the NIP1 ligand via the binding site to downstream factors in the signal-transduction pathway leading to resistance. Also, the apparent unexpected results with the S23P and G45R mutations are being discussed.

This thesis provides a detailed insight into the molecular basis of the interaction between *R. secalis* and its major host, barley. The central role of NIP1 in this interaction both as an elicitor of defense response in *Rrs1*-barley, and as a virulence factor in *rrs1*-barley is discussed. A working model based on biochemical and structural data is proposed in chapter 6. The results presented in this thesis shed more light on the early events in the interaction between a plant and a pathogen. In addition, it could contribute to the isolation of the NIP1 receptor from barley and establish its connection to the "pathogenesis" pathway in *rrs1*-barley or the "active resistance" pathway in *Rrs1*-containing plants. The possibilities can potentially be explained by the guard hypothesis (Van der Biezen and Jones, 1998).

Chapter 2

A dual role of microbial pathogen-derived effector proteins in plant disease and resistance

CHAPTER 2

A DUAL ROLE OF MICROBIAL PATHOGEN-DERIVED EFFECTOR PROTEINS IN PLANT DISEASE AND RESISTANCE

MANY PROTEINS FROM PLANT PATHOGENS AFFECTING THE INTERACTION WITH THE HOST PLANT HAVE DUAL FUNCTIONS; THEY PROMOTE VIRULENCE ON THE HOST SPECIES AND THEY FUNCTION AS AVIRULENCE DETERMINANTS BY ELICITING DEFENSE REACTIONS IN HOST CULTIVARS EXPRESSING THE APPROPRIATE RESISTANCE GENES. IN VIRUSES, ALL PROTEINS ENCODED BY THE SMALL GENOMES CAN BE EXPECTED TO BE ESSENTIAL FOR VIRAL DEVELOPMENT IN THE HOST. HOWEVER, IN DIFFERENT PLANTS SURVEILLANCE SYSTEMS HAVE EVOLVED THAT ARE ABLE TO RECOGNIZE MOST OF THESE PROTEINS. BACTERIA AND FUNGI HAVE SPECIALIZED PATHOGENICITY AND VIRULENCE GENES. MANY OF THE LATTER WERE ORIGINALLY IDENTIFIED THROUGH THE RESISTANCE GENE-DEPENDENT ELICITOR ACTIVITY OF THEIR PRODUCTS. THEIR ROLE IN VIRULENCE BECAME ONLY APPARENT WHEN THEY WERE INACTIVATED OR TRANSFERRED TO DIFFERENT MICROBES OR UPON THEIR ECTOPIC EXPRESSION IN HOST PLANTS. MANY MICROBES APPEAR TO MAINTAIN THESE GENES DESPITE THEIR DISADVANTAGEOUS EFFECT, INTRODUCING ONLY FEW MUTATIONS TO ABOLISH THE INTERACTION OF THEIR PRODUCTS WITH THE PLANT RECOGNITION SYSTEM. THIS HAS BEEN INTERPRETED AS BEEN INDICATIVE OF A VIRULENCE FUNCTION OF THE GENE PRODUCTS THAT IS NOT IMPAIRED BY THE MUTATIONS. ALTERNATIVELY, IN PARTICULAR IN BACTERIA THERE IS NOW EVIDENCE THAT PATHOGENICITY WAS ACQUIRED THROUGH HORIZONTAL GENE TRANSFER. GENES SUPPORTING VIRULENCE IN THE DONOR ORGANISM'S ORIGINAL HOST APPEAR TO HAVE TRAVELED ALONG. BEING GRATUITOUS IN THE NEW SITUATION, THEY MAY HAVE BEEN INACTIVATED WITHOUT LOSS OF ANY BENEFICIAL FUNCTION FOR THE PATHOGEN.

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Crit. Rev. Plant Sci. in press

I. INTRODUCTION

Human civilization has always cared for the development of agriculture to improve the efficiency of food production. However, as confirmed already by ancient writings and Biblical references, the history of agriculture has also been a story of devastating plant diseases. Crop breeding aims at higher yields as well as adaptation to various soils and other environmental factors, frequently at the expense of disease resistance. The vulnerability of crop plants to diseases is further intensified by agricultural practices such as monocultures, vegetative propagation, tillage, fertilization, irrigation and unfavorable sites (Yarwood, 1970). As the consequence, substantial crop failures have occurred that occasionally resulted in starvation tragedies, such as the famous Irish potato famine, caused by a severe *Phytophthora infestans* epidemic on potato in 1845 and 1846 (Schumann, 1991), and the less well-known but even more catastrophic Great Bengal Famine resulting from the almost complete destruction of the 1942 rice harvest due to a *Helminthosporium oryzae* epidemic (Horsfall and Cowling, 1978; Klinkowski, 1970).

Plants are constantly exposed to attack by viruses, bacteria, fungi, Oomycetes, nematodes, and insects. Wild plant populations can normally cope with these threats. However, the dense planting of genetically uniform crops over large areas, for instance, virtually invites epidemics through selection of virulent pathogens, and expensive plant protection schemes are required to reduce yield losses. Today, means to control plant diseases cost billions of dollars per year and a solution to the problem is not in sight. Therefore, the need for the development of novel strategies for plant protection is obvious. Prerequisite is a much profounder understanding of the molecular processes leading to plant disease and resistance.

The communication between plants and microbial pathogens through the exchange of signal molecules has been the focus of extensive research over the last decade. In order to exploit the host as a nutrient source, plant-pathogenic microbes need to overcome the plant external barriers including the cuticle and the epidermal cell walls. Bacteria and viruses, as well as some fungal parasites, depend on natural openings such as stomata or wounds for invasion. In contrast, most phytopathogenic fungi and Oomycetes as well as nematodes and insects are able to actively gain entrance to host tissues by means of secreted hydrolytic enzymes, mechanical penetration or a combination of both. Pathogen genes functioning in the process that renders a microbe pathogenic on a plant species are called pathogenicity (*Pth*) genes. In contrast, virulence (*Vir*) genes encode factors that interfere with cellular functions in host cultivars for the purpose of optimizing the pathogen's nutritional situation. In necrotrophic pathogens, virulence factors may be toxins that kill host cells. In contrast, biotrophic pathogens need to control the plant defense response and modulate the host metabolism in their favor.

Despite the major differences between classes of microbial plant pathogens there is one common theme; the need of a pathogen to secrete effector molecules and, hence, to leave cover also automatically includes the risk of being picked up by the plant surveillance system. Therefore, research on pathogen virulence factors is likely to improve our understanding of those basic plant cellular processes that are manipulated by the pathogen. In addition, our knowledge on resistance-related signaling pathways will be increased in those cases where a virulence factor is utilized by the plant as a signal in the recognition process that leads to the stimulation of defense reactions.

II. ELICITORS AND PLANT RESISTANCE

Secreted or surface-localized molecules of pathogen origin that trigger defense reactions in plants have been known for a long time. They are of different chemical nature and comprise carbohydrates, lipids and proteins (Knogge, 2002). One of the earliest characterized elicitors is a specifically 1,3-1,6-branched hepta-β-glucan from cell walls of the soybean pathogen *Phytophthora sojae* (Sharp *et al.*, 1984). Despite substantial research on its function including the identification of its receptor in the host plant (Mithöfer *et al.*, 2000; Umemoto *et al.*, 1997), its role during the interaction with the host and, hence, its biological relevance for plant resistance remains to be demonstrated. However, glucan perception appears to vary in different plant species, because the hepta-glucan was not active in parsley (Parker *et al.*, 1988) and rice, whereas a 1,3-1,6-branched tetra-glucan from cell walls of the rice pathogen *Magnaporthe grisea* is active in rice, but not in soybean (Yamaguchi *et al.*, 2000). *P. sojae* was the source of another extracellular elicitor, a 42-kDa glycoprotein (Parker *et al.*, 1991; Sacks *et al.*, 1995). Its protein moiety triggers defense reactions in parsley and potato (S. Rosahl and T. Nürnberger, personal communication), the elicitor activity residing in a 13-amino acid peptide fragment (Pep-13) that specifically binds to a receptor in parsley plasma membranes (Nennstiel *et al.*, 1998; Nürnberger *et al.*, 1994). The protein has transglutaminase activity and appears to be involved in microbial cell wall biosynthesis. Its gene was found in all *Phytophthora* species analyzed and the deduced proteins show at least two invariant domains essential for enzyme activity (T. Nürnberger, personal communication). One of these domains is almost identical with the Pep-13 sequence suggesting that the plant evolved a recognition system targeting a crucial portion of the microbial enzyme.

A similar strategy appears to have led to the development of a plant perception system for bacterial flagellin that specifically targets the most highly conserved domain within the N terminus of the protein (Felix *et al.*, 1999). Synthetic peptides from this domain act as elicitors of defense reactions in cells of tomato and several

other plant species. In contrast, the respective peptides from the plant-associated bacteria *Agrobacterium tumefaciens* and *Rhizobium meliloti* are elicitor-inactive. In *Arabidopsis thaliana* a locus, termed *FLS1*, was detected that determines flagellin sensitivity (Gomez-Gomez *et al.*, 1999). In addition, a second locus, *FLS2*, contains a ubiquitously expressed gene encoding a receptor kinase with an extracellular leucine-rich repeat (LRR) domain and an intracellular serine/threonine protein kinase domain (Gomez-Gomez and Boller, 2000). This receptor kinase shows structural homology with plant resistance (*R*) gene products (s. III, Table 1), in particular with the product of the rice *R* gene, *Xa21* (Song *et al.*, 1995). Two mutant alleles conferring flagellin insensitivity were identified, *fls2-24* and *fls2-17*, that have a point mutation in the LRR domain and the kinase domain, respectively (Gomez-Gomez and Boller, 2000). Both are also compromised in flagellin binding (Gomez-Gomez *et al.*, 2001). This is no surprise in case of the mutation in the extracellular LRR domain, supposed to interact with the ligand. However, the requirement of a functional kinase domain for flagellin recognition suggests that auto-phosporylation is necessary for the proper assembly of a functional receptor complex, that may comprise the product of the *FLS1* gene and a negative regulator, the kinase-associated protein phosphatase (Gomez-Gomez *et al.*, 2001).

In these cases, the molecules utilized by the plants to mediate recognition have basic indigenous functions for the microbes and therefore should not be called virulence factors. Flagellin and other conserved microbial products, which are invariant among diverse groups of microorganisms, such as bacterial lipopolysaccharide, bacterial peptidoglycan or fungal constituents such as chitin, glucan, lipids or proteins have been recently referred to as pathogen-associated molecular patterns (PAMPs). These PAMPs are also recognized by the innate immune systems of insects and vertebrates (Aderem and Ulevitch, 2000; Akira *et al.*, 2000; Hayashi *et al.*, 2001), and the striking similarities of the molecular basis of immunity in different kingdoms were highlighted in a number of recent reviews (Cohn *et al.*, 2001; Nürnberger and Scheel, 2001; Takken and Joosten, 2000). It appears that PAMP recognition is mediated through phylogenetically conserved Toll-like receptors (Hayashi *et al.*, 2001) that activate plant species (non-host) or basic resistance mechanisms.

Whereas PAMPs act at the species or higher level, many molecules have been identified that specify resistance at the host cultivar level. The current view is that the acquisition of virulence factors has enabled pathogen strains to overcome plant species resistance. In turn, these factors haven driven the co-evolution of plant *R* genes and, thus, the development of phylogenetically more recent cultivar-specific disease resistance to specific pathogen races. In this process, pathogen virulence factors that are recognized through plant *R* genes become avirulence determinants and

Table 1: Isolated plant resistance genes (modified after Baker *et al*., 1997). *Avr* genes in brackets have not been cloned to date. CC; coiled coil domain, CC*; putative leucine zipper domain, NBS; nucleotide binding site, LRR; leucine-rich repeats, PK; protein kinase, TM; transmembrane domain.

Class	*R gene*	*Plant species*	*Pathogen*	*Avr gene*	*Structure of R protein*	*Reference*
1-I	*N*	tobacco	Tobacco mosaic virus	Replicase	TIR-NBS-LRR	Whitham *et al*., 1994
	RPS4	*A. thaliana*	*P. syringae* pv. *tomato*	*avrRps4*	TIR-NBS-LRR	Gassmann *et al*., 1999
	RPP1,10,14	*A. thaliana*	*Peronospora parasitica*	?	TIR-NBS-LRR	Botella *et al*., 1998
	RPP5	*A. thaliana*	*Peronospora parasitica*	(*avrRpp5*)	TIR-NBS-LRR	Parker *et al*., 1997
	L6, L1-12	flax	*Melampsora lini*	(*AL6*)	TIR-NBS-LRR	Lawrence *et al*., 1995
	M	flax	*Melampsora lini*	(*AM*)	TIR-NBS-LRR	Anderson *et al*., 1997
1-II	*RPS2*	*A. thaliana*	*P. syringae* pv. *tomato*	*avrRpt2*	CC*-NBS-LRR	Bent *et al*., 1994
	RPS5	*A. thaliana*	*P. syringea pv. tomato*	*avrPphB*	CC*-NBS-LRR	Warren *et al*., 1998
	RPM1	*A. thaliana*	P. syringae pv. maculicula	*avrRpm1; avrB*	CC*-NBS-LRR	Grant *et al*., 1995
	RPP8	*A. thaliana*	*Peronospor*a partasitica	(*avrRpp8*)	CC*-NBS-LRR	McDowell *et al*., 1998
	HRT	*A. thaliana*	Turnip crinkle virus	Coat protein	CC*-NBS-LRR	Cooley *et al*., 2000
	Prf	tomato	*P. syringae* pv. *tomato*	*avrPto*	CC*-NBS-LRR	Salmeron *et al*., 1994
	Mi-1	tomato	*Meloidogyne incognita;*	? (nematode)	CC*-NBS-LRR	Milligan *et al*., 1998;
			Marcosiphum euphorbiae	? (aphid)		Rossi *et al*., 1998;
						Vos *et al*., 1998
	I2	tomato	*Fusarium oxysporum*	?	CC*-NBS-LRR	Simons *et al*., 1998
	Rx1	potato	Potato virus X	Coat protein	CC*-NBS-LRR	Bendahmane *et al*., 1999
	Rx2	potato	Potato virus X	Coat protein	CC*-NBS-LRR	Bendahmane *et al*., 2000
	Gpa2	potato	*Globodera pallida*	?	CC*-NBS-LRR	van der Voort *et al*., 1999
	Dm3	lettuce	*Bremia lactucae*	?	CC-NBS-LRR	Meyers *et al*., 1998
	Bs2	pepper	*X. campestris* pv. *vesicatoria*	*avrBs2*	CC-NBS-LRR	Tai *et al*., 1999
	Sw-5	tomato	Tospoviruses	?	CC-NBS-LRR	Brommonschenkel *et al*., 2000;
						Spassova *et al*., 2001
	Xa1	rice	*X. oryzae* pv. *oryzae*	?	CC-NBS-LRR	Yoshimura *et al*., 1998

	Pib	rice	*Mangnaporthe grisea*	?	CC-NBS-LRR	Wang *et al*., 1999
	Pi-ta	rice	*Magnaporthe grisea*	*AVR-Pita*	CC-NBS-LRR	Bryan *et al*., 2000
	Cre3	wheat	*Heterodera avenae*	?	CC-NBS-LRR	Lagudah *et al*., 1997
	Mla1	barley	*B. graminis* f.sp. *hordei*	(*AvrMla1*)	CC-NBS-LRR	Zhou *et al*., 2001
	Mla6	barley	*B. graminis* f.sp. *hordei*	(*AvrMla6*)	CC-NBS-LRR	Halterman *et al*., 2001
	Rp1-D	maize	*Puccinia sorghi*	?	CC-NBS-LRR	Collins *et al*., 1999
2	*Pto*	tomato	*P. syringae* pv. *tomato*	*avrPto*	PK	Martin *et al*., 1993
3	*Cf-9*	tomato	*Cladosporium fulvum*	*Avr9*	LRR-TM	Jones *et al*., 1994
	Cf-4	tomato	*Cladosporium fulvum*	*Avr4*	LRR-TM	Thomas *et al*., 1997
	Cf-2	tomato	*Cladosporium fulvum*	(*Avr2*)	LRR-TM	Dixon *et al*., 1996
	Cf-5	tomato	*Cladosporium fulvum*	(*Avr5*)	LRR-TM	Dixon *et al*., 1998
	Hcr9-4E	tomato	*Cladosporium fulvum*	(*Avr4E*)	LRR-TM	Takken *et al*., 1999
	Hs1 $^{pro-1}$	sugar beet	*Heterodera schachtii*	?	LRR-TM	Cai *et al*., 1997
4	*Xa21*	rice	*X. oryzae* pv. *oryzae*	?	LRR-TM-PK	Song *et al*., 1995
5	*Hm1*	maize	*Cochliobolus carbonum*	-	HCT reductase	Johal and Briggs, 1992
6	*Mlo*	barley	*B. graminis* f.sp. *hordei*	?	7 TM protein	Bueschges *et al*., 1997

their genes avirulence (*Avr*) genes. Expression of an *Avr* gene triggers the defense response exclusively in host cultivars expressing the matching *R* gene, the absence of either of these genetic determinants leading to disease (gene-for-gene interaction; (Flor, 1955; 1971). Hence, *Avr* genes are first of all defined through their negative impact on the ability of pathogen strains to infect host cultivars.

To date, avirulence determinants have been identified in viruses, bacteria, fungi and Oomycetes, and a candidate gene was recently isolated from a nematode. In addition, genes related to *Pth* and *Avr* genes of pathogens have been identified in *Rhizobium* species, suggesting their involvement in symbiotic interactions as well (Baron and Zambriski, 1995; Ciesiolka *et al.*, 1999; Viprey *et al.*, 1998). Clearly, the expression of *Avr* genes is disadvantageous to plant pathogenic microbes, resulting in host range restriction. While some *Avr* genes are dispensable, their deletion not causing any observable phenotype, many of these genes are maintained. Instead they are rendered inactive by introducing point mutations that translate into single amino acid alterations in the gene products. The gratuitous effect of *Avr* genes on single host cultivars may therefore be over-compensated by indigenous functions that are beneficial to the microbe on the host species (Vivian and Gibbon, 1997; White *et al.*, 2000). Alternatively, in particular in bacteria there is growing evidence that *Avr* genes may have been acquired through horizontal gene transfer where they have traveled along with *Pth* genes (Gabriel, 1999). In this scenario, they may have had a selective value for the donor species.

III. PLANT RESISTANCE GENES

The elicitor-receptor model has been employed to explain the recognition of a pathogen by a host plant based on the *Avr*-*R* gene interaction (de Wit, 1997; Gabriel and Rolfe, 1990; Keen, 1990; Lamb, 1996). This model predicts that microbial *Avr* gene products directly or indirectly interact with receptors encoded by host *R* genes. Perception of the AVR signal by the plant R protein initiates the plant defense response that often, but not always, is characterized by a hypersensitive response (HR). The HR is a form of programmed cell death occurring at the site of infection (Heath, 2000; Morel and Dangl, 1997). Other typical resistance reactions include a burst of active oxygen species, the stimulation of ion fluxes, changes in protein phosphorylation, and the synthesis of pathogenesis-related proteins (for recent reviews see Bolwell, 1999; Ebel and Mithöfer, 1998; Grant and Loake, 2000; Hutcheson, 1998; Muthukrishnan *et al.*, 2001; Richberg *et al.*, 1998; Rushton and Somssich, 1999; Sessa and Martin, 2000). Therefore, detection of an AVR signal by the host plant constitutes the pivotal event in a successful defense response. Localization of the receptors in the plant plasma membrane was originally expected to

allow an early interception of invading pathogens. From information gained by the analysis of cloned plant *R* and pathogen *Avr* genes, however, a more complex picture of related-related signal perception and transduction emerged.

In 1992, the first plant *R* gene was isolated encoding an enzyme that inactivates a host-specific toxin (Johal and Briggs, 1992). Since then more than thirty *R* genes have been cloned from various plants (reviewed by Bent, 1996; Hammond-Kosack and Jones, 1997; Martin, 1999; Takken and Joosten, 2000). Although effective against a variety of different pathogens such as viruses, bacteria, Oomycetes, fungi, nematodes and even aphids, the structure of *R* gene products show remarkable similarities, most of them containing a leucine-rich repeat (LRR) domain along with a small number of other putative signaling domains (Ellis and Jones, 1998; Jones and Jones, 1997; Martin, 1999; Michelmore and Meyers, 1998; Takken and Joosten, 2000; van der Biezen and Jones, 1998b). These structural features allow their grouping into several classes (Tab. 1). LRRs have been known from proteins of other organisms, where these domains are involved in protein-protein interactions (Kajava, 1998; Kobe and Deisenhofer, 1995). In R proteins they are believed to directly interact with AVR factors, thereby determining recognition specificity (Bent, 1996; Jones and Jones, 1997; Martin, 1999). Direct proof is however often lacking. Another R protein feature is the nucleotide-binding site (NBS) that consists of kinase 1a (P-loop), 2 and 3a motifs (Traut, 1994), and several other conserved sequence motifs with unknown function (van der Biezen and Jones, 1998a). A similar domain was found in proteins that regulate programmed cell death in *Caenorhabditis elegans* and mammalians suggesting an analogous function in plant R proteins. In an intriguing scenario, the R proteins may be part of a membrane-attached protein complex in plants that is activated by AVR signals. The LRR domains may ensure that activation of the complex occurs only upon recognition. Ligand-induced conformational changes in the R proteins may enable the cleavage of ATP or GTP, which then allows the N-terminal domains to activate downstream effectors (van der Biezen and Jones, 1998a).

The majority of *R* genes cloned to date encodes cytoplasmic proteins of the NBS-LRR type (Tab. 1). These can be divided into two major groups (Pan *et al.*, 2000a). Group I NBS domains are always linked with an N-terminal domain with similarity to the cytoplasmic portion of the *Drosophila* Toll and the mammalian interleukin-1 receptor (TIR). The latter proteins are involved in innate immunity in the respective organisms suggesting an evolutionary relationship between plant and animal basic related systems (Aderem and Ulevitch, 2000; Aravind *et al.*, 1999; Cohn *et al.*, 2001). The N-terminus of group II NBS domain proteins potentially adopts coiled-coil (CC) structures, *i.e.*, bundles of α-helices showing a distinctive packing of amino acid side chains at their interfaces. A subset of the CC domains represents putative leucine zipper domains. This suggests the presence of at least two distinctive basic signaling pathways mediated by the TIR and CC domains, respectively (Aarts *et*

al., 1998; Parker *et al.*, 2000). Group II genes were detected in mono- and dicotyledonous species. In contrast, group I genes appear to lack from the genomes of major cereal species, indicating divergent evolution of R genes in the major groups of land plants (Pan *et al.*, 2000b). One of the first cloned *R* genes, *Pto*, encodes a protein deviating from most R proteins by the absence of LRRs (Martin *et al.*, 1993). Instead, the gene product is a serine/threonine-specific protein kinase that is part of a plant defense-signaling cascade. Only relatively few *R* genes isolated to date encode transmembrane proteins with extracellular LRR domains. Of these, however, only the product of the rice *Xa21* gene, a transmembrane receptor protein kinase, complies with the original expectation of a surface-localized AVR receptor with a cytoplasmic signal transduction domain (Song *et al.*, 1995).

IV. VIRAL ELICITORS

Viral genomes are very small and encode only a limited number of proteins. Each protein in this minimal set can be expected to be essential for viral replication and systemic infection of the host. Since basic development and virulence cannot be separated in viruses each viral protein can be assumed to be a virulence factor. However, viral proteins that are expressed *in planta* are also good candidates for recognition signals able to trigger the host related response if recognized by a plant R protein. Consequently, many viral proteins have been identified to function as AVR determinants. In addition, detailed molecular analysis of mutations in these proteins demonstrates the basic need of the pathogen to evade recognition by the host.

1. Tobamovirus Replicase Proteins

The tobamoviruses are among the best-studied plant viruses. Viral gene products involved in each of the major steps of the tobamovirus life cycle have been found to determine resistance in combination with specific host *R* genes. The genome of *Tobacco mosaic virus* (TMV) encodes at least four proteins: two replicase proteins of 126 kDa and 183 kDa, respectively, a 30-kDa movement protein and a 17.5-kDa coat protein (Goelet *et al.*, 1982; Zaitlin, 1999). The 183-kDa protein is the product of a read-through at the amber termination codon of the 126-kDa protein. Both proteins contain domains similar to the methyl transferase and helicase domains of the replicase-associated proteins of other RNA viruses, whereas the 183-kDa protein in addition comprises the polymerase domain. It is suggested that both proteins form a heterodimer for efficient replication (Lewandowski and Dawson, 2000).

Tobacco (*Nicotiana tabaccum*) cultivars carrying the *R* gene *N* are resistant to TMV and all known tobamoviruses except strain TMV-Ob (Padgett and Beachy,

1993). After isolating the *N* gene its product was shown to be a cytoplasmic protein of the TIR-NBS-LRR class (Whitham *et al.*, 1994). This suggested that TMV is detected upon translation of viral proteins inside the plant cell. Sequence analysis of HR-inducing mutants of TMV-Ob revealed amino acid alterations in the C-terminal region of the helicase region of the 126/183 kDa replicase proteins (Padgett and Beachy, 1993) suggesting these proteins to determine avirulence. Further confirmation came from experiments using chimeric virus genomes in which the genes encoding the 126/183-kDa proteins of TMV and TMV-Ob were exchanged (Padgett *et al.*, 1997). Using a modified TMV-Ob as a vector to express various replicase fragments, it was determined that a C-terminal 425 amino acid portion of the replicase is sufficient to cause HR. To exclude the possible involvement of other viral factors in HR elicitation, TMV cDNA fragments were transferred into tobacco via *Agrobacterium tumefaciens*-mediated transformation. Again, a C-terminal 50 kDa region of the replicase was sufficient to trigger the HR in an *N*-dependent manner, thus pinpointing this region as the TMV elicitor domain (Erickson *et al.*, 1999a). Consistent with the helicase function, the 50-kDa fragment was shown to have ATPase activity. However, a point mutation abolishing the enzyme activity did not lead to a loss in HR induction (Erickson *et al.*, 1999b). This suggests that features independent of the protein's function are required for recognition by the *N* gene product.

The replicase proteins of TMV and its close relative, *Tomato mosaic virus* (ToMV), are also AVR determinants in a different host plant. In tomato (*Lycopersicon esculentum*), three *R* genes, *Tm-1*, *Tm-2*, and its allele, *Tm-2*^{2}, have been recognized to confer resistance to TMV and ToMV (Hall, 1980; Pelham, 1966). A TMV strain breaking the *Tm-1*-based resistance was isolated and its genomic sequence compared to the wild-type strain (Meshi *et al.*, 1988). Of the four nucleotide exchanges detected only the two in the replicase protein led to amino acid alterations. *In vitro* constructed viral RNAs, each carrying only one of the mutations, revealed that the $Gln_{979} \rightarrow Glu$ substitution plays an essential role in overcoming *Tm-1* resistance. Originally, a change in local net charge in this protein region was suggested to affect the electrostatic interaction with a *Tm-1* host R factor. Recently, however, viral mutants were constructed that contained other substitutions at position 979 (Hamamoto *et al.*, 1997a). Not only an Asp mutant but also a Lys, an Asn, and an Arg mutant were able to multiply in *Tm-1* tomato protoplasts, whereas propagation of a His mutant was virtually inhibited. These results cast doubts on a simple charge-based mechanism, but rather suggest another factor such as a conformational change of the replicase proteins. Surprisingly, an Ile_{979} mutant that multiplied like the wild-type strain in tobacco was virtually unable to replicate in tomato cells independent of the presence or absence of the *Tm-1* gene (Hamamoto *et al.*, 1997b). The normal replication of this mutant in tobacco indicates that the basic function of the replicase proteins is not affected by the substitution. However, in tomato cells lacking the *Tm-1*

gene the 126- and 183-kDa proteins appear to interact with an unknown host factor in the same manner as with Tm-1. Since the involvement of (a) host-encoded factor(s) in forming the *in vivo* replicase complex has been postulated (Ishikawa *et al.*, 1993), amino acid 979 may play an essential role in this interaction of the replicase proteins and the host factor(s). In tomato, but not in tobacco, the interaction may be destabilized in the Ile_{979} mutant. *Tm-1* may therefore encode an altered form of the tomato host factor that is unable to properly interact with the wild-type viral replicase proteins, but can interact with the $Gln_{979}\rightarrow$Glu substituted protein (Dawson and Hilf, 1992).

2. Tobamovirus Coat Proteins

The TMV coat protein (CP) was among the first elicitors defined for gene-for-gene-type interactions. The wild-type protein is inactive but a number of mutants were isolated or generated that specifically trigger an HR in tobacco (*N. sylvestris*) carrying the *N'* gene (Culver and Dawson, 1989; Knorr and Dawson, 1988; Saito *et al.*, 1989). This *R* gene has not been isolated to date, but substantial information exists on the CP. Its three-dimensional structure has long been known (Bloomer *et al.*, 1978; Franklin, 1956; Namba *et al.*, 1989; Stubbs *et al.*, 1977; Watson, 1954). At the core of the protein is a right-handed helical bundle of four α-helices. The position of the helices is maintained by a number of inward-facing hydrophobic residues as well as by a β-sheet region at the outer end of the helical bundle. Mutations that disrupt the normal association of the four helices were shown to interfere with *N'*-mediated recognition (Taraporewala and Culver, 1997). Amino acid substitutions that occur within the interface regions in ordered CP aggregates lead to *N'*-based elicitation of the HR, the strength of the response depending on the degree of interference with the CP quarternary structure (Culver *et al.*, 1994). In contrast, mutations disrupting the CP tertiary structure were elicitor-inactive. Finally, the CP elicitor site was narrowed down to the right face of the helical bundle, and regions outside this site do not contribute to HR elicitation (Taraporewala and Culver, 1996; Taraporewala and Culver, 1997). Chimeric TMV cDNA constructs carrying the CP open reading frame of *Odontoglossum ringspot virus* (ORSV) and *Cucumber green mottle mosaic virus* (CGMMV), respectively, were found to also encode elicitor active proteins, suggesting that the elicitor active site is conserved in the CPs of other tobamoviruses (Taraporewala and Culver, 1997). Therefore, *N'* gene-mediated recognition appears to target a conserved site on tobamovirus CPs that is essential for CP aggregation and that is normally buried in the quarternary structure, but that is exposed by the disassembly of CP aggregates.

In *Capsicum* spp. four allelic genes, L^1-L^4, govern resistance against tobamoviruses. The L^3 gene-mediated resistance is only overcome by isolates of

Pepper mild mottle virus (PMMV). The viral CP has been identified as the HR elicitor by analyzing chimeras between an avirulent and a virulent European strain on *C. chinense* (L^3L^3), a single amino acid substitution, Asn_{138}→Met, rendering the protein elicitor-active (Berzal-Herranz *et al.*, 1995). In contrast, a double substitution in the CP sequence, Thr_{43}→Lys and Asp_{50}→Gly, was found to be responsible for the loss of elicitor activity in a Japanese strain (Tsuda *et al.*, 1998). Based on the three-dimensional model of TMV CP, residue 138 was located in the elicitor region in an exposed position in a β-turn that connects two of the CP α-helices (Berzal-Herranz *et al.*, 1995). In contrast, residues 43 and 50 reside in one of the helices, most likely involved in CP aggregation (Taraporewala and Culver, 1997). Met_{138} may directly interact with the *R* gene product. However, this amino acid is not conserved in the CPs of all tobamoviruses known to trigger the L^3-mediated related response. Therefore, a change in the local hydropathy profile was discussed as causing the recognition by the plant R factor. It cannot be excluded however that Asn_{138} has a role in stabilizing the CP quarternary structure and thus preventing the recognition by the host cell. A similar destabilizing effect may cause the recognition of the AVR determinant in the double substitution mutant (Tsuda *et al.*, 1998).

Analysis of viral chimeras involving the genomes of a virulent PMMV strain and an avirulent *Paprika mild mottle virus* (PaMMV) also resulted in identifying the viral CP as the factor recognized by the L^2 gene in *Capsicum frutescens* (de la Cruz *et al.*, 1997). At present it is not known however which part of the protein is involved in the recognition event. All tobamoviruses that elicit the L^2 gene-mediated HR also elicit the HR in L^3 plants. However, PMMV is recognized by the L^3 gene but not by the L^2 gene. This finding may argue for an evolution of the *L* genes towards recognizing a wider spectrum of tobamoviral CPs (de la Cruz, 1997).

Different plant species appear to target different CP features for recognition. When CPs from different tobamoviruses were expressed from a *Potato virus X* (PVX) vector on eggplant (*Solanum melongena*), the proteins from TMV-U2 and ORSV were found to induce an HR similar to the CP from wild-type TMV-U1 (Dardick and Culver, 1997). In comparison, the CP from CGMMV did not elicit an HR but induced spreading chlorosis in inoculated leaves. In contrast, the CGMMV CP elicits the *N'* gene-mediated HR in tobacco, while the TMV-U1 CP fails to induce this response (Taraporewala and Culver, 1997) suggesting that tobacco and eggplant differ in the recognized CP features.

3. Tobamovirus Movement Proteins

The fourth protein encoded by the tobamovirus genome is the 30-kDa movement protein (MP) that potentiates viral cell-to-cell movement but is dispensable for replication (Meshi *et al.*, 1987). Unlike the *Tm-1*-based resistance that is mediated

through mutations in the viral replicase gene product (Meshi *et al.*, 1988), the resistance encoded by *Tm-2* and *Tm-2²* is expressed only in whole plants but not in protoplasts. In addition, *Tm-2* resistance can be broken by host pre-inoculation with the taxonomically different PVX, suggesting that resistance operates at the level of viral cell-to-cell movement. Sequence comparison of the genes encoding the MP of a wild-type TMV strain and a *Tm-2* resistance-breaking strain identified two amino acid substitutions (Meshi *et al.*, 1989). Strains with either one of the two mutations did not overcome resistance as efficiently as a double mutant. Another resistance-breaking strain shared one of the amino acid alterations in the 30-kDa protein, but differed in the second mutation. The amino acid exchanges occurred at or near the conserved regions of the 30-kDa protein suggesting that the mechanism of *Tm-2* resistance may be closely related to the original function of the protein in viral cell-to-cell movement.

Comparison of the MP sequences from ToMV strains breaking the resistance conferred by the *Tm-2* and *Tm2²* alleles with a wild-type strain revealed a number of additional mutations involving mostly charged amino acids. Hence, alterations in the local charge of the protein may affect the ability of the host *R* gene product to interact with the viral MP (Calder and Palukaitis, 1992). In the MP of a *Tm2²* resistance-breaking strain two amino acid alterations were detected in the C-terminal region, which displays a high variability among different tobamoviruses. This protein portion has been shown to be dispensable for virus transport in tobacco suggesting that resistance does not interfere with the basic function of the protein in viral movement. Rather, a 30-amino acid C-terminal domain of the movement protein serves as a specific recognition target in the *Tm-2²*-mediated resistance (Weber and Pfitzner, 1998; Weber *et al.*, 1993).

4. Potato Virus X Proteins

The interaction of PVX with several potato *R* genes provides another system in which the recognition molecules have been extensively characterized. Two types of resistance to PVX have been identified in potato (*Solanum tuberosum*). The genes *Rx1* (originating from *S. andigena*) and *Rx2* (originating from *S. acaule*) mediate “extreme resistance” that leads to the rapid arrest of PVX accumulation in the initially infected cell without triggering an HR. In contrast, the genes *Nb* and *Nx* control an HR-associated resistance. Both *Rx* genes were recently cloned and encode proteins with 95 % sequence identity. Their products belong to the CC-NBS-LRR class of plant R proteins (Bendahmane *et al.*, 1999; Bendahmane *et al.*, 2000). Most of the amino acid differences occur in the N-terminal region including the NBS domain, while the LRR domains differ only in a few amino acids. The resistance response mediated by both proteins requires the same domain of the PVX CP (Bendahmane *et al.*, 1995; Querci *et al.*, 1995) and is also effective against viruses unrelated to PVX (Köhm *et al.*,

1993). The nature of the *Rx* response is determined by the mode of expression of the CP elicitor. CP produced from the virus causes extreme resistance without HR, whereas CP expressed *in planta* elicits an HR, with extreme resistance being epistatic to HR. This is interpreted such that viral expression leads to an early and rapid elicitation of related reactions that also inhibit the production of high CP levels. This primary response is unlikely to include *de novo* protein synthesis (Gilbert *et al.*, 1998) and is active against other viruses as well. In contrast, expression from a transgene leads to a continuing build-up of CP resulting in the activation of the HR as a secondary response (Bendahmane *et al.*, 1999).

Interestingly, *Rx1* is a member of an *R* gene cluster of four highly homologous genes on potato chromosome 12. Another member of this cluster is the *Gpa2* gene conferring resistance to populations of the potato cyst nematode *Globodera pallida* (van der Vossen *et al.*, 2000). The products of these genes and that of the *Rx2* gene on chromosome 5 display >88 % sequence identity. This sequence conservation suggests a direct evolutionary relationship between the Rx proteins that "learned" to recognize different elicitor molecules.

The *Rx2* locus on chromosome 5 is also linked to several resistance genes including *R1* against *Phytophthora infestans* and *Gpa3* against *G. pallida*, but also the *Nb* gene conferring HR resistance to PVX (de Jong *et al.*, 1997; van der Voort *et al.*, 1998). In contrast to the *Rx* genes, the *Nb* gene targets the PVX MP and a single amino acid position, Ile_6, is required for recognition (Malcuit *et al.*, 1999). Finally, the *Nx* gene (Cruz and Baulcombe, 1995) that was mapped to potato chromosome 9 (Tommiska *et al.*, 1998) provides another example for the PVX CP being the principal AVR determinant.

5. Avirulence Determinants from Other Viral Groups

Avirulence determining proteins that usually differ in single amino acid proteins from the wild-type proteins have also been described from other plant virus groups (Culver, 1996). The N terminus of the CP of *Turnip crinkle virus* is the AVR factor recognized by *A. thaliana* carrying the *R* gene *HRT* (Dempsey *et al.*, 1997) that encodes a CC-NBS-LRR-class protein (Cooley *et al.*, 2000). The 2a polymerase protein of *Cucumber mosaic virus* functions as an HR elicitor (Karasawa *et al.*, 1999; Kim and Palukaitis, 1997) in a cowpea (*Vigna unguiculata*) cultivar carrying the *Cry* locus conferring resistance to viral strain Y (Nasu *et al.*, 1996). The 22p MP of *Tomato bushy stunt virus* is an avirulence determinant in *Nicotiana edwardsonii* (Chu *et al.*, 1999; Scholthof *et al.*, 1995). BV1, another movement-associated protein of *Bean dwarf mosaic virus*, is an HR and avirulence determinant in bean (*Phaseolus vulgaris*; (Garrido-Ramirez *et al.*, 2000). Likewise, single amino acid substitutions in the small 6-kDa protein (6K2) and viral genome-linked protein (VPg) of *Potato virus*

A have demonstrated these proteins to trigger resistance in *Nicandra physaloides* (Rajamäki and Valkonen, 1999). The resistance mediated through the recessive *R* gene *va* in tobacco was overcome by mutations occurring in the VPg protein of *Tobacco vein mottling virus* (Nicolas *et al.*, 1997). A protein of unknown function, the gene VI product of *Cauliflower mosaic virus*, determines avirulence in *N. clevelandii* in combination with the *ccd1* gene (Kiraly *et al.*, 1999; Schoelz, 1986). In addition, the cylindrical inclusion (CI) protein of *Turnip mosaic virus* (TuMV) was recently identified as the AVR factor in oilseed rape (*Brassica napus*) (Jenner *et al.*, 2000) carrying the *R* gene *TuRB01*, the first gene for host resistance to TuMV to be mapped in a Brassica crop (Walsh *et al.*, 1999).

These examples confirm that basically each protein encoded by a viral genome has the potential to trigger the host defense response. However, the recognition system of different plants target different proteins or even different regions of a given viral protein. The recognition-causing single amino acid substitutions can either directly enable an interaction with a plant R factor or cause conformational changes that expose the target region to access by the R factor.

V. BACTERIAL ELICITORS

Compared to viruses, bacterial plant pathogens exhibit a several fold higher degree of complexity. Consequently, bacterial pathogenicity- and virulence-associated functions are encoded by sets of specialized genes that are often located on plasmids and can usually be clearly separated from housekeeping functions that are encoded by separate sets of genes. Since 1984, when the first bacterial *Avr* gene was cloned from *Pseudomonas syringae* (Staskawicz *et al.*, 1984), the ease with which molecular techniques can be applied to bacteria has led to the isolation of more than 40 *Avr* genes from Gram-negative pathogens of the genera *Pseudomonas*, *Xanthomonas*, *Erwinia* and *Ralstonia* (for review see Dangl, 1994; Leach and White, 1996; Vivian and Gibbon, 1997). In addition, deletion or mutation of several of these genes resulted not only in loss of avirulence in the presence of the respective plant *R* genes but simultaneously in a reduction of virulence on susceptible host plants (White *et al.*, 2000). Furthermore, several *Avr* genes when expressed in the host lacking the corresponding *R* gene induced disease-like symptoms suggesting their products to be directly involved in promoting virulence (Kjemtrup *et al.*, 2000). This review will mainly focus on these latter proteins.

1. The Type III Secretion System

The finding that almost all plant *R* genes acting against bacteria encode putative cytoplasmic proteins prompted the question as to whether the bacterial signal enters

the plant cells for direct interaction with the R protein. Attempts to answer this question have identified a surprising homology between animal and plant pathogenic bacteria, a highly specialized protein secretion system, termed type III. It has long been known that Gram-negative bacterial pathogens require *hrp* genes for HR triggering and pathogenicity. The *hrp* gene locus has now been shown to encode proteins that control the production of the type III secretion system (Alfano and Collmer, 1996; Bonas and van den Ackerveken, 1997; Cornelis and van Gijsegem, 2000; Galán and Collmer, 1999; Mudgett and Staskawicz, 1998). Delivery of effector proteins into host cells appears to be mediated by a filamentous surface appendage, called the Hrp pilus (Roine *et al.*, 1997). Inside the host cells the translocated bacterial proteins can interfere with cellular functions to modulate the host metabolism. Components of the type III secretion system are related to components of the bacterial flagellar biogenesis complex indicating an evolutionary adaptation of the flagellar export apparatus to secrete virulence factors (Van Gijsegem *et al.*, 1995). Type III secretion systems operate host cell contact-dependent (Aldon *et al.*, 2000) and export proteins lacking any characteristic secretion motif. Instead, the *in vitro* secretion signal appears to reside in the tertiary 5' mRNA structure suggesting a mechanism in which translation is coupled to the translocation of the nascent protein (Anderson *et al.*, 1999; Anderson and Schneewind, 1999b). In addition, however, results from recent experiments using a genomic, single-copy *Avr* gene integration system instead of a plasmid-based technique suggest that the N-terminal region of the Avr protein, AvrRpm1, contains information required for the *in vivo* translocation into host cells. In contrast, the defense-activating domain resides in the C-terminal region indicating a bimodular structure of some bacterial AVR proteins (Guttman and Greenberg, 2001). This is reminiscent of the YopE effector from *Yersinia* spp. for which different signals are required for *in vitro* secretion and *in vivo* translocation into animal cells (Cheng *et al.*, 1997). Although the type III-secreted proteins are structurally diverse, they can be translocated by very different bacteria suggesting that the components of the type III secretion machinery that recognize the mRNA secretion signal are conserved among bacterial species (Collmer *et al.*, 2000; Galán and Collmer, 1999; Kjemtrup *et al.*, 2000).

The basic process of type III secretion was first identified and analyzed in bacterial pathogens of animals (Anderson and Schneewind, 1999a; Cornelis and van Gijsegem, 2000; Hueck, 1998). However, meanwhile there is also direct evidence for type III-dependent translocation of AVR proteins into host cells by plant pathogenic bacteria. The AVR protein AvrRpt2 from *Pseudomonas syringae* pv. *tomato* was shown to be secreted from an *E. coli* strain carrying the *hrp* gene cluster from *Erwinia chrysanthemi* (E_{ch} *hrp*), but not if the E_{ch} *hrp* gene cluster was secretion-defective (Mudgett and Staskawicz, 1999). Likewise, secretion-mutant strains of *P. syringae* pv. *syringae* expressing the *avrRpt2* gene were unable to cause an HR on *A. thaliana*

plants carrying the complementary *R* gene, *RPS2I*, suggesting that a functional secretion apparatus is required for AvrRpt2 activity. Furthermore, independent of the presence of *RPS2* N-terminal processing of the AvrPt2 protein was observed in *A. thaliana* and *N. tabacum* inoculated with bacteria. A very similar processing was found when the *avrRpt2* gene was expressed *in planta*. *In vitro* this cleavage occurred only when purified AvrRpt2 protein was incubated with crude *A. thaliana* homogenate, not however with apoplastic fluids or upon infiltration of the protein into the extracellular spaces of leaves. This suggests that the N-terminal processing of AvrRpt2 is catalyzed by a plant protease after translocation into the host cell (Mudgett and Staskawicz, 1999).

2. Dual function AVR proteins from the genus *Pseudomonas*

Despite the number of isolated bacterial *Avr* genes the mechanisms underlying their impact on plants as well as their targets within host cells remain elusive (Tab. 2). An exception is the *Avr* gene *avrPto* from *P. syringae* pv. *tomato*, the causal agent of the tomato speck disease. Its product, AvrPto, is a 164-amino acid, hydrophilic protein (Ronald *et al.*, 1992) that was the first AVR protein demonstrated to physically interact with the corresponding R gene product, Pto, in the yeast two-hybrid system (Scofield *et al.*, 1996; Tang *et al.*, 1996). *avrPto* has been submitted to extensive mutational analysis resulting in the identification of a core region of the gene product, here in particular of 6 amino acid residues, to be involved in the interaction with Pto (Chang *et al.*, 2001). A strict correlation was observed between AvrPto/Pto interaction in the two-hybrid system and biological activity of the mutant proteins *in planta*.

Pto encodes a serine/threonine-specific protein kinase that specifically phosphorylates a second serine/threonine kinase, Pti1, (Zhou *et al.*, 1995). In addition, Pto interacts with three putative transcription factors, Pti4, Pti5 and Pti6, which bind to the 'PR box' present in the promoter region of a large number of genes encoding pathogenesis-related proteins (Jia and Martin, 1999; Zhou *et al.*, 1997), the DNA binding of Pti4 being enhanced upon specific phosphorylation by Pto (Gu *et al.*, 2000). Therefore, Pto mediates resistance in tomato by a phosphorylation cascade that is triggered by the bacterial AvrPto protein. However, this resistance is dependent on a second host gene, *Prf*, encoding a member of the NBS-LRR class of R proteins (Chang *et al.*, 2000; Oldroyd and Staskawicz, 1998; Salmeron *et al.*, 1994; Salmeron *et al.*, 1996; Tobias *et al.*, 1999). Constitutive expression of *Pto* induced a *Prf*-dependent HR in the absence of *avrPto* (Rathjen *et al.*, 1999). In addition, Pto does not require AvrPto for its interaction with the Pti proteins in the yeast two-hybrid system, or for Pti1 phosphorylation *in vitro* (Zhou *et al.*, 1995). It was therefore speculated that the Pto-initiated phosphorylation cascade may be involved in basic

resistance of tomato against *P. syringae* pv. *tomato*. AvrPto originally might have been a virulence factor targeting the Pto kinase and abolishing the interaction between Pto and the Pti proteins, required for the basal resistance conferred by Pto (van der Biezen and Jones, 1998b). Although the function of Prf in the signaling process is still unknown, in this scenario it is suggested that Prf may have evolved to detect the complex of virulence factor and target molecule. However, only unraveling of the Prf function will allow the development of a model for the specific *avrPto/Pto*-mediated resistance. Nevertheless, these and data from an increasing number of other pathosystems (*cf.* VI.1.) have led to the more general "guard hypothesis" for *R* gene function. This hypothesis describes the inter-relationship between AVR proteins, R proteins, and AVR protein binding proteins (receptors) by assuming that the products of pathogen virulence genes interact with host proteins to promote disease. R proteins detect this association of plant pathogenicity targets with pathogen virulence factors, thereby re-defining them as AVR factors, and initiate the plant defense response (van der Biezen and Jones, 1998b).

Additional evidence for a role of *avrPto* beneficial for the pathogen came from the observation that the gene enhances the virulence of *P. syringae* pv. *tomato* in a strain-dependent manner in tomato plants lacking *Pto* (Chang *et al.*, 2000). The enhanced necrosis correlated with a small increase in bacterial growth. When the virulence activity of a group of AvrPto mutants was examined that carry single amino acid substitutions and lack the AVR activity on tomato plants (Shan *et al.*, 2000a), three mutants were identified that clustered in the center of AvrPto and that exhibited virulence activity in tomato plants with or without *Pto*. These data contrast with results from another study (Chang *et al.*, 2001), although different amino acid alterations were used at the three amino acid positions in question. Nevertheless, the finding may indicate that the AVR function of AvrPto can be structurally separated from the virulence function.

For many bacterial *Avr* genes it is difficult to assess an intrinsic property because they show a pronounced effect on virulence only when present in particular strains or pathovars or on particular host species or cultivars. For example, the virulence effect of *avrPto* was only detected when present in strain T1 of *P. syringae* pv. *tomato* (Chang *et al.*, 2000; Shan *et al.*, 2000a). Likewise, the genes *avrE* and *avrA* from *P. syringae* pv. *tomato* quantitatively contribute to virulence on tomato only in strain PT23 but not in DC3000 (Lorang and Keen, 1995; Lorang *et al.*, 1994). Downstream of *avrE* a second gene, *avrF*, was detected (Bogdanove *et al.*, 1998b). Interestingly, the *avrEF* locus is homologous to the *dspEF* (syn. *dspAB*) locus that is required for pathogenicity of *Erwinia amylovora* (Bogdanove *et al.*, 1998b; Gaudriault *et al.*, 1997). *dspE* and *avrE* encode hydrophilic proteins of 198 kDa and 195 kDa, respectively, with 30 % identity. In contrast, the products of *dspF* and *avrF* are small, acidic proteins of 16 kDa and 14 kDa showing 43 % identity. Both proteins

Table 2: Bacterial *Avr* genes with demonstrated or presumed virulence activity. For members of the *YopJ* family cf. text.

Pathogen	*Gene*	*Avr function*	*Vir function*	*Reference*
P s. pv. *tomato*	*avrPto*	HR in Pto tomato plants that also carry Prf	enhanced growth and necrosis in susceptible tomato lines carrying *Prf*, necrosis upon expression in tomato	Chang *et al*., 2000; Ronald *et al*., 1992; Tobias *et al*., 1999
	avrA	HR elicitor	small reduction in virulence in tomato	Lorang and Keen, 1995; Lorang *et al*., 1994
	avrD	enzyme catalysing synthesis of HR-eliciting syringolides in RPG4 soybean	highly conserved non-functional alleles in virulent pathogen races	Kobayashi *et al*., 1990
	avrE	HR elicitor in soybean and tobacco, functional homolog of dspE	*avrE* mutant shows greatly decreased virulence, restores pathogenicity of *dspE*-deficient *E. amylovora*, necrosis upon expression in *A. thaliana*	Kjemtrup *et al*., 2000; Lorang and Keen, 1995; Lorang *et al*., 1994
	avrF		functional homolog of *dspF*	Bogdanove *et al*., 1998b
	avrRpt2	HR elicitor in RPS2 soybean and RPS2 *A. thaliana*	growth promotion in *A. thaliana* lacking *RPS2*, more severe disease symptoms	Bent *et al*., 1992; Chen *et al*., 2000; McNellis *et al*., 1998; Whalen *et al*., 1991
P.s. pv. glycinea	*avrA*	HR elicitor in RPG2 soybean		Napoli and Staskawicz, 1987
	avrB	HR elicitor in RPG1 soybean and RPM1 *A. thaliana*	chlorosis and browning upon expression in *A. thaliana*	Kjemtrup *et al*., 2000; McNellis *et al*., 1998; Staskawicz *et al*., 1987; Tamaki *et al*., 1988; Wanner *et al*., 1993
	avrC	HR elicitor in RPG3 soybean		Staskawicz *et al*., 1987; Tamaki *et al*., 1988
P.s. pv. maculicola	*avrRpm1*	HR elicitor in RPM1 *A. thaliana* and RPG1 soybean	neither growth nor generation of disease symptoms by avrRpm1 mutants on *A. thaliana*	Ritter and Dangl, 1995
P.s. pv. phaseolicola	*avrPphB*	HR elicitor in R3 bean and	weak browning upon expression in bean	Puri *et al*., 1997;

		RPS5 *A. thaliana*		Stevens *et al*., 1998
	avrPphC	HR elicitor in soybean (avrC homolog)		Yucel *et al*., 1994
	avrPphE	HR elicitor in R2 bean	highly conserved non-functional alleles in virulent pathogen races, weak browning upon expression in bean	Mansfield *et al*., 1994; Stevens *et al*., 1998
	avrPphF	HR elicitor in R1 bean	confers virulence in bean and soybean	Jackson *et al*., 1999; Tsiamis *et al*., 2000
	virPphA	HR elicitor in soybean	quantitative contribution to virulence in bean	Jackson *et al*., 1999
P.s. pv. pisi	*avrPpiA*	HR in R2 pea	loss of pathogenicity towards susceptible *A. thaliana* ecotypes	Vivian *et al*., 1989
P.s. pv. syringae	*hopPsyA* (syn. *hrmA)*	HR in tobacco	part of a pathogenicity island	Alfano *et al*., 1997; Heu and Hutcheson, 1993; van Dijk *et al*., 1999
Erwinia amylowora	*dspEF* (syn. *dspAB)*	quantitative contribution to HR elicitation in tobacco, converts *P. syringae* pv. *glycinea* to avirulence in soybean	required for pathogenicity on pea, apple, cotoneaster	Bogdanove *et al*., 1998a; Gaudriault *et al*., 1997
X.c. pv. vesicatoria	*avrBs1*	HR in Bs1 pepper	non-functional allele improves bacterial survival in the soil	O'Garro *et al*., 1997; Ronald and Staskawicz, 1988
	avrBs2	HR in Bs2 pepper	strains lacking the gene show reduced virulence	Kearney and Staskawicz, 1990
	avrRxv	HR in Rxv bean and in tomato	member of the YopJ gene family	Ciesiolka *et al*., 1999; Whalen *et al*., 1993
	avrXv3	HR in Xv3 tomato	member of the YopJ gene family	Astua-Monge *et al*., 2000a
	avrXv4	HR in Xv4 tomato	member of the YopJ gene family	Astua-Monge *et al*., 2000b
	avrBsT	HR in pepper	member of the YopJ gene family	Ciesiolka *et al*., 1999; Minsavage *et al*., 1990

have predicted C-terminal amphipathic α-helices and resemble the Syc proteins that function as chaperones for virulence factors secreted by type III secretion systems of animal pathogens (Wattiau *et al.*, 1996). The *dspEF* locus, like *avrE*, conferred avirulence on soybean when expressed in *P. syringae* pv. *glycinea*. Similarly, *avrE* restored pathogenicity of *E. amylovora dspE* mutants on pear, although virulence was lower as compared to wild-type bacteria. Therefore, *dspEF* and *avrE* have similar dual functions and can operate transgenerically (Bogdanove *et al.*, 1998b).

avrRpm1 from *P. syringae* pv. *maculicola* and *avrPpiA* from *P. syringae* pv. *pisi* encode proteins with 97 % identity (Dangl *et al.*, 1992). However, while *avrRpm1* is required for full bacterial virulence on susceptible *A. thaliana* (Ritter and Dangl, 1995), no such function could be demonstrated for *avrPpiA* on pea (Gibbon *et al.*, 1997). Both homologous AVR proteins as well as the sequence-unrelated product of the *avrB* gene determine resistance of *A. thaliana* to *P. syringae* in the presence of the *R* gene, *RPM1* (Grant *et al.*, 1995). The product of this *R* gene is a member of the CC-NBS-LRR class of R proteins and has been localized to the inner cytoplasmic membrane. Due to the lack of a transmembrane segment or of other motifs for membrane attachment, its association with the plasma membrane may be based on the interaction with an unidentified integral membrane protein (Boyes *et al.*, 1998). A possible candidate for such a membrane-anchoring protein is the product of the *NDR1* gene encoding a small protein with two putative transmembrane regions (Century *et al.*, 1997) that is required for the function of *RPM1* and other *R* genes in *A. thaliana* (Century *et al.*, 1995). An additional candidate protein that is characterized by several transmembrane segments was identified when the N-terminal portion of RPM1 was used as bait in the yeast two-hybrid system (Boyes *et al.*, 1998).

Together with AvrB, AvrC, AvrPto and a proteolytic cleavage product of AvrPphB, the AvrRpm1 protein belongs to a subset of AVR proteins containing N-terminal fatty acid acylation motifs (myristoylation and palmitoylation sites). These covalent modifications that occur on a wide variety of cellular signaling proteins such as protein kinases, G proteins and transmembrane receptors have been shown to promote plasma membrane targeting and binding (Resh, 1999) and to influence protein-protein interactions and cellular signal transduction (Johnson *et al.*, 1994; Resh, 1996). For AvrRpm1 and AvrB the consensus myristoylation sites were shown to be required for maximal function in virulence and avirulence (Nimchuk *et al.*, 2000). In addition, an epitope-tagged RPM1 that recognizes both AVR proteins was found to be a peripheral membrane protein that likely resides on the cytoplasmic face of the plasma membrane (Boyes *et al.*, 1998). For AvrPto it could be demonstrated that mutating the myristoylation site abolishes its avirulence activity (Shan *et al.*, 2000b). Interestingly, the corresponding R protein Pto also contains a myristoylation site that has been suggested to play a role in signaling (Martin *et al.*, 1993). In contrast to AvrPto and Pto, however, a direct interaction between AvrRpm1 or AvrB and

RPM1 has not been demonstrated. Nevertheless, it is tempting to speculate that these AVR signals are recognized by plasma membrane-anchored receptor complexes. The targeting of the AVR proteins to this cellular site suggests that the virulence function of these proteins is also associated with the membrane, possibly through interference with receptor-linked signaling pathways. However, the relatively weak cytotoxic effects observed upon expression of *avrB* (Gopalan *et al.*, 1996) and *avrRpt2* (McNellis *et al.*, 1998) in plants lacking the respective *R* genes, was interpreted as the AVR proteins having additional targets within the plant cells.

The *avrRpt2* gene from *P. syringae* pv. *tomato* was used in another study to analyze its effect on bacterial virulence (Chen *et al.*, 2000). Strain DC3000 expressing *avrRpt2* grew to significantly higher levels and often resulted in the formation of more severe disease symptoms on several *A. thaliana* lines lacking the cognate *R* gene, *RPS2*, as well as on two *A. thaliana* mutant lines exhibiting enhanced resistance. Bacterial strains expressing *avrRpt2* (or *avrRpm1*) from a single genomic copy showed stronger avirulence and virulence effects as compared to strains with plasmid-borne genes (Guttman and Greenberg, 2001). Interestingly, the virulence effect of AvrRpt2 on *A. thaliana* plants was dramatically increased in the presence of the *R* gene, *RPM1*, whereas no effect was observed in *RPS4* or *RPS5* plants. In transgenic *RPM1* plants lacking the *RPS2* gene expression of *avrRpt2* appeared to completely inhibit both *RPM1*-mediated HR and restriction of pathogen growth. In addition, bacteria carrying *avrRpt4* or *avrPphB* showed an enhanced growth in transgenic plants expressing *avrRpt2*. Therefore, two different mechanisms were hypothesized for virulence promotion by *avrRpt2*. The first one is interference with the resistance mediated specifically by RPM1 resulting in complete inhibition of resistance. In contrast, the second mechanism resulting in increased bacterial growth may target a more common component of the plant defense response.

In bacterial pathogens of animals *Vir* genes are frequently part of gene clusters termed pathogenicity islands (PAI; (Hacker *et al.*, 1997). PAIs occupy up to 200 kb of genomic DNA, but have also been found on plasmids (Hu *et al.*, 1998). They are selectively present in pathogenic strains, are bordered by tRNA genes and contain DNA sequences such as flanking direct repeats, insertion sequences and transposases indicative of gene mobility. In addition, they often differ in their G+C content from the rest of the genome suggesting that they were acquired through horizontal gene transfer. In phytopathogenic bacteria, PAIs were detected in *P. syringae* pvs. *syringae* and *tomato* (Alfano *et al.*, 2000). Several *Avr* genes (*avrE*; Lorang and Keen, 1995, *avrPphE*; Mansfield *et al.*, 1994, *hopPsyA* [syn. *HrmA*]; Heu and Hutcheson, 1993; van Dijk *et al.*, 1999) have been shown to be part to these gene clusters. Other *P. syringae Avr* genes are located elsewhere in the genome or on plasmids (Leach and White, 1996). In the bean pathogen *P. syringae* pv. *phaseolicola*, a PAI with three putative virulence genes is located on a 154-kb plasmid that also contains previously

identified *Avr* genes (*avrD*; Kobayashi *et al.*, 1990, *avrPphC*; Yucel *et al.*, 1994b, *avrPphF*; Jackson *et al.*, 1999). Plasmid-cured bacterial strains lost virulence towards bean and, instead, caused HR in previously susceptible cultivars suggesting the presence of "masked" *Avr* genes on the chromosome or other plasmids. These genes that can only be detected in the absence of the PAI were designated β *Avr* genes. Other *Avr* genes, such as *avrPphB* or *avrPphE*, that continued to function in the presence of the PAI were classified as α *Avr* genes (Jackson *et al.*, 1999).

One of the *Vir* genes in the PAI, *virPphA*, was isolated and shown to partially restore virulence towards bean of the plasmid-cured strains (Jackson *et al.*, 1999). This gene provides an excellent example for a link between *Avr* and *Vir* gene function because its product was demonstrated to act as an HR activator in soybean (Jackson *et al.*, 1999). From additional analysis of the *Avr* genes *avrPphF* and *avrPphC* an even more complex pattern emerged (Tsiamis *et al.*, 2000). *avrPphF* was identified as an α *Avr* gene that is organized into an operon with two open reading frames (ORF). In contrast to the two-ORF operon of *avrBs1* from *X. campestris* pv. *vesicatoria* where only the second ORF is required for avirulence (Ronald and Staskawicz, 1988), both *avrPphF* ORFs are needed for function (Tsiamis *et al.*, 2000). However, *avrPphF* was also found to confer specific virulence on a bean cultivar and, in the absence of the PAI, acted as a β *Avr* gene in a cultivar that is susceptible to all known bacterial races. The gene that masks this *Avr* activity turned out to be *avrPphC*, which had previously been found to act as an *Avr* gene in soybean cultivars (Yucel *et al.*, 1994b). This prompts the question as to how *Vir* genes may act to block the phenotypic expression of *Avr* genes that function only after the loss of the PAI (Jackson *et al.*, 1999).

The different *avrD* alleles isolated from *P. syringae* pathovars exemplify a different type of interaction between products of an *Avr* and an *R* gene. The *avrD* gene that was first cloned from *P. syringae* pv. *tomato* encodes an enzyme that directs the synthesis of C-glycosyl lipids, named syringolides, in Gram-negative bacteria. These compounds but not the *Avr* gene product itself, function as HR elicitors in soybean cultivars carrying the complementary *R* gene, *Rpg4* (Keen *et al.*, 1990; Kobayashi *et al.*, 1990; Midland *et al.*, 1993). A 34-kDa protein with syringolide binding activity that does not contain typical R protein domains was identified in soybean cultivars carrying or lacking the *Rpg4* gene (Ji *et al.*, 1998). This suggests that the initial signal perception may not occur through the product of the *R* gene. Two classes of *avrD* alleles were defined that differ not only in sequence homology but also in function, directing the synthesis of different elicitors of the soybean HR. The originally cloned *avrD* gene along with a highly homologous (95 % identity) *avrD* allele identified in *P. syringae* pv. *lachrymans* were combined in class I (Yucel *et al.*, 1994a). Other functional *avrD* alleles from *P. syringae* pvs. *lachrymans* and *phaseolicola* (Keith *et al.*, 1997) and several non-functional alleles from different races of *P. syringae* pv. *glycinea* (Keith *et al.*, 1997; Kobayashi *et al.*, 1990) were

grouped into class II (Yucel *et al.*, 1994a). A function of *avrD* beneficial for the bacteria has not been shown yet. It is tempting to speculate however that the presence of highly conserved non-functional *avrD* alleles in multiple races of *P. syringae* pv. *glycinea* that differ from functional alleles only in a very limited number of mutations indicate an abolishment of the avirulence function, but the maintenance of an independent unidentified beneficial function. Interestingly, the syringolides are structurally similar to signal molecules from other microbes as well as from plants (Leach and White, 1996). This suggests that products of the *avrD*-involving bacterial secondary biosynthetic pathway(s) are modulators of as yet unknown host cellular functions.

3. Dual function AVR proteins from the genus *Xanthomonas*

The first evidence that *Avr* genes encode additional biochemical activities or disease-promoting functions was reported for *avrBs2* from *Xanthomonas campestris* pv. *vesicatoria* (Kearney and Staskawicz, 1990). This gene controls resistance in the host plant, pepper (*Capsicum annuum*), carrying the *R* gene *Bs2* that encodes a member of the CC-NBS-LRR-class of R proteins (Tai *et al.*, 1999). However, spontaneous and induced mutations in the *avrBs2* gene substantially reduced bacterial growth on susceptible plants in comparison with the wild-type strain (Kearney and Staskawicz, 1990; Swords *et al.*, 1996). The *avrBs2* gene encodes an 80-kDa protein with homology to the agrocinopine synthase from *A. tumefaciens* and to the glycerophosphoryl diester phosphodiesterase (UgpQ) from *E. coli*. Both enzymes are involved in the synthesis or hydrolysis of phosphodiester linkages suggesting a possible enzymatic function as a phosphodiesterase for the AvrBs2 protein (Swords *et al.*, 1996). A homologous gene encoding a protein with 84 % similarity was identified in the *Brassica* pathogen *X. campestris* pv. *campestris*. This gene restored *Bs2*-specific resistance and enhanced virulence to *avrBs2* mutants of *X. campestris* pv. *vesicatoria* demonstrating functional conservation (Swords *et al.*, 1996). In addition, sequences related to *avrBs2* occur in all strains of *X. campestris* pv. *vesicatoria* (Minsavage *et al.*, 1990) and in many other pathovars of *X. campestris* (Kearney and Staskawicz, 1990) and *X. oryzae* (Mazzola *et al.*, 1994). This widespread occurrence of *avrBs2* suggests a highly conserved role of the gene in the virulence of *X. campestris*. Furthermore, since the *R* gene *Bs2* has been shown to also be functional upon transfer into other solanaceous species (Tai *et al.*, 1999), the *avrBs2*-based resistance may be exploited to engineer more durable resistance in this group of plants. A large family of homologous *Avr* genes was identified in *Xanthomonas* spp. (Leach and White, 1996; Vivian and Gibbon, 1997) and named after its first member, *avrBs3*, from *X. campestris* pv. *vesicatoria* (Bonas *et al.*, 1989). Although most members of this family were identified based on their avirulence function, several

Table 3: **The *Avr*/*Pth* gene family from *Xanthomonas***

Pathogen	*Gene*	*Avr function*	*Reference*
X.c. pv. *vesicatoria*	*avrBs3*	in Bs3 pepper	Bonas *et al*., 1989; Herbers *et al*., 1992
	avrBs4 (syn. *avrBs3-2*, *avrBsP*)	in Bs4 tomato	Ballvora *et al*., 2001; Bonas *et al*., 1993; Canteros *et al*., 1991
X.c. pv. *malvacearum*	*avrBn*	in cotton	Gabriel *et al*., 1986
	avrB4	in B1 and B4 cotton	De Feyter and Gabriel, 1991
	avrb6	in B1 cotton	De Feyter and Gabriel, 1991
	avrb7	in cotton	De Feyter and Gabriel, 1991
	avrBIn	in cotton	De Feyter and Gabriel, 1991
	avrB101	in cotton	De Feyter and Gabriel, 1991
	avrB102	in B1 cotton	De Feyter and Gabriel, 1991
	avrB103	in cotton	Yang *et al*., 1996
	avrB104	in cotton	Yang *et al*., 1996
	avrB5	in cotton	Yang *et al*., 1996
	pthN		Chakrabarty *et al*., 1997
	pthN2		Chakrabarty *et al*., 1997
X.c. pv. *aurantifolii*	*pthB*		Gabriel *et al*., 1996
	pthB2		Gabriel *et al*., 1996
	pthC		Gabriel *et al*., 1996
	pthC2		Gabriel *et al*., 1996
X. oryzae	*avrxa5*	in xa-5 rice	Hopkins *et al*., 1992
	avrXa7	in Xa-7 rice	Hopkins *et al*., 1992
	avrXa10	in Xa-10 rice	Hopkins *et al*., 1992
X. citri	*pthA*	in bean and cotton when present in *Xc* pvs. *phaseoli* and *malvacearum*	Gabriel *et al*., 1986
	avrXc1		Gabriel *et al*., 1986
	avrXc2		Gabriel *et al*., 1986
	avrXc3		Gabriel *et al*., 1986
X. phaseoli	*avrXp1*		Gabriel *et al*., 1996

genes encode pathogenicity/virulence factors (Tab. 3). The gene products are highly identical (> 90 %), their key feature being a central, highly conserved 34-amino acid repeat domain (102-bp repeats). This domain that comprises varying numbers of repeats in the different proteins specifies resistance on the host plants (Ballvora *et al.*, 2001; Bonas *et al.*, 1993; Bonas *et al.*, 1989; Canteros *et al.*, 1991; De Feyter *et al.*, 1993; Gabriel *et al.*, 1986; Hopkins *et al.*, 1992; Swarup *et al.*, 1991; Yang and Gabriel, 1995b). Replacement of the repeat-coding region in *avrXa10* with the corresponding region of *avrXa7* resulted in gain of avirulence toward the *R* gene *Xa7*

and loss of activity toward *Xa10* (Zhu *et al.*, 1998). Exchange of the repeat domains of *avrb6* and *pthA* demonstrated that the repeat domain also controls virulence specificity on the respective hosts (Yang *et al.*, 1994). This role in virulence was also shown when an *avrXa7* construct could not only restore the avirulence specificity of an *avrXa7* mutant strains of *X. oryzae* pv. *oryzae* for *Xa7*, but also re-established full virulence on susceptible rice (Yang *et al.*, 2000). Deletion of repeat units generates new avirulence specificities, but deletion variants of the same length have also different specificities (Herbers *et al.*, 1992). This suggests that position and sequence of the repeat units and not overall length are critical for determining avirulence and virulence.

In the C-terminal regions of the predicted proteins of the AvrBs3-type heptad repeats similar to leucine zippers and three putative nuclear localization signals (NLSs) were identified (van den Ackerveken *et al.*, 1996; Yang and Gabriel, 1995a; Zhu *et al.*, 1998). The coding sequences for the C-terminal regions of two members of the gene family, *avrb6* and *pthA*, were fused to a β-glucuronidase (GUS) reporter gene. When introduced into onion cells, both translational fusions were transiently expressed, and GUS activity was specifically found in the nuclei of transformed cells indicating the functionality of the NLSs (Yang and Gabriel, 1995a). Mutations in all three NLS sequences of *avrXa10* caused a loss in avirulence and virulence activities on rice (Yang *et al.*, 2000; Zhu *et al.*, 1998). Both were restored upon addition of the NLS motif from SV40 T-antigen (Yang *et al.*, 2000).

Additional support for a putative intra-nuclear function of the AvrBs3-type proteins came from the identification of structural similarity of the C-terminus of AvrXa10 to the acidic activation domain of many eukaryotic transcription factors (Zhu *et al.*, 1998). Deletion of the putative activation domain in AvrXa10, AvrBs3 and AvrXa7 without removal of the NLSs resulted in the loss of avirulence activity. In addition, the corresponding region of AvrXa10 can replace the C-terminal coding regions of AvrBs3 and AvrXa7, and the genes retained specificity for the resistance genes *Bs3* in pepper and *Xa7* in rice, respectively. The transcriptional activation potential of AvrXa10 was demonstrated using fusions of the protein to the Gal4 DNA-binding domain. The hybrid protein was capable of activating transcription of reporter genes in yeast and *A. thaliana*. In contrast, removal of the carboxyl region severely reduced transcriptional activation (Zhu *et al.*, 1998). Using an amino acid replacement strategy, mutants were identified that are defective both for transcriptional activation in yeast and avirulence activity in rice. In addition, the activation domain from the herpes virus protein VP16 could replace the endogenous activation domain of AvrXa10; the hybrid protein elicited a resistance reaction specifically in the presence of *Xa10* (Zhu *et al.*, 1999). The activation domain was also required for virulence activity. Here, however, the VP16 domain could not substitute for the endogenous domain indicating that virulence and avirulence have different cellular requirements.

Furthermore, in gel shift assays AvrXa7 bound to double-stranded DNA (Yang *et al.*, 2000). These results indicate that products of the *avrBs3* gene family are virulence factors that are targeted to host cell nuclei. Their mode of action appears to reside in an interaction with the host DNA and a modulation of the host transcriptional machinery. However, the NLSs of AvrBs4 are not required for HR elicitation in the tomato host suggesting that it is recognized before reaching the nucleus and, hence, two different pathways are mediating HR induction and virulence (Ballvora *et al.*, 2001).

Although most members of this gene family were identified as *Avr* genes, some were isolated based on their function in virulence or pathogenicity. In particular, the gene *pthA* from *X. citri* was identified in a 'virulence enhancement' approach (Swarup *et al.*, 1991). Upon transformation with *pthA*, strains of *X. campestris* that are normally only weakly virulent on citrus developed the typical citrus canker symptoms including the rupturing of epidermal layers and the release of bacteria for dispersal. The increase of bacterial numbers on the leaf surface that was also seen with the *avrb6* gene from *X. campestris* pv. *malvacearum* (Yang *et al.*, 1994) is thought to contribute to a more efficient dissemination of bacteria. Transient expression of the *pthA* gene in citrus leaves also induced canker symptoms (Duan *et al.*, 1999). In contrast, no such phenotype was observed when the 97 % identical *avrb6* gene replaced the *pthA* gene (Yang and Gabriel, 1995b) suggesting host specificity of the virulence effect. When *pthA* was expressed in tobacco, bean, and cotton an HR was induced (Duan *et al.*, 1999). The latter response also occurred when *pthA* was transferred into bean- or cotton-specific xanthomonads upon inoculation of the respective hosts (Swarup *et al.*, 1992). This demonstrates that *pthA* encodes a citrus-specific virulence factor that functions as an AVR factor in plants other than citrus. The observation that protein extracts containing PthA elicited no disease symptoms when inoculated onto plants, the requirement of a functional type-III secretion system, and the nuclear targeting of the protein suggest that the virulence function is also mediated through affecting the transcriptional machinery of the host plant (Yang and Gabriel, 1995a).

From *X. campestris* pv. *vesicatoria* four other *Avr* genes, *avrRxv* (Whalen *et al.*, 1993), *avrBsT* (Minsavage *et al.*, 1990), *avrXv3* (Astua-Monge *et al.*, 2000b) and avrXv4 (Astua-Monge *et al.*, 2000a), were isolated that do not belong to the *avrBs3* family. However, the presence of putative NLS sequences suggests their activity to also target the host cell nucleus. The amino acid sequences of the gene products show high similarity (Astua-Monge *et al.*, 2000b; Ciesiolka *et al.*, 1999). Furthermore, their sequences are similar to the virulence factors YopJ and YopP from the mammalian pathogen *Yersinia enterocolitica*, to the YopJ homologue AvrAS with unknown function from *Salmonella typhimurium* and to the ORF called Y4LO of unknown function from plant symbiontic *Rhizobium* sp. Members of the YopJ family were

shown to be cysteine proteases that act on highly conserved ubiquitin-like substrates covalently added to numerous regulatory proteins (Orth *et al.*, 2000). Disrupting this posttranslational modification results in the induction of programmed cell death in animal cells. Macrophage apoptosis early in the infection process is advantageous to establishment of *Yersinia* and *Salmonella* infection (Mills *et al.*, 1997; Monack *et al.*, 1997; Monack *et al.*, 1996). In contrast, HR in plants is clearly associated with plant resistance. Nevertheless, it is tempting to speculate that pathogenic bacteria use similar strategies to elicit programmed cell death in both plant and animal hosts (Dangl *et al.*, 1996; Mills *et al.*, 1997).

4. Harpins

Harpins constitute another group of bacterial effector proteins that elicit a number of defense-associated reactions including the HR in plants. They are heat stable, rich in glycine and/or serine, lack cysteine, and differ in their primary sequences. They lack an N-terminal signal peptide and are secreted via the type III apparatus, presumably into the extracellular compartment, not into the host cytoplasm. The first of these proteins was identified in *Erwinia carotorova*, the causal agent of the devastating fire-blight disease on many rosaceous plants, such as apple and pear (Wei *et al.*, 1992). $HrpN_{Ea}$, the structural gene encoding $harpin_{Ea}$, is located within the *hrp* gene cluster. A homologous gene, $hrpN_{Ech}$, encoding the structurally similar $harpin_{Ech}$ was identified in the soft-rot pathogen *E. chrysanthemi* (Bauer *et al.*, 1995). *E. carotorova* $hrpN_{Ea}$ mutants were non-pathogenic to pear (Wei *et al.*, 1992) or showed a substantially reduced phenotype on host plants (Barny, 1995). Similarly, *E. chrysanthemi* $HrpN_{Ech}$ mutants displayed a reduced ability to incite infections in witloof chicory leaves, suggesting that the products of both genes have an important role in bacterial pathogenicity or virulence.

The harpins, PopA1 and its degradation derivative PopA3, encoded by the *popA* gene in the tomato pathogen *Ralstonia solanacearum* are structurally dissimilar to the *Erwinia* spp. *hrpN* products (Arlat *et al.*, 1994). In addition, a *popA* mutant remained fully pathogenic on sensitive plants, indicating that this gene is not essential for pathogenicity. The isolated *popA* products elicited the HR specifically in those *Petunia* lines that were resistant to bacterial infection, suggesting that these harpins may be host specificity determinants.

In contrast to the *hrpN* and *popA* genes that are located near their respective *hrp* gene clusters, the harpin genes of *Pseudomonas syringae* pvs. *syringae* ($hrpZ_{Pss}$), *glycinea* ($hrpZ_{Psg}$) and *tomato* ($hrpZ_{Pst}$) reside within *hrp* operons (He *et al.*, 1993; Preston *et al.*, 1995). The gene products show sequence similarities between 63 and 79 %. Interestingly, the $HrpZ_{Pst}$ gene contains an insertion encoding a 24-amino acid, glycine-rich stretch with homology to a sequence in PopA1 (Preston *et al.*, 1995). The

host range of *P. solanacearum* and *P.s*. pv. *tomato* overlap and it is therefore tempting to speculate that this region, presumably obtained through horizontal gene transfer, has some significance for bacterial pathogenesis on tomato. Although the role of the HrpZ proteins in compatible interactions remains unclear, it was recently demonstrated that the product of the $hrpZ_{Psph}$ gene from *P. syringae* pv. *phaseolicola* binds to a non-proteinaceous binding site in tobacco plasma membranes (Lee *et al.*, 2001a) and stably associates to synthetic lipid bilayers (Lee *et al.*, 2001b). Furthermore, the $HrpZ_{Psph}$ protein and its homologues from *P. syringae* pvs. *syringae* and *tomato* triggered ion currents of similar extent. The ion-conducting pores were permeable for cations, but not for Cl^-. Such pore-forming activity may indicate a role of the HrpZ protein in nutrient release and/or delivery of virulence factors during bacterial pathogenesis (Lee *et al.*, 2001b).

In *E. amylovora* and *P. syringae* pv. *tomato* a second harpin was identified that is encoded by the *hrpW* gene (Charkowski *et al.*, 1998; Kim and Beer, 1998). The HrpW proteins are composed of two domains, an N-terminal harpin-like domain with sequence similarity to HrpN, HrpZ, and PopA and a C-terminal domain homologous to pectate lyases from plant pathogenic fungi and from *E. carotorova* (Kim and Beer, 1998). In the presence of Ca^{2+} HrpW was shown to bind to pectate. However, no enzyme activity could be detected and the pectate lyase domain is not required for HR elicitation (Charkowski *et al.*, 1998). Nevertheless, these findings support the notion that this type of harpin has a site of action in the plant cell walls (Hoyos *et al.*, 1996), possibly helping the Hrp pilus (Roine *et al.*, 1997) to pass through.

VI. FUNGAL ELICITORS

In comparison to bacterial *Avr* genes the number of characterized *Avr* genes from phytopathogenic fungi has remained low. This is mainly due to the substantially larger sizes of fungal genomes that prevent simple methods such as shotgun cloning in bacteria. In addition, many important fungal pathogens are strict biotrophs that are unable to grow outside their host plants and, thus, are not easily amenable to molecular genetic approaches. During recent years, mutation strategies were applied to different fungal pathogens that have been successful in identifying several *Pth* genes, but not *Avr* genes. An alternative would be positional cloning of *Avr* genes. However, many important plant pathogens lack a known sexual stage, thus preventing such kind of genetic approach. In contrast to viruses and bacteria, therefore, the products of putative *Avr* genes were mostly isolated prior to gene cloning and proof of function as elicitors of plant resistance reactions.

Fungi growing extracellularly can be assumed to secrete proteins that are involved in killing plant cells to gain access to the host nutrient supply or in

modulating the host metabolism in favor of the pathogen. Consequently, these proteins were the first targets to identify AVR factors from the tomato leaf mould fungus, *Cladosporium fulvum*, and from the barley leaf scald fungus, *Rhynchosporium secalis*. In contrast, from the rice blast fungus *Magnaporthe grisea* that is accessible to genetic analysis, positional cloning strategies allowed the isolation of several genes involved in determining/restricting the fungal host range. Some of these genes act at the species level, whereas recently a race-specific *Avr* gene was cloned.

1. Fungal AVR proteins: host cultivar-specific elicitors

The interaction of *C. fulvum* with its host, tomato, was the first fungal pathosystem shown at the molecular level to comply with the gene-for-gene hypothesis (de Wit, 1992). In apoplastic fluids from infected susceptible host lines the fungal cultivar-specific peptide elicitor, AVR9, was identified that triggers the HR on host plants expressing the *R* gene *Cf-9* (Scholtens-Toma and de Wit, 1988). The *Avr9* gene encodes a pre-pro-protein of 63 amino acids, including a signal peptide characteristic for extracellular targeting (van den Ackerveken *et al.*, 1992). Upon secretion, a 40-amino acid protein is further processed by the action of fungal and plant proteases to yield the mature 28-amino acid AVR9 peptide (van den Ackerveken *et al.*, 1993b). For the subcellular localization of the corresponding R protein, CF9, conflicting results were presented. Functional, c-myc-tagged CF9 was found to be plasma-membrane localized and highly glycosylated (Piedras *et al.*, 2000). In contrast, a C-terminal dilysine motif (KKRY) suggested CF9 to reside in the ER. Indeed, fusions between GFP and the CF9 transmembrane domain were targeted to this membrane system in yeast, *A. thaliana* and tobacco cells (Benghezal *et al.*, 2000). Recently, however, it was shown that an AARY mutant of CF9 is still functional in mediating AVR9 recognition indicating that other proteins may mask the dilysine motif of CF9 (van der Hoorn *et al.*, 2001).

Injection of the purified AVR9 peptide into the apoplast of *Cf9*-expressing tomato plants or potato virus X-based expression of the AVR9 cDNA (Laugé *et al.*, 2000) is sufficient to cause the HR demonstrating that this AVR factor acts at the extracellular level. This was further confirmed by identifying a high-affinity AVR9-binding site on tomato plasma membranes. However, the binding site is also present on membranes of susceptible plants as well as of other solanaceous species (Kooman-Gersmann *et al.*, 1996). In addition, binding studies using CF9-producing COS and insect cells, membrane preparations from such cells failed to detect specific binding. Furthermore, experiments employing surface plasmon resonance and surface-enhanced laser desorption and ionization for studying the binding of AVR9 to solubilized microsomal proteins from a transgenic, c-myc-CF9-expressing tobacco line did not provide any evidence for specific binding (Luderer *et al.*, 2001). This

suggests that the product of the *Cf9* gene alone is not sufficient for AVR9 perception, and elicitor binding is not sufficient for HR induction. Different models have been proposed to explain signal perception and transduction in the AVR9/CF9-mediated HR induction (Joosten and de Wit, 1999). AVR9 may bind to the high-affinity binding site enabling this complex to interact with CF9, which then triggers the HR. Alternatively, an unidentified low-affinity binding site is assumed to play a role in resistance, whereas the identified high-affinity binding site is involved in processes facilitating virulence. The low-affinity binding site may be the CF9 protein itself that upon AVR9-binding, due to the very short cytoplasmic tail of the R protein, needs to interact with a transmembrane protein for signal transduction. Finally, in analogy to the mechanisms by which CLAVATA proteins regulate developmental processes in *A. thaliana*, in a third model an unknown transmembrane receptor-like protein kinase is the low-affinity binding site that upon AVR9-binding is activated by CF9. All models have in common that a trimeric protein complex involving the product of the *Cf9* gene is formed at the host membrane that contains a domain activating a cytoplasmic signaling pathway.

The *Avr9* gene is identical in all analyzed fungal strains that cause resistance of *Cf-9* plants, but absent from virulent strains (van Kan *et al.*, 1991). The gene does not share any significant homology with gene sequences present in the databases. However, ^{1}H-NMR analysis of the protein and biochemical assignment of the disulfide bonds revealed its structural similarity with members of the cystine knot protein family (Vervoort *et al.*, 1997). This family consists of small, cysteine-rich proteins such as serine protease inhibitors, ion channel blockers and growth factors (Isaacs, 1995). AVR9 having a compact β-sheet region of three antiparallel strands and two solvent-exposed loops is most related to a member of the inhibitor cystine knot subgroup, potato carboxypeptidase inhibitor (Joosten and de Wit, 1999; Rees and Lipscomb, 1982; Vervoort *et al.*, 1997), suggesting an inhibitory function of AVR9. Mutational and functional analysis using the PVX expression system (Chapman *et al.*, 1992) as well as chemically synthesized mutant proteins (Mahé *et al.*, 1998) indicated the hydrophobic β-loop region of the AVR9 peptide to be crucial for necrosis-inducing activity (Kooman-Gersmann *et al.*, 1997) as well as for membrane binding (Kooman-Gersmann *et al.*, 1998).

All *C. fulvum* races virulent on *Cf9* plants lack the entire *Avr9* gene (van Kan *et al.*, 1991). In addition, *Avr9* disruption mutants were not affected in their growth pattern *in vitro* or on *Cf0* plants (Marmeisse *et al.*, 1993). This suggests that, at least under laboratory conditions, the pathogen does not require AVR9 for pathogenicity and full virulence. Since expression of the *Avr9* gene is regulated by the nitrogen response factor NRF1 (Perez-Garcia *et al.*, 2001) a putative involvement in the nitrogen metabolism of the fungus has been proposed (van den Ackerveken *et al.*,

1994). However, the intrinsic function of the AVR9 protein remains yet to be determined.

The *Avr4* gene from *C. fulvum* causing HR on tomato plants that carry the *Cf-4* gene encodes a pre-pro-protein of 135 amino acids (Joosten *et al.*, 1994). Cleavage of a secretory signal sequence yields a mature elicitor protein of 86-88 residues (Joosten *et al.*, 1997). In contrast to the *Avr9* situation, all fungal strains virulent on *Cf-4* plants contain an *Avr4* allele. These inactive *avr4* alleles differ from the *Avr4* gene by single point mutations that are assumed to affect the biochemical properties of the gene products such that their stability *in planta* is drastically reduced (Joosten *et al.*, 1994; Joosten *et al.*, 1997). Interestingly, cysteines frequently form a target for mutations, thereby disrupting one of the four disulfide bridges of AVR4. It was suggested that these mutations allow plant or fungal proteases to degrade the AVR4 isoforms, and thereby preventing recognition by plant factors (Joosten *et al.*, 1997). On *Cf-0* tomato plants, however, no difference in virulence between *C. fulvum* races with *Avr4* or *avr4* was observed, indicating that AVR4 is dispensable for virulence. Like AVR9, AVR4 does not share any significant homology to sequences in the databases. Current studies to elucidate the three-dimensional structure of AVR4 (H. van den Burg, J. Vervoort, personal communication) may however provide an idea of the putative intrinsic function of this protein.

In contrast to AVR9 and AVR4, two other extracellular proteins, ECP1 and ECP2, have been shown to affect fungal virulence (Laugé *et al.*, 1997; van den Ackerveken *et al.*, 1993a). Both proteins, although also relatively cysteine-rich, do not share sequence homology with the *C. fulvum* AVR proteins. The *Ecp1* gene encodes a precursor protein of 96 amino acids that is processed to result in a 65-amino acid mature protein. The *Ecp2* gene encodes a 165-amino acid protein that upon processing yields a 142-amino mature acid protein. Both *Ecp* genes are present in all tested fungal strains and are highly expressed in fungal mycelia growing inside the host resulting in abundance of the proteins in apoplastic fluids from infected tomato leaves (Joosten and de Wit, 1988; Wubben *et al.*, 1994). This *in planta* expression may indicate that plant factors are required for the induction of *Ecp1* and *Ecp2* during compatible interactions, comparable to the *Agrobacterium*- and *Rhizobium*-plant interactions (Peters *et al.*, 1986; Stachel *et al.*, 1985).

C. fulvum mutants deficient of the *Ecp1* or *Ecp2* genes, obtained by a gene replacement strategy, are virulent on tomato seedlings, indicating that these genes are not essential for pathogenicity (Laugé *et al.*, 1997; Marmeisse *et al.*, 1994). The *Ecp1*-deficient strain was not affected in its ability to invade, to colonize and to re-emerge from the host, but conidiophore formation is drastically reduced. In contrast, the *Ecp2*-deficient strain was inhibited during development, hardly produced any conidia, and the phenotype of a double mutant closely resembled that of the *Ecp2*-deficient strain. These data indicate that absence of one of the two proteins cannot be

compensated for by the presence of the other one (Laugé *et al.*, 1997). It remains to be determined how ECP1 and ECP2 contribute to virulence. It has been hypothesized however that the ECPs are suppressors of host defense reactions (Laugé *et al.*, 1997).

Recently, three additional proteins, ECP3, ECP4 and ECP5, were isolated (Laugé *et al.*, 2000). Subsequently, the elicitor activity of the proteins was tested in a "reverse screening" approach to identify *R* genes either by injecting the purified ECPs into plant leaves or by using nucleotide sequences encoding the ECPs in the PVX expression system *in planta*. Among a total of 28 tomato breeding lines, none responded to ECP1 or ECP4, whereas ECP3 and ECP5, respectively, were elicitor-active on two lines. Four tomato lines were identified in which ECP2 triggers an HR (Laugé *et al.*, 1998). A single dominant gene, *Cf-ECP2*, which had been introgressed into these lines from the same *L. pimpinellifolium* accession, controls this resistance. An *Ecp2*-disruption strain is pathogenic, although only weakly virulent, on *Cf-ECP2* plants demonstrating that resistance is solely based on ECP2 recognition by the host. Since the ECP2 protein was found in all 25 *C. fulvum* strains from a worldwide collection, the *Cf-ECP2*-mediated resistance appears to operate through recognition of an important fungal virulence factor.

L. pimpinellifolium has been the source of most tomato *Cf* genes. Therefore, screening for HR-inducing activity of the ECPs and AVRs from *C. fulvum* was extended to 40 accessions of this wild relative (Laugé *et al.*, 2000). Six accessions were identified that recognize AVR9, but none that responds to AVR4. ECP5 triggered HR in three lines, ECP4 in two lines, with one line recognizing both proteins. In addition, a single line each responded to treatment with ECP1, ECP2 and ECP3, respectively. As for ECP2, ECP1- and ECP4-mediated resistance is inherited through single dominant genes, *Cf-ECP1* and *Cf-ECP4*, respectively. In addition, two accessions of the non-host species *Nicotiana paniculata* were found to specifically respond with an HR to ECP2, whereas the interaction of the various proteins with other non-host species did not produce a phenotype. These data confirm the high likelihood of identifying an AVR function of extracellular virulence factors of pathogens, which therefore can be used as tools to identify new *R* genes.

Similar to the ECPs from *C. fulvum* the AVR factor NIP1 from *R. secalis*, the causal agent of leaf scald on barley, rye and several other grasses, was first identified through its virulence activity. The protein belongs to a family of small, necrosis-inducing proteins (NIPs) that were purified from fungal culture filtrates and found to be non-specifically toxic when injected into leaves of barley and other cereals (Wevelsiep *et al.*, 1991). Two of these proteins, NIP1 and NIP3, but not NIP2, indirectly stimulate the activity of the K^+-stimulated, Mg^{2+}-dependent H^+-ATPase in isolated plasma membrane vesicles (Wevelsiep *et al.*, 1993). Their toxic activity suggests a role of these proteins in fungal virulence. This was further supported by the observation that disruption of the *NIP1* gene results in a weak reduction in symptom

expression on susceptible plants (W. Knogge, unpublished data). Cloning of the *NIP2* and *NIP3* genes and generation of replacement mutants will allow an assessment of their importance during pathogenesis.

While for NIP2 and NIP3 a function in resistance induction has not been established yet, NIP1 was shown to be a cultivar-specific elicitor of defense reactions in barley cultivars carrying the *R* gene *Rrs1* (Hahn *et al.*, 1993). All fungal races avirulent on *Rrs1* plants carry and express the *NIP1* gene, whereas virulent races lack the gene or possess *NIP1* alleles encoding elicitor-inactive proteins (Knogge *et al.*, 1999). Fungal complementation and gene replacement mutants demonstrated that NIP1 is the product of the *Avr* gene complementary to the *Rrs1* gene (Knogge *et al.*, 1999; Rohe *et al.*, 1995).

The *NIP1* gene encodes an 82-amino acid protein, including a 22-amino acid signal sequence (Rohe *et al.*, 1995). The protein contains 10 cysteine residues forming 5 intramolecular disulfide bonds (K. A. E. van 't Slot, unpublished results). No sequences homologous to NIP1 have been found in the databases. The spacing of the first 8 cysteines in NIP1 however is reminiscent of a group of fungal proteins known as hydrophobins (Wessels, 1994; 1996; 1997). In addition, the predicted hydropathy profiles of NIP1 and the hydrophobin cerato-ulmin are strikingly similar. In contrast, assignment of the NIP1 cysteine bonds revealed a pattern different from the hydrophobin disulfide bridging. Although NIP1 is hence unlikely to be a functional hydrophobin it may nevertheless have a common ancestor with these proteins.

In fungal isolates four different types of NIP1 have been identified that are characterized by single amino acid alterations (Rohe *et al.*, 1995). While NIP1-I and NIP1-II differ in their elicitor activities, NIP1-III and NIP1-IV are inactive. Introduction of the single NIP1-III- and NIP1-IV-specific amino acids into the NIP1-I sequence and expression of the proteins using a heterologous system (Chapter 3) demonstrated their essential role for elicitor activity (K. A. E. van 't Slot, unpublished results). These NIP1 types were also non-toxic and attempts to structurally separate both functions of the protein have failed, suggesting that elicitor and toxic activity are mediated through the same receptor (Knogge, 1996). Recently, a single class of high-affinity NIP1-binding sites was identified on microsomal membranes from *Rrs1*- and *rrs1*-barley as well as from rye and the non-hosts wheat and oats, not however on membranes from *A. thaliana* (K. A. E. van 't Slot, unpublished results). This indicates that the NIP1 receptor is probably not encoded by the *Rrs1* gene and models similar to those suggested for the AVR9/CF9 interaction may be suitable to interpret the molecular interactions. The three-dimensional structure of NIP1 that is being solved based on ^{1}H-NMR and ^{15}N-NMR spectroscopy is expected to shed more light on signal perception at the host cell surface.

Although the mechanism underlying signal perception and transduction is not fully established yet, it is clear that binding of the AVR proteins from *C. fulvum* and

R. secalis to their putative receptors occurs on the extracellular surface of the host plasma membranes. In contrast, the rice blast fungus *M. grisea* provides an example for an intracellular AVR/R protein interaction. Crosses between strains of *M. grisea* exhibiting differential virulence on various rice cultivars have revealed the existence of several gene-for-gene interactions with cultivar specificity between the fungus and rice (Silué *et al.*, 1992; Valent and Chumley, 1991; 1994). Rice cultivars carrying the *R* gene *Pi-ta* are resistant to fungal races carrying the *Avr* gene, *AVR-Pita* (syn. *AVR2-YAMO*; Jia *et al.*, 2000). *AVR-Pita*, which was isolated by positional cloning, encodes a 223-amino acid protein with a signal peptide. The deduced amino acid sequence shows similarity with fungal Zn^{2+} proteases (Jia *et al.*, 2000; Valent, 1997). Although direct biochemical evidence is still missing, this function is supported by the identification of several point mutations within the putative active site of the protein from virulent strains that render the gene product inactive in the recognition process. In addition, it was recently shown that the protein is further processed to yield a 176-amino acid elicitor-active form, AVR-Pita$_{176}$, which corresponds to the processed versions of known fungal metalloproteases (Jia *et al.*, 2000). This indicates that AVR-Pita may indeed function through its enzyme activity. Surprisingly, application of AVR-Pita$_{176}$ to the apoplast of rice leaves failed to trigger a defense response. In contrast, using a transient expression system it was demonstrated that AVR-Pita$_{176}$, but not AVR-Pita$_{223}$, elicits an HR when expressed *in planta* in the presence of the *Pi-ta* gene, suggesting a cytoplasmic site of action (Jia *et al.*, 2000).

The *Pi-ta* gene that was isolated through a map-based cloning strategy encodes a putative cytoplasmic protein of 928 amino acids with a centrally localized NBS domain and a C-terminal leucine-rich domain (LRD) that does however not fit to any previously reported LRR consensus (Bryan *et al.*, 2000). When N-terminal deletions of the Pi-ta protein were expressed in the yeast two-hybrid system, only the LRD domain interacted with AVR-Pita$_{176}$, not however with products of *avr-pita* alleles. Far-western analysis (Chen and Evans 1995) confirmed this binding specificity; AVR-Pita specifically bound to the LRD domain of Pi-ta, whereas a point mutation in the LRD domain as well as a Met$_{178}$-Trp substitution in AVR-Pita$_{176}$ abolished the interaction. This high specificity of the physical interaction between the LRD domain of Pi-ta and AVR-Pita$_{176}$ suggests that the AVR protein is the signal molecule that is directly perceived by the *R* gene product. Interestingly, the presence of Zn^{2+} is required for the interaction, underlining the metalloprotein nature of the *Avr* gene product. AVR-Pita$_{176}$ may therefore be a fungal protease involved in releasing host nutrients. The protein may trigger the plant defense response by simply binding to the *R* gene product. Alternatively, the fungal protein may activate the Pi-ta protein by specific cleavage. The discrimination between these two mechanisms needs to await future experiments. In addition, it remains an open question how the fungal protein is translocated into the plant cytoplasm.

2. Fungal AVR determinants acting at the species level

The *PWL* genes of *M. grisea* govern pathogenicity toward weeping lovegrass (*Eragrostis curvula*). *M. grisea* is pathogenic to more than 50 grass species. However, genetically distinct populations of the fungus exist that infect and colonize different grass species. *PWL2* was identified in a strain virulent on rice, but non-pathogenic to *E. curvula*. After isolating the gene by positional cloning (Sweigard *et al.*, 1995) three additional genes, *PWL1* and *PWL3* from a finger millet (*Eleusine coracana*) pathogen and *PWL4* from a weeping lovegrass pathogen, were identified by their homology to *PWL2* (Kang *et al.*, 1995). Transformation experiments revealed that *PWL1* and *PWL2*, but not *PWL3* and *PWL4*, abolish the ability of *M. grisea* to infect weeping lovegrass without loosing pathogenicity to other hosts. The inactivity of *PWL4* appears to be caused by its improper expression, because it was rendered functional when controlled by the *PWL1* or *PWL2* promoters.

The four *PWL* genes encode proteins of 147 (*PWL1*), 145 (*PWL2*), 137 (*PWL3*) and 138 (*PWL4*) amino acids, respectively. The gene products show sequence homologies between 50 and 75 % and have several characteristics in common. They all have 21-amino acid putative signal sequences indicating their extracellular localization. They are proline-rich (5-7.5 %) and many glycine residues (17-19 %) are evenly distributed along the primary sequence, suggesting that the presence of α-helices is unlikely. They all have a high percentage of charged amino acids (26-28 %) resulting in a net charge of +2 for PWL1 and –5 for the three other proteins. The allele *pwl2-2* from a pathogenic fungal strain was found to encode a protein with a single amino acid exchange resulting in the loss of function (Sweigard *et al.*, 1995). This indicates that a highly specific interaction of the PWL protein with a plant factor reminiscent of AVR/R protein interactions is required for plant resistance. However, preliminary attempts to demonstrate elicitor activity of PWL2, as expressed in *E. coli*, have failed suggesting that it may not function through interaction with a plant surface receptor (Sweigard *et al.*, 1995).

Although nothing is known about a possible role of the PWL proteins in fungal virulence, a mutation frequency higher than in the rest of the *M. grisea* genome indicates that the gene family is highly dynamic and rapidly evolving (Kang *et al.*, 1995). Spontaneous *PWL2* deletions did not impair fungal growth and development under laboratory conditions, but only one field isolate lacking *PWL*-homologous DNA was detected (Sweigard *et al.*, 1995). This is interpreted as indirect evidence for a putative beneficial function of the *PWL* genes when the fungus is growing under natural conditions in the field.

VII. ELICITORS FROM OTHER PHYTOPATHOGENIC MICROBES

1. Elicitins of *Oomycetes*

In *Phytophthora* culture filtrates a class of proteins was identified that show two distinct effects on the mostly used test plant, tobacco (Bonnet *et al.*, 1985; Ricci *et al.*, 1989). They cause extended leaf necrosis reminiscent of the HR as well as systemic acquired resistance to normally virulent pathogens (Bonnet *et al.*, 1996; Kamoun *et al.*, 1993; Keller *et al.*, 1994; 1996; Ricci *et al.*, 1989; Yu, 1995). In addition, at sub-necrotic concentrations they induce defense-related plant reactions such transmembrane ion fluxes, generation of reactive oxygen, protein phosphorylation, ethylene production, phytoalexin and PR protein biosynthesis (Blein *et al.*, 1991; Keller *et al.*, 1994; Milat *et al.*, 1991; Ricci *et al.*, 1993; Viard *et al.*, 1994). Elicitins and elicitin-encoding genes have been detected in all *Phytophthora* species as well as in a few species of the related genus *Pythium*, while they are lacking in other oomycete families (Gayler *et al.*, 1997; Huet *et al.*, 1995; Panabières *et al.*, 1997). The original elicitin proteins were characterized by their size of typically 98 amino acids, by their high serine and threonine content and their lack of tryptophane, histidine and arginine and by the occurrence of three invariant disulfide bonds.

Due to their high sequence conservation the tertiary structures of the different elicitins can be expected to be similar to the recently solved structure of cryptogein, the elicitin from *P. cryptogea* (Boissy *et al.*, 1996; Fefeu *et al.*, 1997; Gooley *et al.*, 1998). It is a globular protein displaying a novel fold with five α-helices positioned on one face of the molecule. The other face consists of an antiparallel two-stranded β-sheet and an Ω-loop with one edge of the β-sheet and the adjacent Ω-loop forming a hydrophobic cavity. Recently it was shown that this cavity is a high-affinity binding site for sterols (Lascombe *et al.*, 2000; Mikes *et al.*, 1998; Mikes *et al.*, 1997; Vauthrin *et al.*, 1999). Linoleic acid was able to bind to the same site, although with lower affinity (Osman *et al.*, 2001). Two additional elicitins have been crystallized, β-cinnamomin from *P. cinnamomin* (Archer *et al.*, 2000) and oligandrin from *Py. oligandrum* (Lascombe *et al.*, 2000). With the latter a sterol complex was also obtained. These data suggest an intrinsic function of the elicitins as extracellular carriers of sterols and other lipids. In addition, the interaction of elicitins with high-affinity binding sites identified on tobacco plasma membranes (Bourque *et al.*, 1999) appears to require the formation of a sterol-elicitin complex (Ponchet *et al.*, 1999). Many Oomycetes are devoid of sterol biosynthesis and may need to acquire these lipids from the host plant. However, to what extent these organisms indeed require sterols remains unclear.

A substantial amount of data on elicitins has been accumulated meanwhile without, however, generating a clear-cut picture of the role of these proteins in

pathogenesis or resistance (Grant *et al.*, 1996; Ponchet *et al.*, 1999). An analysis of *Phytophthora* isolates from different plant species revealed that elicitin production is almost ubiquitous (Kamoun *et al.*, 1994; Ricci *et al.*, 1992), the major exception being tobacco isolates of *P. parasitica* (Kamoun *et al.*, 1994; Pernollet *et al.*, 1993). The HR induced in tobacco by all tested elicitins suggests that these proteins play a major role in the basic resistance of this plant to *Phytophthora* spp. (Kamoun *et al.*, 1994). However, the identification of a few elicitin-producing isolates that are virulent on tobacco does not argue for the hypothesis that elicitins are species-specific avirulence determinants (Bonnet *et al.*, 1994; Mouton-Perronnet *et al.*, 1995). Despite some debate in the literature, with the exception of the genus *Nicotiana* most plant species lack the capacity to respond to elicitins. In addition, some but not all cultivars of two Brassicacean species, radish (*Raphanus sativus*) and turnip (*Brassica campestris*; Kamoun *et al.*, 1993) developed necrosis. It was therefore concluded that elicitins might be genus-specific elicitors within the *Solanaceae* and cultivar-specific elicitors within the *Brassicaceae*. Detailed analysis of the elicitin effect on the F_2 offspring from a cross between two radish cultivars, one developing strong necrosis and the other showing accelerated senescence, revealed in addition to the parental phenotypes a null phenotype, all three segregating in a 1:2:1 ratio (Keizer *et al.*, 1998). Only protoplasts from the necrosis-inducing plants responded to elicitin treatment with the activation of defense reactions that can be suppressed by phosphorylation inhibitors. This indicates a linkage between the presence of a functional signal perception system and necrosis induction.

In *P. sojae* elicitin production of different pathogen races did not correlate with the avirulence phenotype (Mao and Tyler, 1996). In addition, the infiltrated elicitins cryptogein and parasiticein were not recognized by any of the 12 *Rps* genes encoding resistance of soybean to *P. sojae*. However, since the purified *P. sojae* elicitins were not used in these experiments their role in determining race-specific resistance cannot entirely be ruled out. In contrast, a role of the elicitin INF1 from *P. infestans* was revealed by gene silencing experiments (van West *et al.*, 1999). This protein was shown to induce programmed cell death, oxidative burst and defense gene expression in tobacco cells (Sasabe *et al.*, 2000). Transformants carrying an *inf1* antisense construct gained the capacity to grow on *N. benthamiana*, but not on *N. rustica* and *N. tabacum* (Kamoun *et al.*, 1998). This suggests that the INF1 protein functions as an AVR factor, probably at the species level, conditioning resistance in *N. benthamiana*. However, further detailed analyses are required to unravel the function of elicitins in the respective host plants.

2. *Avr* gene candidate from nematode species

The *R* genes cloned to date include a few mediating resistance against nematodes and aphids (Rossi *et al.*, 1998). In tomato, the *R* gene *Mi* (Milligan *et al.*, 1998; Vos *et al.*, 1998) controls the major root-knot nematodes *Meloidogyne incognita*, *M. javanica* and *M. arenaria* (Williamson, 1998). Plant resistance involves HR induction in root cells around the invading nematode (Hwang *et al.*, 2000) and the activation of defense-related genes (Lambert *et al.*, 1999). Recently, a cDNA was cloned from avirulent genotypes of *M. incognita* that encodes a secretory protein of 441 amino acids, termed MAP-1 (Semblat *et al.*, 2001). The protein is characterized by two classes of highly repetitive motifs, but its function remains unknown. An abundance of proline and the presence of 17 cysteine residues suggest significant folding of the protein. *Map-1*-specific primers revealed the presence of homologous sequences in several isolates of all three tomato root-knot nematodes, but not in other *Meloidogyne* spp. or other plant-parasitic nematode genera. Therefore, although not proven yet, the *map-1* gene may to be the first *Avr* gene from a plant-parasitic nematode. The protein was localized at the anterior end of the nematodes where it appears to be secreted by the amphids, the primary chemosensory organs near the oral opening. The function of these organs is not fully understood, nor is the function of their secretions (Perry, 1996). Therefore, future experiments are required to unravel the role the MAP-1 protein plays during nematode pathogenesis and HR induction in resistant plants.

VIII. CONCLUSION

Proteins from plant pathogenic microbes that have a demonstrated or presumed role in virulence have proven to be valuable tools in the process to understand the molecular basis of plant-pathogen interactions. One emerging common theme appears to be that most of these proteins have intracellular targets in the host. Hence, in their effort to utilize or manipulate the host physiology in their favor, plant pathogens translocate effector molecules into host cell. Although this could be expected for viral proteins that are produced inside plant cells, identification and characterization of the type-III secretion system in bacterial pathogens of plant and animals represents a major step in our understanding of the underlying molecular mechanisms. In addition, the recent finding of a cytoplasmic interaction between a fungal protein and a plant *R* gene product may open new pathways to study the interaction in particular of biotrophic fungi with their hosts. Future research will aim at identifying the host processes that are affected by the different pathogen factors and studying the impaired processes will in turn lead to an understanding of the physiological “default” situation.

The increasing number of pathogen factors with shown dual function in virulence and recognition by the host suggests that the evolution of a plant recognition system that targets important pathogen virulence factors or even critical domains within these factors appears to be another common theme. This natural strategy provides the blueprint for genetic engineering strategies to improve crop protection by identifying and targeting critical pathogen factors and thereby implementing more durable resistance. A complementing strategy aims at identifying novel plant *R* gene with a high likelihood of durability that have been lost during breeding of the presently grown crop cultivars. In this context, the "reverse screening" for plant lines that recognize secreted, isolate-nonspecific fungal proteins may represent a very successful approach. This strategy may be particularly suitable for identifying *R* genes that are involved in mediating plant resistance at the species level.

Chapter 3

Heterologous expression of the avirulence gene product, NIP1, from the barley pathogen *Rhynchosporium secalis*

CHAPTER 3

HETEROLOGOUS EXPRESSION OF THE AVIRULENCE GENE PRODUCT, NIP1, FROM THE BARLEY PATHOGEN *RHYNCHOSPORIUM SECALIS*

NIP1, THE PRODUCT OF THE AVIRULENCE GENE *AVRRRS1* FROM *RHYNCHOSPORIUM SECALIS*, A FUNGAL PATHOGEN OF BARLEY, IS A SMALL CYSTEINE-RICH PROTEIN. THIS PROTEIN IS ESSENTIAL FOR THE RECOGNITION OF THE FUNGUS BY THE HOST PLANTS CARRYING THE COMPLEMENTARY RESISTANCE GENE *RRS1*. DIFFERENT HETEROLOGOUS EXPRESSION SYSTEMS WERE TESTED TO PRODUCE SUFFICIENT QUANTITIES OF NIP1 TO ALLOW ITS UTILIZATION IN RECEPTOR IDENTIFICATION AND ISOLATION. IN ADDITION, PROTEIN AMOUNTS HIGHER THAN THOSE PRODUCED IN FUNGAL CULTURES ARE REQUIRED TO DETERMINE ITS 3D STRUCTURE AND TO ANALYZE ITS INTERACTION WITH A RECEPTOR. THE SYNTHESIS OF A HIS-TAG FUSION PROTEIN IN *ESCHERICHIA COLI* COMBINED WITH A REFOLDING PROCEDURE YIELDED UP TO 3 MG OF RECOMBINANT NIP1 FROM A 1 LITER BACTERIAL CULTURE. AFTER REMOVAL OF THE HIS-TAG, THE RECOMBINANT PROTEIN SHOWED THE SAME PHYSICO-CHEMICAL CHARACTERISTICS AS THE NATIVE NIP1 AND, MOST IMPORTANTLY, FULL BIOLOGICAL ACTIVITY.

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Protein Expression and Purification **17** (1999): 64-73.

INTRODUCTION

Upon pathogen attack, plants are capable of launching a battery of defense reactions to repulse the invader (Kombrink and Somssich, 1995). Key to plant resistance is a signal perception / transduction system that is initiated by signal molecules displayed by the pathogen (Baker *et al.,* 1997). In plant-pathogen interactions exhibiting host cultivar-specific resistance to particular pathogen races (gene-for-gene interactions, Flor, 1971), the synthesis of these signals is controlled by avirulence genes in the pathogen (Keen, 1982; Knogge, 1996; Knogge and Marie, 1997). The activity of avirulence gene products is based on a direct or indirect interaction with their plant counterparts, the products of resistance genes (Lamb, 1996). In less specific interactions, hydrolytically released typical cell wall components of pathogenic fungi such as β-1,3-glucans (Umemoto *et al.,* 1997) and *N*-acetyl-chitoologosaccharides (chitin fragments; Shibuya *et al.,* 1993) or secreted molecules of unknown function such as a particular glycoprotein (Nürnberger *et al.,* 1994) have been shown to trigger the plant defense through interaction with plasma membrane receptors.

In recent years, resistance genes have been isolated from several plants (Hammond-Kosack and Jones, 1995; Jones, 1996). The primary structures of the deduced products of several of these genes suggest their plasma membrane localization, but a respective function remains to be demonstrated. Other resistance genes, in particular those against bacterial pathogens, encode intracellular proteins that may interact with the avirulence gene products upon their translocation into the plant cytoplasm (Bonas and van den Ackerveken, 1997). However, in many cases the putative ligands are unknown.

Many avirulence genes have been cloned from bacterial plant pathogens (Dangl, 1994). In comparison, only very few of these genes and their products have been isolated and characterized from fungal pathogens (Knogge and Marie, 1997; de Wit *et al.,* 1997). One of these genes is *AvrRrs1* from the imperfect fungus *Rhynchosporium secalis* (Oudem.) J.J. Davis, an important foliar pathogen of barley (Shipton *et al.,* 1974). Host cultivars carrying the resistance gene *Rrs1* recognize fungal races expressing the *AvrRrs1* gene (Rohe *et al.,* 1995). The product of this gene, termed necrosis-inducing protein 1 (NIP1), is a small secreted protein that, upon cleavage of a 22-amino-acid signal peptide, consists of 60 amino acids including 10 cysteines (Rohe *et al.,* 1995). This single protein is capable of eliciting all defense reactions that are required to protect the host against the fungus (Rohe *et al.,* 1995, Hahn *et al.,* 1993). Most fungal races virulent on *Rrs1*-barley completely lack the *AvrRrs1* gene. In races carrying a NIP1-encoding gene, four types of NIP1 isoforms differing in single amino acid positions were found, types I and II showing elicitor activity and types III and IV being inactive (Rohe *et al.,* 1995).

The mode of action of NIP1 is likely to be an interaction with a plant plasma membrane-localized receptor (Knogge, 1996). Therefore, identification, isolation, and cloning of this receptor constitute crucial steps in unraveling the defense-related signal perception / transduction pathway in barley. However, these types of studies require substantial amounts of the ligand protein that cannot be obtained from fungal culture filtrates. Therefore, the establishment of an efficient heterologous expression system is imperative. The present paper describes different attempts to express NIP1 in *Pichia pastoris*, in the baculovirus-insect cell system and in *E. coli*.

RESULTS

Expression of NIP1 in Eukaryotic Cells

The 60-amino-acid sequence of the mature NIP1 contains 10 cysteine residues (Rohe *et al.,*1995). Incubation of the protein with thiol-reducing agents leads to complete loss of elicitor activity (Hahn *et al.,* 1993), indicating that disulfide bridges are pivotal for the protein's functional structure. This was substantiated by the failure to detect free sulfhydryl groups in the recombinant protein using Ellman's reagent (data not shown). In addition, ESI-MS yielded a molecular mass that deviates from the calculated value by –10 Da, suggesting 5 disulfide bonds (see below).

The reducing potential of the cytoplasm disfavors disulfide bond formation and production of eukaryotic proteins in their correct formation in *E. coli* (Hannig and Makrides, 1998). In addition, secretion of heterologous proteins from *E. coli* cells is not a facile task (Makrides, 1996). Therefore, a eukaryotic expression system appeared to be favorable to synthesize NIP1 in a secreted form which would also facilitate purification of the recombinant protein. For expression in the methylotrophic yeast *P. pastoris* two vectors encoding different secretory signal sequences were constructed. While vector pHIL-D2 allows the use of the native NIP1 signal sequence, vector pHIL-S1 carries the signal sequence of the acidic phosphatase gene, *PHO1*, from *P. pastoris*. When the native NIP1 signal sequence was used, NIP1 could not be detected on Western blots of *P. pastoris* culture filtrate proteins. However, in cellular extracts from several tested transformants, the anti-NIP1 antibodies detected low amounts of a protein with the mass of unprocessed NIP1 (data not shown). This observation indicates that the signal sequence from the *R. secalis* gene may not be appropriate for use in *P. pastoris* cells. Alternatively, the protein may be rapidly degraded after secretion due to the activity of extracellular proteases (Sreekrishna *et al.,* 1997).

For further expression studies a construct was generated that contained the sequence encoding the mature NIP1 3' of the *PHO1* signal sequence. Among several transformants containing the NIP1 cDNA, only one was identified that secreted NIP1

into the culture medium. After purification by cation exchange chromatography (FPLC Mono S, Amersham Pharmacia Biotech), 48 µg of elicitor-active NIP1 was obtained from a 3-ml culture (Table 1). However, induction of NIP1 synthesis was only successful with freshly transformed cells and the amount of recombinant protein drastically decreased after transfer to new culture media. The decrease in productivity was accompanied by a phenotypic change of the transformed cells which appeared slimy and resisted sedimentation by centrifugation. Southern blot analysis revealed that the failure the produce NIP1 was not due to a deletion of the *Nip1* gene that was still present in the genome of the *P. pastoris* transformant. As a consequence, expression of NIP1 in *P. pastoris* was abandoned.

TABLE 1: Comparison of NIP1 Yields from Different Heterologous Expression Systems

Expression system	Yield (mg/liter)	Remarks
P. pastoris	(16)	Calculated from a 3-ml culture
Baculovirus/Sf9 Cells	0.02	
E. coli: GST fusion	0.15	Two different forms
E. coli: His-tag	0.10	Strain SG13009
E. coli: His-tag, + refolding	1.80	Strain SG13009 (290 µg/g cells)
E. coli: His-tag, + refolding	3.30	Strain DHB4 (400 µg/g cells)
E. coli: His-tag, + refolding	3.80	Strain AD494 (510 µg/g cells)
R. secalis	0.02	

The baculovirus-based expression system consisting of insect cell cultures infected with recombinant *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) represents an alternative eukaryotic expression system (Smith *et al.,* 1983). The NIP1 cDNA (Rohe *et al.,* 1995) was cloned into two different transfer vectors, pAcYM1 (Matsuura *et al.,* 1987)and pAcC3 (Luckow and Summers, 1988). Homologous recombination with BaculoGold DNA yielded recombinant viruses which were used to infect *S. frugiperda* cells (Sf9). In both vectors, transcription of the *Nip1* gene was under the control of the strong polyhedrin promoter, the NIP1 cDNA being inserted such that all but the first or the first two nucleotides (in pAcYM1 the A and in pAcC3 the AT of the start codon) of the sequence connected to the translation start codon of the polyhedrin gene. Upon infection of Sf9 cells, expression and secretion of elicitor active NIP1 was obtained with both constructs (data not shown). However, amounts were low and the cells displayed an altered morphology compared to cells expression other control proteins. Monolayer cultures appeared patchy and enlarged cells were observed indicating an effect of NIP1 on the

insect cells. In liquid culture, the vitality of the cells was found to be lower rendering them more sensitive to suboptimal culture conditions such as reduced oxygen supply compared to monolayer cultures. Furthermore, serum-free medium could not be used in liquid culture to facilitate the purification of the secreted NIP1 (Krieger *et al.,* 1992). Several experiments with the pAcC3/*Nip1* plasmid revealed that viral propagation was obtained in liquid culture using serum-free Sf-900 II medium, but that NIP1 expression required the transfer of the infected cells into Grace's medium supplemented with 10% fetal calf serum. Under these conditions, 15µg of purified, elicitor-active NIP1 were obtained from 650 ml of cultural supernatant. This is in the order of the yield obtained from *R. secalis* culture filtrates (Table 1) and this approach was therefore not pursued any further.

Expression of NIP1 as a His-tagged fusion protein in E. coli

Due to the low yields obtained from eukaryotic expression systems and bacterial expression of NIP1 fused to glutathione *S*-transferase (GST) and polyhistidine (His_6-tag), was attempted. The vectors were constructed such that the mature NIP1 sequence was synthesized as the C-terminal portion of the recombinant fusion protein. In addition, in both cases the tag could be proteolytically removed from the recombinant protein by thrombin and factor Xa protease respectively.

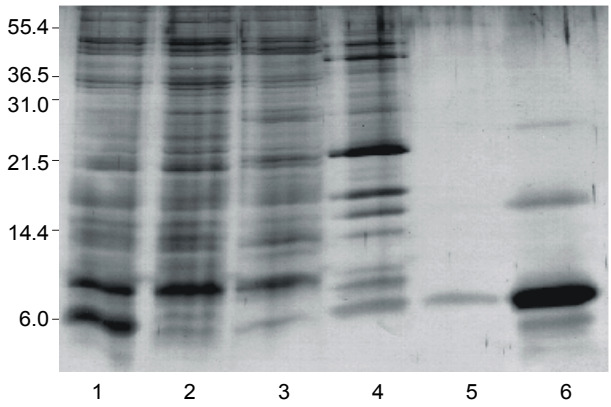

FIG. 1. Purification of His-tagged NIP1. $AgNO_3$-stained 18% SDS-polyacrylamide gel after electrophoretic separation of aliquots referring to 50 µl of bacterial culture. Lane 1, supernatant of bacterial cell lysate in 6 M guanidinium-HCl, 0.1 M NaH_2PO_4 / 0.01 M Tris, pH 8.0; lane 2, eluate from application of sample to Ni^{2+}-NTA agarose; lane 3, washing with 8 M urea in 0.1 M NaH_2PO_4 / 0.01 M Tris-HCl, pH 8.0 (10 mM 2-mercaptoethanol); lane 4, washing with the same buffer, pH 6.3 (10 mM 2-mercaptoethanol); lane 5, washing with the same buffer, pH 6.3; lane 6, elution with same buffer, pH 4.5. Positions of molecular mass markers (kDa) are indicated.

The highly soluble GST moiety was expected to produce the fusion protein intracellularly in a soluble form (Nygren *et al.,* 1994). Indeed, no inclusion bodies were detected. Extraction of soluble bacterial protein and affinity chromatography on glutathione S-Sepharose yielded a fusion protein of 33 kDa (GST, 26 kDa; NIP1, 6.4

kDa) and two to three additional proteins. Because the recombinant protein was found to be elicitor-inactive (data not shown), the GST portion was removed by thrombin cleavage. Subsequent RP-HPLC yielded two major NIP1 in addition to several minor peaks. Both major peaks were elicitor-active, whereas the minor peaks were inactive (data not shown). Due to the cloning procedure, the mature recombinant NIP1 contained two additional N-terminal amino acids, glycine and serine, which however did not significantly affect elicitor activity. From a 2-l bacterial culture, 7 mg of GST/NIP1 fusion protein were obtained yielding 300 µg of purified elicitor-active NIP1 (Table 1).

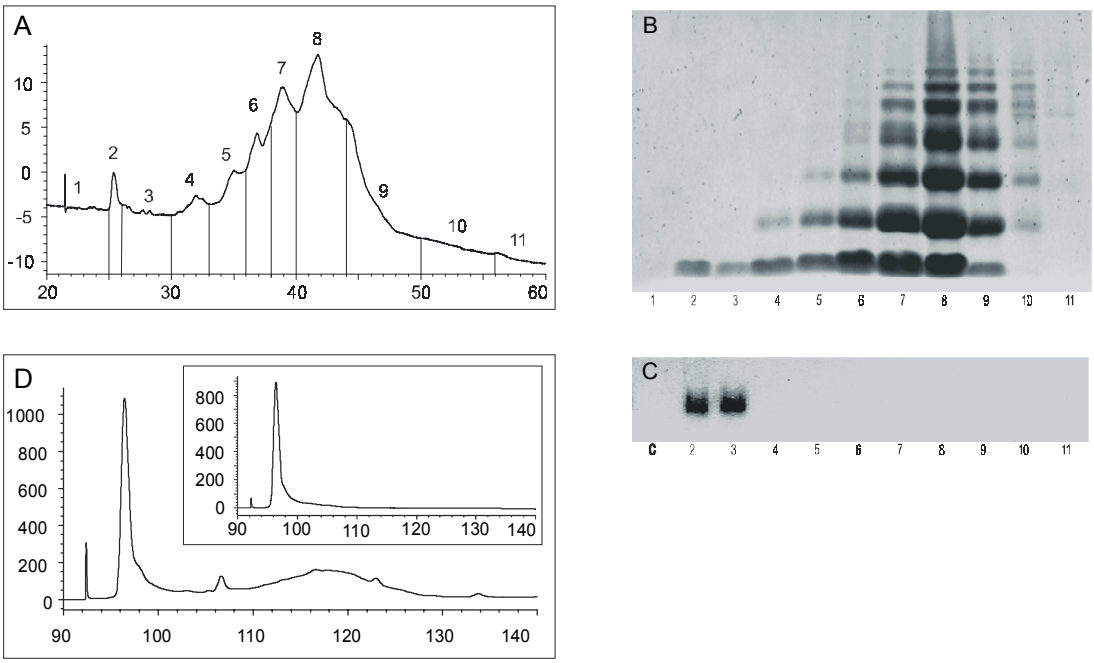

FIG. 2. Purification of the recombinant NIP1 by RP-HPLC. (A) Elution profile of His-tagged NIP1 from an analytical column after affinity purification on Ni^{2+}-NTA agarose. The numbered fractions were collected and subjected tot SDS-PAGE. (B) SYPRO-Red-stained 16% SDS-polyacrylamide gel after electrophoretic separation of aliquots from RP-HPLC fraction 1-11 in the absence of 2-mercaptoethanol. (C) Northern analysis of RNA (15 µg) extracted from barley leaves treated with 100 ng protein from the RP-HPLC fraction dissolved in 10 µl of 0.05% Tween 20. (D) Elution profile of His-tagged NIP1 from a preparative column after refolding. Inset: Rechromatography of the pooled monomer peak. In A and D, ordinate, absorbance at 280 nm (mAU); abscissa, elution time (min).

To further improve the yield, bacterial expression of NIP1 was finally attempted as an N-terminally His-tagged protein. The vector was constructed such that a factor Xa cleavage site precedes the NIP1 sequence, thus permitting the release of mature protein without additional amino acids. In addition to *E. coli* strain SG13009, strains DHB4 and AD494 were used. The latter is a derivative of strain

DHB4 (Boyd *et al.,* 1987) carrying a *trxB::kan* disruption in place of the wild-type *trxB* gene encoding thioredoxin reductase (Russel and Model, 1985). This strategy was meant to generate a less reducing cytoplasmic environment in order to facilitate disulfide bond formation (Derman *et al.,* 1993, Derman and Beckwith, 1995).

Independent of the bacterial strain used, however, NIP1 accumulated exclusively in inclusion bodies. Therefore, the bacterial cells were extracted and the recombinant protein was purified on Ni^{2+}-NTA agarose in the presence of 8 M urea (Fig. 1). In addition to NIP1, the protein bands with higher molecular mass cross-reacted with anti-NIP1 antisera (data not shown). After desalting, several protein-containing fraction eluted from the RP-HPLC column (Fig. 2A). SDS-PAGE in the presence of 2-mercaptoethanol yielded monomeric NIP1 in all fractions (data not shown). In contrast, SDS-PAGE under nonreducing conditions exposed a series of oligomers (Fig. 2B). This oligomerization may be caused by formation of intermolecular disulfide bridges. However, since monomeric NIP1 was also present in most fractions, oligomerization must at last in part be noncovalent. Elicitor activity was found only in those fractions that did not contain oligomeric NIP1 (Fig. 2C). The yield from 1 liter of bacterial culture was 100 µg of monomeric, elicitor-active NIP1.

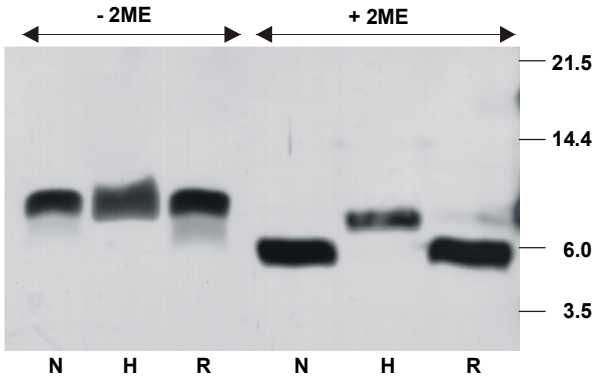

FIG. 3. Apparent molecular mass of NIP1. $AgNO_3$-stained 20% SDS-polyacrylamide gel after separation in the absence and presence of 0.7 M 2-mercaptoethanol. N, native, fungus-derived NIP1; H, His-tagged, refolded NIP1; R, recombinant NIP1 after removal of the His-tag. Positions of molecular mass markers (kDa) are indicated.

A drastic increase in the proportion of monomeric NIP1 was obtained when the total recombinant protein eluting from the Ni^{2+}-NTA agarose was allowed to refold overnight using a cysteine / cystine redox system (Beiboer *et al.,* 1996). Rechromatography of the monomer fraction by RP-HPLC revealed a stable, pure protein (Fig. 2D). From 1 liter of bacterial cell culture, 1.8 mg of His-tagged NIP1 was obtained using strain SG13009, while strains DHB4 and AD494 yielded 3.3 and 3.8 mg/liter of NIP1 respectively (Table 1). Based on PAGE (Fig. 1) and Western blotting (data not shown) the protein eluting from the Ni^{2+}-NTA agarose was

estimated to be >98% pure. Refolding yielded approximately 10% monomeric NIP1 and after removal of the His-tag the final NIP1 yield was 7%.

Apparent Molecular Mass of NIP1

Based on the amino acid sequence a molecular mass of 6443 Da (isoform type I) was calculated. During SDS-PAGE under non-reducing conditions, the protein moved to a higher size position (8.7 kDa) than expected, however, without displaying a difference between the His-tagged (8151 Da) and the mature protein. In contrast, in the presence of 0.7 M 2-mercaptoethanol this mass difference could be observed, but NIP1 showed a slightly higher than expected mobility (6.0 kDa for the native and the recombinant protein, 7.6 kDa for the fusion protein; Fig. 3). In comparison, during gel filtration in both the absence and presence of 10 mM 2-mercaptoethanol NIP1 eluted with a buffer volume corresponding to an apparent molecular mass of 10.4 kDa (data not shown). This deviates substantially from the expected value indicating a possible dimerization of the protein in solution. However, ESI-MS, performed with the type II isoform (6509 Da calculated), yielded single mass peaks of 6509.0 ± 1.5 and 6509.1 ± 0.3 Da for the native and recombinant NIP1, respectively, excluding covalent dimerization through disulfide bonds. Therefore, noncovalent dimerization or, alternatively, structural features of the protein such as a far from globular shape may account for the abnormal behavior during gel chromatography and SDS-PAGE.

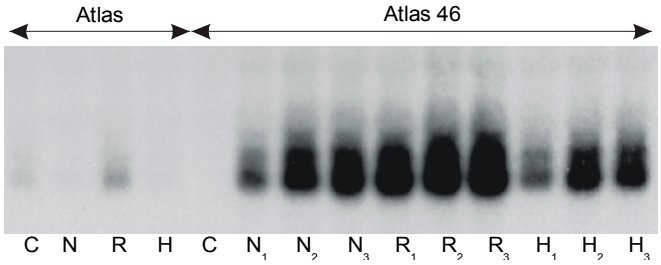

FIG. 4. Elicitor activity of recombinant NIP1. Northern analysis of RNA extracted from NIP1-treated leaves of the near-isogenic barley cultivars Atlas (rrs1) and Atlas 46 (Rrs1) using PR5 cDNA as a probe. For each sample, 3 primary leaves were treated with 10 µl of the following solutions: C, 0.05% Tween 20; N, native, fungus-derived NIP1; R, recombinant NIP1; H, His-tagged, refolded NIP1. The suffix numbers 1, 2, and 3 indicate that 50, 100, or 150 ng of protein was applied to each leaf.

Part of the defense response of barley to infection by *R. secalis* is the synthesis of pathogenesis-related proteins 5 and 9 (PR5, PR9, Hahn *et al.,* 1993). NIP1 purified from fungal culture filtrates elicits the synthesis of PR5 and PR9 mRNA when applied to the surface of leaves specifically from *Rrs1*-barley cultivars in a way very similar to the fungus (Rohe *et al.,* 1995, Hahn *et al.,* 1993). The recombinant NIP1 forms

were elicitor-active on *Rrs1*-barley, but inactive in the absence of the *Rrs1* gene. While His-tagged NIP1 showed a reduced elicitor activity, proteolytic release of the mature protein fully reestablished the activity (Fig. 4).

DISCUSSION

NIP1, the product of the avirulence gene *AvrRrs1* from *R. secalis* (Rohe *et al.,* 1995), is synthesized and secreted in relatively small amounts into the culture medium (Wevelsiep *et al.,* 1991). The protein is a cultivar-specific elicitor of defense reactions that is active only in barley cultivars carrying the resistance gene *Rrs1* (Hahn *et al.,* 1993). This genotype-specificity along with a quantitatively similar elicitor activity compared to the native NIP1 are crucial criteria for the qualitative evaluation of recombinant NIP1. When the His-tagged and the protease-released mature protein from *E. coli* were compared with the native NIP1, only the processed form showed full elicitor activity.

The efficacy of NIP1 production varied greatly in the tested expression systems. Due to the presence of five intramolecular disulfide bonds eukaryotic systems secreting the recombinant protein appeared most promising. However, neither *P. pastoris* nor the baculovirus / insect cell system yielded reproducibly sufficient amounts of NIP1. In both cases, the protein appeared to have a negative effect on the physiology of the expressing cells by inhibiting growth and changing the phenotype of the cells.

Soluble GST/NIP1 fusion protein was successfully synthesized in *E. coli*. The inability of the fusion protein to elicit plant defense reactions may be due to its rather large size (33 kDa). To exert its elicitor activity the protein needs to cross the plant cell wall to access its target on the plasma membrane. This process may be hindered by the size of the fusion protein. Alternatively, the conformation of the fusion protein may impede NIP1 activity. However, proteolytic release of mature NIP1 resulted in active elicitor protein. Nevertheless, the yield obtained was only approximately 10 times higher than that by purification from fungal cultures.

In contrast to the GST/NIP1 fusion protein, the His-tagged NIP1 accumulated exclusively in inclusion bodies in all bacterial strains tested. The majority of the protein occurred as different-sized oligomers and only a small percentage of active, monomeric NIP1 could be isolated directly. Yields were however drastically improved by subjecting the affinity-purified protein to a refolding procedure, thus shifting the ratio from oligomers to the monomeric form. No significant difference was observed when thioredoxin reductase-deficient strain AD494 was used in comparison with its parent strain DHB4 (Boyd *et al.,* 1987), indicating that the redox state of the cytoplasm does not interfere with the synthetic capacity of the cells.

However, the yield in these two strains was approximately 2-fold higher than that in strain SG13009, thus representing a 2- to 300-fold increase of NIP1 yield compared to fungal cultures.

NIP1 does not show amino acid sequence similarity with other proteins in the databases. However, the first eight cysteines are spaced in a way characteristic for a family of fungal proteins termed hydrophobins. These proteins are cell wall components and have been identified in various fungi (Wessels, 1997). In addition to the conserved cysteine pattern, they have a small size and a similar arrangement of hydrophobic domains in common (Wessels, 1994), whereas their amino acid sequences are very diverse. Class I hydrophobins are capable of self-assembly at water-gas interfaces to form SDS-insoluble membranes displaying a rodlet pattern similar to the surface of aerial fungal hyphae (Wessels, 1996). Cerato-ulmin from *Ophiostoma ulmi* (Stringer and Timberlake, 1993), cryparin from *Cryphonectria parasitica* (Zhang *et al.,* 1994), and HBF1 from *Trichoderma reesei* (Nakari-Setälä *et al.,* 1996) constitute the class II hydrophobins. These proteins are moderately hydrophobic and deviate in their hydropathy profiles from the class I hydrophobins. They also self-aggregate, but aggregates dissolve in SDS.

The hydropathy pattern of NIP1 (data not shown) resembles that of the class II hydrophobins (Wessels, 1994), although the spacing of 5 amino acids between cysteines 5 and 6 of NIP1 is characteristic for class I hydrophobins. Another similarity of NIP1 and class II hydrophobins is the noncovalent oligomerization under denaturing conditions. For cerato-ulmin, a higher than expected molecular mass of about 13 kDa was originally determined by SDS-PAGE, while ESI-MS yielded 7.6 kDa (Yaguchi *et al.,* 1993). Similar results were described for HFB1 (Nakari-Setälä *et al.,* 1996) and for cryparin (Zhang *et al.,* 1994, Carpenter *et al.,* 1992). This raises the question of a possible dimerization of these proteins. For NIP1 an answer to this question must await further studies. However, if dimerization of the protein in solution is required for its biological activity, the reduced elicitor activity observed with the His-tagged protein may be due to a different aggregation behavior in comparison to the native and the processed protein.

With the establishment of an effective heterologous expression system, sufficient NIP1 amounts can now be obtained that allow to address several crucial questions. Self-aggregation experiments will be performed to further investigate the hydrophobin nature of NIP1. Furthermore, NMR-based 3D structural analysis of the protein is expected to shed light on the molecular interaction between NIP1 and its receptor. In particular, it will be interesting to unravel the reason for the inactivity of two NIP1 forms from virulent fungal races that differ only in single amino acids from the active forms (Rohe *et al.,* 1995). Most importantly, however, the protein will be used as a ligand for binding studies and for affinity chromatography to identify and isolate the NIP1 receptor.

MATERIALS AND METHODS

Fungal, Insect, and Bacterial Cell Lines

Expression studies in *P. pastoris* were performed in strain GS111 *his4* (Invitrogen, San Diego, CA). For baculovirus expression, the *Spodoptera frugiperda*-derived cell line Sf9 was used. A glutathione *S*-transferase (GST)/NIP1 fusion protein was expressed in *E. coli* strain M15 (Villarejo and Zabin, 1974), a His-tag/NIP1 fusion protein in strain SG13009 (Qiagen, Hilden, Germany), DHB4 (Boyd *et al.,* 1987) and AD494 (E. Steward, Harvard Medical School, Boston, MA).

Expression in P. pastoris

Vector pHIL-D2 (Invitrogen) was digested with *Eco*RI and the protruding ends were filled in with Klenow polymerase. The NIP1 cDNA comprising 37 bp of 5' noncoding sequence, the coding sequence including the native signal sequence, and 267 bp of 3' noncoding sequence was excised from plasmid pHH-NIP1L (Rohe *et al.,* 1995) by *Eco*RI and *Xho*I digestion. After fill-in of protruding ends the cDNA was ligated with the vector pHIL-D2. Orientation was analyzed by restriction analysis and sequencing.

For cloning of the NIP1 cDNA into the vector pHIL-S1 (Invitrogen), the native signal was removed. For this purpose, the sequence encoding the mature protein was amplified by PCR using primers that introduced a 5'-*Xho*I site (5'-GCCG*CTCGAG*ATCGATGCA GATACACCCTTTG-3') and a 3' *Eco*RI site (5'-CCGG*GAATTC*TTAACATTGGCGGTA TCCCGTC-3'). The PCR product was blunt-ended and cloned into the *Sma*I site of pUC18. Subsequently, the insert was excised with *Xho*I and *Eco*RI and ligated into the pHIL-S1 vector.

Transformation of *P. pastoris*, strain GS111 *his4*, through electroporation, selection of transformants, and expression of NIP1 were carried out according to the manufacturer's protocol (Invitrogen).

Expression in Insect Cells Using a Baculovirus Vector

Two different vectors were chosen in an attempt to generate NIP1 in the baculovirus expression system. The vector pAcYM (Matsuura *et al.,* 1987) carries the entire 5' noncoding sequence of the polyhedrin gene including the A of the translation start codon, followed by a *Bam*HI site. The NIP1 cDNA-harboring plasmid pHH-NIP1L (Rohe *et al.,* 1995) was linearized with *Xho*I. Protruding ends were filled in with Klenow polymerase and phosphorylated. After subsequent ligation of *Bam*HI linkers the NIP1 cDNA was excised from the plasmid as *Bam*HI/*Bam*HI fragment and cloned into the *Bam*HI site of pAcYM1. Recombinant clones with the correct orientation were identified by restriction analysis.

The vector pAcC3 (Luckow and Summers, 1988) contains the 5' noncoding sequence of the polyhedrin gene including the ATG start codon as part of a singular *Nco*I restriction site. pHH-NIP1L (Rohe *et al.,* 1995) was linearized by *Eco*RI digestion. The coding region and the 3' noncoding sequence was amplified by PCR using the T7 primer and a *Nip1*-specific primer complementary to the six 5' terminal codons of the *Nip1* sequence (5'-CGCG*TCATGA*AATTCCTCGTACTG-3'). The ATG region was modified to introduce a *Bsp*HI restriction site (marked in italics). *Bsp*HI and *Dra*I digestion of the 580-bp PCR

product released a 400-bp fragment containing the NIP1 coding sequence but lacking the polyadenylation signal. This fragment was cloned into the *Nco*I/*Sma*I-digested pAcC3 vector.

Recombinant baculoviruses were produced by homologous recombination according to the BaculoGold transfection kit manual (PharMingen, San Diego, CA). Sf9 cells (1.6×10^6) were cotransfected with 0.4 µg of vector DNA 0.1 µg of BaculoGold virus DNA. Isolation of recombinant baculoviruses expressing NIP1 was carried out according to previously described protocols (Stabel *et al.,* 1991, Krieger *et al.,* 1992). Production of recombinant protein was attempted in Sf9 cell suspension cultures using six recombinant viral clones for each construct. Culture supernatants were collected 3-4 days post infection by centrifugation and analyzed on Western blots for the presence of NIP1.

Expression as Glutathione S-Transferase Fusion Protein in E. coli

To generate NIP1 as fusion protein with the 26-kDa glutathione *S*-transferase (GST) from *Schistosoma japonicum*, the pGEX-2T expression vector (Amersham Pharmacia Biotech, Freiburg, Germany) was used. For this purpose, a 39-nucleotide PCR primer was synthesized that contains a *Bam*HI site immediately 5' of the sequence encoding the N-terminal 10 amino acids of the mature NIP1. Using this primer in combination with the T7 reverse primer and the linearized plasmid pSR-NIP1 (Rohe *et al.,* 1995) as a template, a 215-bp fragment was amplified and cloned. The *Nip1* sequence contains an internal *Sph*I site. Therefore, the *Bam*HI/*Sph*I fragment (60 bp) was isolated from the cloned PCR fragment and ligated into the *Sph*I/*Xho*I fragment (415 bp) from the NIP1 cDNA clone pHH-NIP1L (Rohe *et al.,* 1995). An *Xho*I/*Eco*RI adapter was ligated with the resulting *Bam*HI/*Xho*I fragment (475 bp) to enable insertion of the construct into pGEX-2T vector using the *Bam*HI and *Eco*RI sites. Transformation of *E. coli* strain M15 (Villajero and Zabin, 1974) yielded a GST/NIP1 fusion protein in which the amino acid sequence of the mature NIP1 was N-terminally extended by a glycine an serine residue encoded by the *Bam*HI restriction sequence. Purification of the recombinant protein was achieved through affinity chromatography on glutathione *S*-Sepharose 4B according to the manufacturer's protocol (Amersham Pharmacia Biotech). The eluting GST/NIP1 fusion protein was lyophilized, dissolved in a small volume of 50 mM Tris/HCl (150 mM NaCl, 2.5 mM $CaCl_2$), pH 8.3, and subjected to thrombin cleavage (1% (w/v) thrombin, 37°C, 5 h). Finally, NIP1, GST, thrombin, and uncleaved fusion protein were separated by reversed-phase (RP) HPLC.

Expression as His-tag Fusion Protein en E. coli

The vector pQE-30 (Qiagen, Hilden, Germany) was used to place a 6x His-tag at the N-terminus of the recombinant NIP1. The sequence encoding the mature protein was PCR-amplified using a 5' primer containing a *Bam*HI site (italics), followed by the sequence encoding a factor Xa cleavage site (underlined) immediately upstream of the codons for the 9 N-terminal NIP1 amino acids (5'-GCGC*GGATCC*<u>ATCGAAGGTAGA</u>GATCGATGCAGAT ACACCCTTTGTTGC-3'). In the 3' primer containing a 23-nt sequence complementary to the 3' end of the *Nip1* coding sequence (5'-GCGG*CCCGGG*TTAACATTGGCGGTATCCCGT CG-3'), a *Sma*I site (italics) was following the stop codon to allow the ligation of the PCR product into the *Bam*HI/*Sma*I sites of the dephosphorylated pQE-30 vector. Correct cloning was verified by sequencing. For transformation the *E. coli* strains SG13009, DHB4 and

AD494 were used. For NIP1 expression, 1 liter of LB medium (100 µg/ml ampicillin) was inoculated with 40 ml of a bacterial overnight culture in LB medium. When the bacterial culture had reached an optical density of 0.7-0.9, 2mM IPTG was added. After incubation at 37°C (200rpm) for 3.5 h cells were sedimented from 250 ml culture aliquots and frozen at -20°C. Bacterial pellets were resuspended in 20 ml of 6 M guanidinium-HCL, 0.1 M Na_2HPO_4, 0.01 M Tris, pH 8.0, and vigorously shaken for 60 min. Cellular debris was removed at 10,000g for 20 min and to the combined supernatants (80 ml), 8 ml of a 50% Ni^{2+}-nitrilotriacetic acid (NTA) agarose slurry was added. After gentle mixing for 60 min the mixture was transferred into a column and affinity chromatography was carried out under denaturing conditions (8 M urea) using the pH shift elution procedure according to the manufacturer's protocol (Qiagen).

Denaturing and Refolding of Recombinant NIP1

The His-tag/NIP1 fusion protein, eluting from the Ni^{2+}-NTA agarose with 8 M urea in 0.1 M Na_2HPO_4 and 0.01 M Tris, pH 4.5, was diluted with 8M urea to a concentration of 0.4 g/l and the pH of the solution was adjusted to 8.7. Subsequently, 3 vol. of refolding buffer (25 mM boric acid, 8 mM cysteine, 1 mM cystine, 5 mM EDTA, pH 8.7) were added (Beiboer *et al.,* 1996). After incubation with gentle stirring overnight at 4°C in the dark, the solution was desalted using a SepPak C18 column (Waters, Eschborn, Germany) and subjected to RP-HPLC.

Removal of the His-tag

The purified His-tag/NIP1 (0.5 mg/ml) was incubated in 50 mM Tris/HCl (100 mM NaCl, 1 mM $CaCl_2$), pH 8.9, at 28°C for 48 h in the presence of factor Xa protease (50 µg/ml). Subsequently, NIP1 was separated from the cleaved His-tag, the protease and the uncleaved fusion protein by RP-HPLC.

Protein Purification

Unless otherwise stated, purification of the recombinant proteins was achieved by RP-HPLC on an HP 1090 chromatograph (Hewlett Packard, Waldbronn, Germany). Analytical-scale purification of expression products was performed on an analytical C4 RP column (Vydac Protein C4, Macherey and Nagel, Düren, Germany, 0.47 × 25 cm) using buffer A (0.1% trifluoroacetic acid) and buffer B (70% isopropanol in buffer A) and a gradient of 0-74% of buffer B for 122 min with a flow rate of 0.5 ml/min. Depending on the expression system and the resulting protein, minor adjustments of this procedure were required. The His-tagged NIP1 fusion protein was purified on a preparative C4 RP column (Vydac Protein C4, 2.2 × 25 cm) using the elution system: 40 min buffer A, 5 min 0-20% buffer B, 60 min 20-58% B, 1 min 58-100% B with a flow rate of 1.5 ml/min. After factor Xa treatment the recombinant NIP1 was purified on a semipreparative C4 RP column (Vydac Protein C4, 1 × 25 cm) using the elution system: 4 min 1-13% buffer B, 21 min 13-20% buffer B, 6 min 20-22% buffer B, 2 min 22-100% buffer B with a flow rate of 1.5 ml/min.

Determination of the Apparent Molecular Mass of NIP1

The apparent molecular mass of the recombinant NIP1 was determined by SDS-PAGE on 20% (w/v) acrylamide gels using the Mark12 Wide Range Protein Standard (NOVEX, San Diego, CA). In addition, FPLC gel filtration (Amersham Pharmacia Biotech) was performed using a Fractogel EMD BioSec Superformance column (1.6 x 60 cm; Merck, Darmstadt, Germany) with 20 mM Na phosphate buffer (100 mM NaCl, 10 mM 2-mercaptoethanol), pH 7.2, with carbonic anhydrase (29.0 kDa), cytochrome *c* (12.4 kDa) and aprotinin (6.5 kDa) as molecular mass standard.

In addition, electrospray ionization mass spectrometric analyses (ESI-MS) were performed on a Finnigan (San Jose, CA) MAT 700 mass spectrometer, equipped with a custom-made electrospray interface. The samples were dissolved in a mixture of methanol / 1% acetic acid (80/20, v/v) to a final concentration of 10 pmol/µl (0.3 mg/ml). The spectra were collected during constant infusion of the samples with a Harvard 2400 syringe pump at a flow rate of 2 µl/min. The molecular mass was calculated using the deconvolution program BIOMASS.

Elicitor Assay

Elicitor activity of the recombinant NIP1 was assayed by treating primary leaves of the near-isogenic barley cultivars Atlas 46 (*Rrs1*) and Atlas (*rrs1*) with 10 µl aliquots of protein solution. 24 h later, RNA was extracted and subjected to Northern hybridization using PR5 cDNA as a probe (Hahn *et al.,* 1993).

Analytical methods

Protein was routinely quantified according to Bradford (Bradford, 1976) using bovine serum albumin as a standard. After proteolytic release of the His-tag and final purification of the recombinant mature NIP1, protein amounts were in addition determined optically using the calculated molar absorption coefficient ε_{280} = 4400 cm^2/mol. SDS-PAGE was performed according to Laemmli (Laemmli, 1970) in discontinuous gels (4% stacking and 16, 18, or 20% separating gels). Proteins in gels were stained with SYPRO-Red (Molecular Probes, Eugene, OR) and detected using a phosphoimager (Molecular Dynamics, Krefeld, Germany) or with the $AgNO_3$ method (Blum *et al.,* 1987). Western analyses were performed after electrophoretic transfer of proteins to nitrocellulose membranes. For NIP1 detection an antiserum raised against the protein from fungal cultures (Wevelsiep *et al.,* 1991) was used along with secondary anti-rabbit antibodies raised in goats and conjugated to horseradish peroxidase. Northern analyses were performed after transfer of RNA to Hybond-N membranes (Amersham Pharmacia Biotech; Sambrook *et al.,* 1989). Free sulfhydryl groups were analyzed using 5,5'-dithio-bis(2-nitrobenzoic acid) (Ellman, 1959) with reduced glutathione as a standard.

ACKNOWLEGEMENTS

Drs. Imre E. Somssich and E. Kombrink are gratefully acknowledged for critically reading the manuscript and Dr. Silvia Stabel for her help with the baculovirus vector expression system. In addition, we thank Dr. E. Steward (Harvard Medical SChool, Boston, MA), for providing us with the E. coli strains DHB4 and AD494. Furthermore, Dr. U.R. Tjaden at the Leiden / Amsterdam Center for Drug Research, Division of Analytical Chemistry, Leiden, The Netherlands, is thanked for performing the ESI-MS analyses. Parts of the project were funded by Grant 0136101A from the Bundesministerium für Forschung und Technologie and by Grant Kn225/3 from the Deutsche Forschungsgemeinschaft. C.M. was funded by the European Commission through a contribution to the Human Capital and Mobility network ERBCHRXCT930244.

Chapter 4

Binding of the Avirulence Protein NIP1 from *Rhynchosporium secalis* to Barley Membranes is not sufficient for Plant Defense Activation

CHAPTER 4

BINDING OF THE AVIRULENCE PROTEIN NIP1 FROM *RHYNCHOSPORIUM SECALIS* TO BARLEY MEMBRANES IS NOT SUFFICIENT FOR PLANT DEFENSE ACTIVATION

THE AVIRULENCE GENE PRODUCT, NIP1, FROM THE BARLEY PATHOGEN *RHYNCHOSPORIUM SECALIS* SPECIFICALLY INDUCES THE SYNTHESIS OF DEFENSE-RELATED PROTEINS IN BARLEY CULTIVARS EXPRESSING THE COMPLEMENTARY RESISTANCE GENE, *RRS1*. IN ADDITION, IT IS TOXIC TO LEAF TISSUES OF BARLEY AND OTHER CEREAL PLANT SPECIES (WHEAT, RYE, OATS) INDUCING NECROTIC LESIONS IN A GENOTYPE-UNSPECIFIC MANNER. THE ELICITOR-ACTIVE NIP1 ISOFORMS TYPE I AND TYPE II AND THE NON-ACTIVE MUTANT ISOFORMS TYPE III* AND TYPE IV* WERE PRODUCED IN *E. COLI*. TYPE I WAS LABELLED WITH IODINE-125 AND USED IN BINDING STUDIES. BINDING OF ^{125}I-NIP1 TO MICROSOMES FROM RESISTANT (*RRS1*) BARLEY WAS SPECIFIC, REVERSIBLE AND SATURABLE. FROM SATURATION LIGAND BINDING EXPERIMENTS A K_D OF 6.0 NM WAS CALCULATED, AND THE APPARENT CONCENTRATION OF A SINGLE CLASS OF BINDING SITES WAS FOUND TO BE 255 FMOL.MG^{-1} OF BARLEY MICROSOMAL PROTEIN. ON MEMBRANES FROM NEAR-ISOGENIC SUSCEPTIBLE (*RRS1*) BARLEY, A BINDING SITE WITH IDENTICAL BINDING CHARACTERISTICS WAS DETECTED. IN ADDITION, A BINDING SITE WAS PRESENT IN WHEAT, RYE, OATS AND MAIZE, BUT NOT IN *ARABIDOPSIS THALIANA*. THEREFORE, THE PRESENCE OF THIS BINDING SITE APPEARS TO CORRELATE WITH THE TOXIC ACTIVITY OF NIP1. IN CONTRAST, COMPETITIVE BINDING EXPERIMENTS USING THE DIFFERENT NIP1 ISOFORMS REVEALED THAT HIGH-AFFINITY BINDING OF THE PROTEIN TO ITS BINDING SITE WAS NOT CORRELATED WITH PR5-INDUCING ACTIVITY. BINDING IS THEREFORE NOT SUFFICIENT TO ACTIVATE THE PLANT DEFENSE RESPONSE IN *RRS1*-BARLEY AND THE *RRS1* GENE IS UNLIKELY TO ENCODE THE PRIMARY NIP1 RECEPTOR. IN THE PRESENCE OF THE R PROTEIN, BINDING OF THE CORRESPONDING ELICITOR TO THE VIRULENCE TARGET WILL RESULT IN THE ONSET OF DEFENSE RESPONSES, WHEREAS IN SUSCEPTIBLE PLANTS BINDING WILL RESULT IN TRANSMISSION OF ITS VIRULENCE FUNCTION.

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To be submitted

INTRODUCTION

Induction of the defense response is the consequence of a plant recognizing an attacking pathogen. Frequently, this recognition is controlled by pairs of complementary genes, a pathogen avirulence (*Avr*) gene and a matching plant resistance (*R*) gene. Consequently, in interactions between plant cultivars and pathogen races, plant resistance is an active process resulting from the expression of both types of genes. It seemed therefore reasonable to assume that this genetic gene-for-gene model, originally proposed for the flax-flax rust interaction (Flor, 1955, 1971), complies with a receptor-ligand model; the products of the *R* gene encoding a membrane-localized receptor and of the *Avr* gene encoding a ligand interact, thereby initiating a cascade of defense reactions (Baker *et al*., 1997).

Cloning and characterization of *R* genes from several plant species revealed, however, that many *R* gene products are cytoplasmic, rather than membrane proteins (Takken and Joosten, 2000). The first example of a direct interaction between *Avr* and *R* gene products was demonstrated in a bacterial pathosystem involving the AVR protein, AvrPto, from *Pseudomonas syringae* pv. *tomato*, the agent of bacterial speck disease, and its matching tomato R protein, Pto, a protein kinase (Scofield *et al*., 1996; Tang *et al*., 1996). In addition, the direct interaction of AVR-Pita, an AVR protein from the rice blast fungus, *Magnaporthe grisea* (Orbach *et al*., 2000), and the product of the rice *R* gene, Pi-ta (Bryan *et al*., 2000), was recently reported (Jia *et al*., 2000). Bacteria possess a type III secretion system that mediates the delivery of bacterial effector proteins into the cytoplasm of plant cells (Galán and Collmer, 1999), thus explaining the intracellular interaction of AVR and R proteins. It remains however an open question how a fungal protein may be translocated into the host cell in those cases where interaction between AVR and R proteins are supposed to interact in the cytoplasm of the plant.

The group of *R* genes encoding membrane-localized products contains the tomato *Cf* genes conferring resistance to races of the leaf mold fungus, *Cladosporium fulvum* (Jones and Jones, 1997; Takken and Joosten, 2000). The interaction of one of the *R* gene products, Cf-9, and the corresponding fungal AVR protein, AVR9, was studied in detail. In contrast to AvrPto-Pto, however, no direct interaction between the *Avr* and the *R* gene products could be demonstrated (Luderer *et al*., 2001). Furthermore, plasma membranes from both resistant (Cf-9) and susceptible (Cf-0) plants display high-affinity AVR9-binding sites with similar properties. In addition, an AVR9-binding site was detected on membranes of other solanaceous species (Kooman-Gersmann *et al*., 1996). These data suggest that the *R* gene *Cf-9* does not encode the primary AVR9-binding site. Therefore, the recently proposed models for the AVR9-induced resistance response assume the formation of a heterotrimeric signal perception complex involving the AVR ligand, a non-specific primary receptor

and a specific signal transducer to initiate the signal transduction in resistant (Cf-9) plants that leads to the activation of defense-related genes (Joosten and de Wit, 1999; Kooman-Gersmann *et al*., 1998; Luderer *et al*., 2001).

The interaction between the imperfect fungus *Rhynchosporium secalis* and its host plant, barley (*Hordeum vulgare* L.), also complies with the gene-for-gene scheme (Knogge and Marie, 1997). The *Avr* gene, *AvrRrs1*, from *R. secalis* is one of the still very limited numbers of fungal *Avr* genes isolated to date. The product of this gene, termed NIP1, induces resistance-related reactions in barley cultivars carrying the *R* gene, *Rrs1* (Hahn *et al*., 1993; Rohe *et al*., 1995). However, unlike in many other plant-pathogen systems, a hypersensitive response is not observed in this model system (Lehnackers and Knogge, 1990). In addition to its role as AVR factor, NIP1 is a member of a small group of secreted toxic proteins that cause scald-like lesions in a cultivar-unspecific manner when injected into leaves of barley (Wevelsiep *et al*., 1991) and other cereal plant species such as wheat, rye and oat (W. Knogge, unpublished results). In addition, NIP1 indirectly stimulates the plasma membrane-localised K^+-stimulated, Mg^{2+}-dependent H^+-ATPase independent of the plant resistance genotype (Wevelsiep *et al*., 1993). Therefore, a role for NIP1 in fungal virulence has been proposed (Knogge and Marie, 1997). Indeed, disruption of the *NIP1* gene in a fungal strain reduced its virulence on susceptible plants compared to the NIP1-expressing wild-type strain (Knogge and Marie, 1997).

R. secalis strains virulent on *Rrs1*-barley plants either lack the *NIP1* gene or carry point mutations in the gene resulting in single amino acid alterations that strongly reduce or abolish the biological activity of the gene product (Rohe *et al*., 1995). Comparison of the amino acid sequences encoded by *NIP1* alleles from several fungal strains so far led to the classification of 4 NIP1 isoforms (Rohe *et al*., 1995). Isoforms type I and type II are elicitor-active on *Rrs1*-barley plants with type II showing somewhat lower activity than type I, whereas type III and type IV are less active or inactive (Rohe *et al*., 1995). Interestingly, the elicitor activity appears to correlate with toxic lesion-inducing activity of the four NIP1 isoforms in barley (Knogge *et al*., 1999). These observations strongly suggest that the elicitor receptor (triggering resistance) and the toxin receptor (conditioning disease) are the same.

Here we describe the presence of a single class of high-affinity NIP1-binding sites on membranes of barley and other cereal plant species. NIP1 binding occurs independent of the plant resistance genotype. In addition, the affinity of the putative NIP1 receptor for the different NIP1 isoforms as assayed in heterologous competitive binding experiments does not correlate with elicitor activity suggesting that the *Rrs1* gene product is not the resistance-mediating primary receptor.

RESULTS

Expression and purification of NIP1 isoforms

Expression and purification of NIP1, type I, has been previously reported (Chapter 3). cDNA encoding the type II protein was obtained by RT-PCR using RNA from *R. secalis*, race AU1 (Rohe *et al*., 1995), as a template. Types II, III and IV have 3 amino acid alterations in common that distinguish them from type I. In addition, the elicitor-inactive isoforms types III and IV are specified by the single amino acid alterations S23→P and G45→R, respectively (Rohe *et al*., 1995). These unique amino acids were introduced into the type I sequence by site-directed mutagenesis to yield the proteins type III* and type IV*. All constructs were cloned into the pQE-30 expression vector (Qiagen, Hilden, Germany) with a sequence encoding factor Xa cleavage site 5' of the sequence coding for the mature NIP1, which allowed the removal of the His tag (Chapter 3). All types of NIP1 were expressed in *E. coli*, purified from inclusion bodies and subsequently refolded (Chapter 3). Purity and masses of the proteins were confirmed by MALDI-TOF yielding 6433.66 Da for type I, 6508.0 Da for type II, 6442.60 Da for type III* and 6531.84 Da for type IV*. All values are in agreement with the expected values.

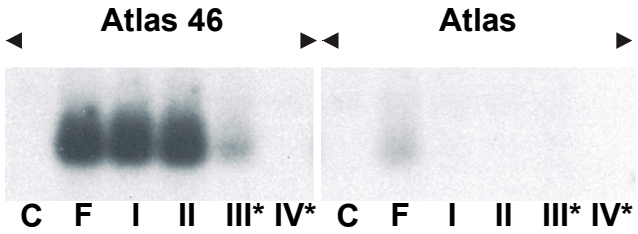

Figure 1: RNA gel blot containing RNA from primary leaves of barley cultivars Atlas 46 (*Rrs1*) and Atlas (*rrs1*) 24 h post treatment with NIP1. Leaves were inoculated with spores of fungal race UK7 (F), containing the *NIP1* gene, or treated with 10 µl of a NIP1 solution containing 150 ng of isoforms type I, II, III*, IV*, respectively, in of 0.05 % Tween-20. As a control (C), leaves were treated with 10 µl of 0.05 % Tween-20.

Elicitor activity of the NIP1 types

Infection of Rrs1-barley leaves by avirulent *R. secalis* races induces the rapid and strong accumulation of mRNA encoding pathogenesis-related protein 5 (PR5). This response is also triggered upon application of purified or recombinant NIP1, type I, to the leaf surface of Rrs1-barley, but not after application to rrs1-barley (Chapter 3). Purified NIP1, type II, induced the same *Rrs1*-specific PR5 mRNA accumulation, albeit to a lower level, whereas the purified protein types III and IV were inactive as elicitors (Chapter 3). The induction of PR5 mRNA accumulation was again used to characterize the elicitor activity of the different recombinant isoforms of NIP1. To

detect any minor activity a saturating concentration of 2.33 µM of the recombinant proteins was used. Under these conditions, NIP1 types I and II exhibited the same *Rrs1*-specific elicitor activity (Fig. 1). While type III* retained only a very weak activity on cultivar Atlas 46 (*Rrs1*), type IV* did not show any elicitor activity. In addition, both isoforms were inactive on cultivar Atlas (*rrs1*; Fig. 1). These results indicate that the three alterations specifying type II do not significantly affect elicitor activity whereas the two independent mutations specifying type III and type IV, respectively, are sufficient to drastically reduce or even abolish elicitor activity.

Iodination of NIP1

To study the binding of NIP1 to plant plasma membranes the protein was radioactively labelled with iodine-125. The lactoperoxidase/glucose oxidase method was used, which directly labels the protein by substituting ^{125}I ortho to the hydroxyl group of tyrosine phenolic rings (McFarthing, 1992). NIP1 contains three tyrosine residues and, hence, six putative iodination sites that may give rise to mixtures of protein labelled at single or multiple sites. Therefore, the method was developed and optimised using non-radioactive ^{127}I. Several protein peaks eluting from the HPLC column (Fig. 2) were analysed by MALDI-TOF mass spectroscopy. The protein in fraction 1 has a molar mass of 6443 Da corresponding to non-iodinated NIP1, whereas the mass of the protein in fraction 2 determined to be 6563 Da indicates mono-iodination. In contrast, fractions 3 (6564 Da : 6691 Da = 2:5), 4 (6564 Da : 6690 Da = 1:8) were mixtures of mono- and di-substituted NIP1, whereas fraction 5 (6692 Da and 6818 Da) of di- and tri-substituted protein forms.

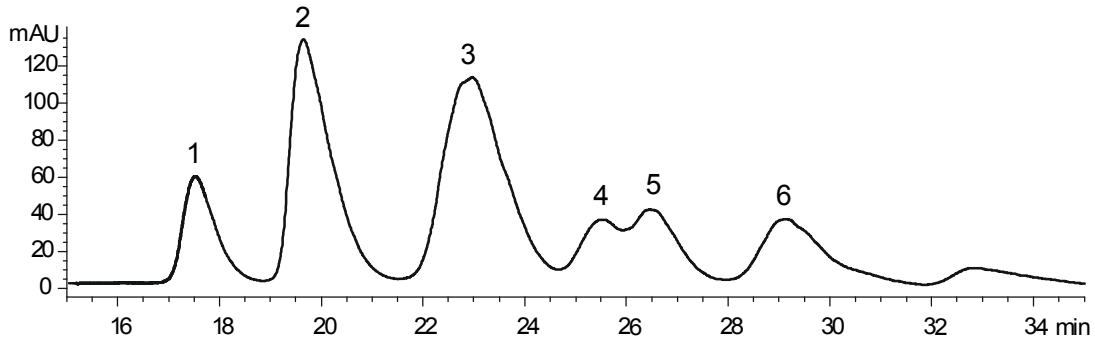

Figure 2: HPLC elution profile of NIP1 iodination products. 1; non-iodinated protein, 2; mono-iodinated protein, 3 and 4; mixtures of mono- and di-iodinated protein, 5; mixture of di- and tri-iodinated protein.

Both fractions 2 and 3 containing mono-iodinated NIP1 yielded sufficient protein to test the elicitor activity. The position of ^{127}I is not known. However, iodination did not significantly affect the capacity of NIP1 to induce PR5 mRNA

accumulation in Rrs1-barley leaves. In addition, structure and activity of the iodinated elicitor were stable in solution for at least three days at room temperature (data not shown). Using the optimised protocol for labelling and purification, ^{125}I was commercially incorporated into NIP1, type I (ANAWA Laboratories Inc., Zuerich, Switzerland), to yield a product with a specific radioactivity of 2,130 Ci/mmol. This ^{125}I-NIP1 co-eluted during RP-HPLC with the ^{127}I-labeled protein eluting in fraction 2.

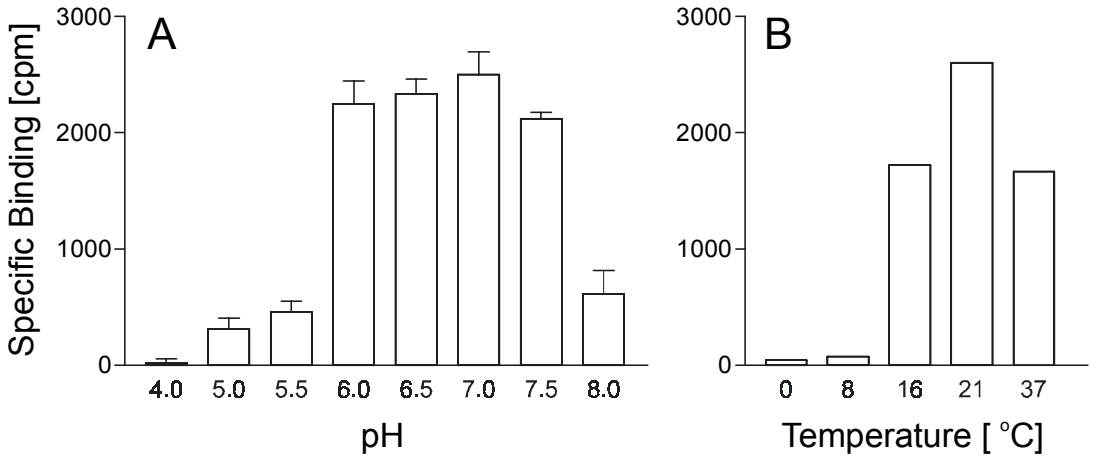

Figure 3: Effects of pH and temperature on binding of NIP1 to barley microsomal membranes. Binding of ^{125}I-NIP1 was measured after 2 h of incubation at room temperature using a concentration of 2.5 x 10^{-10} M and 50 µg x $µl^{-1}$ of microsomal membrane protein in buffers at different pH values (**A**) or at pH 7.0 at different temperatures (**B**).

Binding of ^{125}I-NIP1 to barley microsomes

Binding of ^{125}I-NIP1 to microsomal fractions from primary leaves of barley cultivar Atlas 46 (*Rrs1*) was investigated under different external conditions. A vacuum filtration technique was used to separate free from bound ligand in order to decrease the loss of bound radioactivity caused by rapid dissociation of the receptor-ligand complex. Highest binding activity was found between pH 6 and pH 7.5, while the optimum temperature was around 21°C (Fig. 3). At 37°C, a rapid and irreversible precipitation of the microsomes occurred, independent of the presence of NIP1. Specific binding of ^{125}I-NIP1 to barley microsomes represented about 60 % of total binding at an initial ligand concentration of 2.5 x 10^{-10} M. Less than 5 % of the initially applied ligand eventually bound to microsomal membranes, reducing the possibility that ligand depletion interfered with the characterization of the NIP1 binding site. The amount of specifically bound radioactive ligand increased linearly with increasing amounts of membrane protein, ranging from 30 to 200 µg of protein in the standard assay.

To localize the specific binding site, binding assays were performed using plasma membrane vesicles prepared by phase partition from leaves of barley cultivar Atlas 46 (Kjellbom and Larsson, 1984; Wevelsiep *et al*., 1993). Compared to microsomal membranes, these vesicles showed a two-fold higher binding capacity per milligram of protein indicating that the binding site is also localized on the plasma membrane (data not shown). All further experiments were performed with microsomal membrane fractions.

Characterization of the NIP1 binding site in barley

Analysis of elicitor binding kinetics demonstrated that before an equilibrium is reached, at 125 pM ^{125}I-NIP1 association is faster than dissociation. Half-maximal binding was achieved within 25 min after addition of the ligand, and equilibrium between association and dissociation was reached after 60 min (Fig. 4). Non-specific binding only slightly increased during this time. Addition of a 1,000-fold molar excess of unlabeled NIP1 at t = 60 min initiated a rapid dissociation of bound radioactive NIP1 with a dissociation rate constant (K_{off}) of 6.5 x 10^{-4} s^{-1}, demonstrating that binding of ^{125}I-NIP1 to barley microsomes is a reversible process.

To obtain further information on the nature of the binding site saturation experiments were performed. Microsomal membrane fractions were incubated with increasing amounts of ^{125}I-NIP1. At a concentration of ~60 nM a saturation state was achieved (Fig. 5A). The dissociation constant K_d was determined by non-linear regression to be 5.6 nM (S.E. = 1.0 nM). A linear display of the data in a Scatchard plot (Fig. 5B) suggests a single class of NIP1-binding sites, with concentration of 255 fmol (S.E. = 15 fmol) per mg of microsomal membrane protein.

K_d. Competition of both non-radioactive ^{127}I-NIP1 (not shown) and non-iodinated NIP1 (Fig. 6A) with ^{125}I-NIP1 yielded very similar IC_{50} values. This indicated that iodination does not affect the binding properties of NIP1, thus allowing the use of non-iodinated NIP1 as competitor in binding assays. In contrast, reduced HPLC-purified NIP1 did not compete for binding at a concentration of 1 µM (not shown). This is in agreement with the fact that reduction of NIP1 also abolishes its elicitor activity (Hahn *et al*., 1993). The Hill plot of the competition curve was determined to be on straight curve with a slope -1.03, again indicating a single class of binding sites.

To test whether NIP1-binding correlates with the presence of the *R* gene, *Rrs1*, microsomal membranes of the susceptible barley cultivar Atlas (*rrs1*) were prepared and both time course experiments and competition assays were performed. No significant differences both in the affinity to NIP1 and in the binding kinetics were observed between microsomal fractions from Rrs1- and rrs1-plants (data not shown).

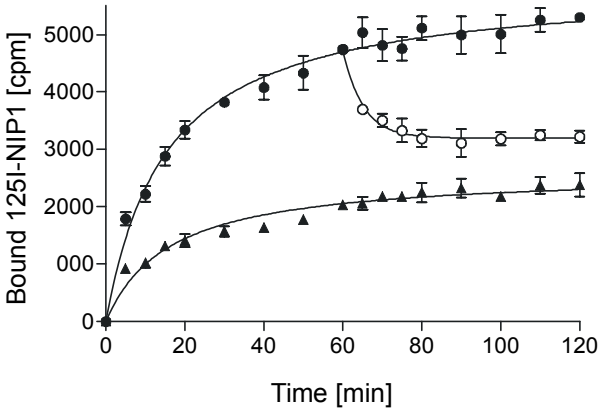

Figure 4: Time courses of specific binding of ^{125}I-NIP1 to microsomal fractions of barley and displacement by unlabeled NIP1. Assays were initiated by the addition of 125 pM ^{125}I-NIP1 to barley microsomes (50 µg membrane protein). Total binding (closed circles), non-specific binding (closed triangles), and binding after displacement of bound ^{125}I-NIP1 by unlabeled NIP1 initiated 60 min after addition of the radio-ligand (open circles) were assayed at the times indicated. Non-specific binding and displacement of bound ^{125}I-NIP1 were determined in the presence of 125 nM unlabeled NIP1. The data points represent the averages of three independent experiments with comparable results.

NIP1 types as competitors in binding experiments

Competitive binding experiments were also performed using the naturally occurring NIP1 type II and the two mutant type III* and type IV* proteins (Figs. 6B-D). The elicitor-active protein type II has an IC_{50} of 671 nM, a value 131-fold higher than that of type I (5.2 nM, Fig. 6E). In contrast, with IC_{50} values of 14.1 and 6.9 nM, respectively, both elicitor-inactive mutant proteins type III* and IV* have apparent affinities for the NIP1-binding site resembling that of type I (Fig. 6F). Very similar results were obtained with microsomes from the susceptible barley cultivar Atlas and from barley plants heterozygous for the *Rrs1* locus (not shown). This demonstrates that binding of NIP1 to microsomal membranes does not correlate with its elicitor activity.

Binding of NIP1 to microsomes from other cereal plant species

NIP1 is a genotype-unspecific toxin inducing scald-like lesions in other cereal plant species including non-hosts such as wheat (Wevelsiep *et al*., 1993). Therefore, microsomal membranes were isolated from wheat, rye, oats and maize as well as from *Arabidopsis thaliana*. Competitive binding experiments revealed the presence of a NIP1 binding site in microsomes from all tested monocotyledonous species with IC_{50} values in a narrow ligand concentration window ranging from 50 % (rye) to 220 % (maize) of that observed for barley (Fig. 7). However, the concentration of binding sites per mg of microsomal protein varied greatly in the different species (wheat: 220

fmol, rye: 150 fmol, oats: 110 fmol, maize: 40 fmol). In contrast, microsomes from the dicotyledonous species *A. thaliana* did not show any affinity for NIP1 (Fig. 7).

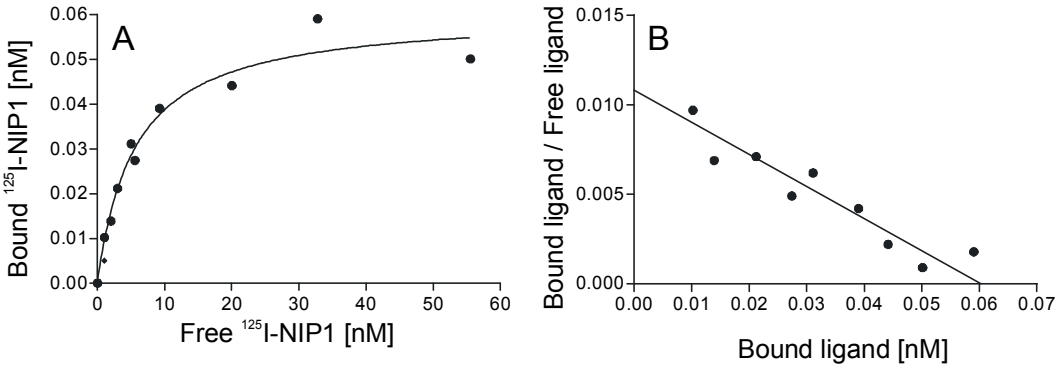

Figure 5: Saturation of the NIP1 binding site in barley microsomal membranes. (A) Specific binding of ^{125}I-NIP1 to microsomal membranes from barley cultivar Atlas 46, using increasing amounts of radio-ligand. **(B)** Scatchard plot displaying the specific binding data derived from **(A)**. K_d (6.0 nM) and number of binding sites (255 fmol x mg^{-1} microsomal protein) were determined by non-linear regression.

DISCUSSION

The fungal *Avr* gene product NIP1 interacts with a single class of high-affinity binding sites

NIP1, a 60 amino acid protein secreted by *R. secalis*, is the product of the *Avr* gene *AvrRrs1* that is complementary to barley resistance gene *Rrs1* (Knogge and Marie, 1997; Rohe *et al*., 1995). The protein triggers defense reactions specifically in resistant (*Rrs1*) barley cultivars but not in susceptible (*rrs1*) cultivars (Hahn *et al*., 1993). Only small amounts of the protein can be purified from fungal culture media (Wevelsiep *et al*., 1991). Therefore, a heterologous expression system was established in *E. coli* that, along with a denaturing/refolding protocol, allows the production of larger amounts of NIP1 (Chapter 3). In addition, this expression system was used to produce NIP1 type III* and type IV* with single amino acid alterations and drastically decreased or no elicitor activity.

The primary function of NIP1 is assumed to relate to its phytotoxic activity (Wevelsiep *et al*., 1991; Wevelsiep *et al*., 1993). Its cultivar-unspecific necrosis-inducing activity and its stimulatory effect on the plant plasma membrane H^+-ATPase suggest NIP1 to be a virulence factor. Evidence for this role was provided by a *NIP1* gene replacement mutant, that exhibited a reduced virulence phenotype producing reduced and delayed lesions on barley leaves compared to the wild-type strain (Knogge and Marie, 1997). The dual functions of NIP1 both as virulence factor and as elicitor prompted to adress the question as to how the protein is perceived by the plant

(Knogge, 1996). The two different activities may be initiated by NIP1 binding to two separate receptors on the host membrane. Alternatively, a single receptor might mediate both functions of the protein. In the latter case, the *R* gene, *Rrs1*, is unlikely to encode the NIP1 receptor. The Rrs1 protein may rather be an additional factor located downstream of the binding site in the signaling pathway. The data presented in this paper indicate the presence of a single class of highly specific NIP1 binding

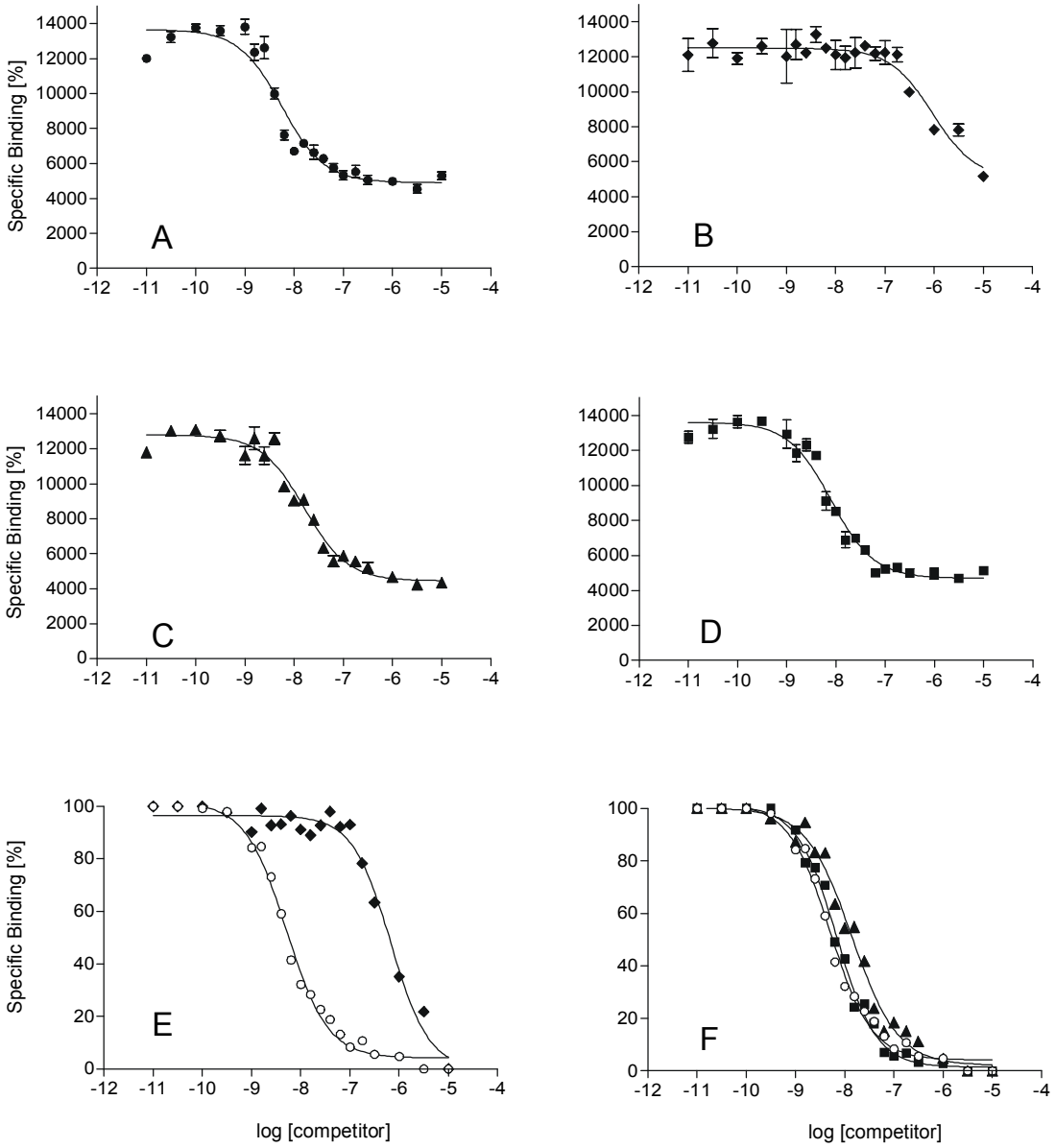

Figure 6: Competition for ^{125}I-NIP1 binding to barley microsomal membranes with different isoforms of unlabeled NIP1 as competitors; **(A)** type I, **(B)** type II, **(C)**, mutant type III*, **(D)** mutant type IV*. All samples were taken from the same experiment. All data points represent the average of 3 independent measurements. Panels **(E)** and **(F)** show normalized competition curves for type I (open circles) and type II (*cf.* panels **(A)** and **(B)**) and for type I (open circles), type III* (filled triangles) and type IV* (filled squares, *cf.* panels **(A)**, **(C)** and **(D)**), respectively. For transparency reasons the error bars are not shown in panel **(E)** and **(F)**.

sites. In conjunction with the finding that amino acid alterations inactivating NIP1 as an elicitor also render the protein non-toxic (Rohe *et al*., 1995), suggesting that the putative receptor is involved both in triggering resistance and in conditioning virulence.

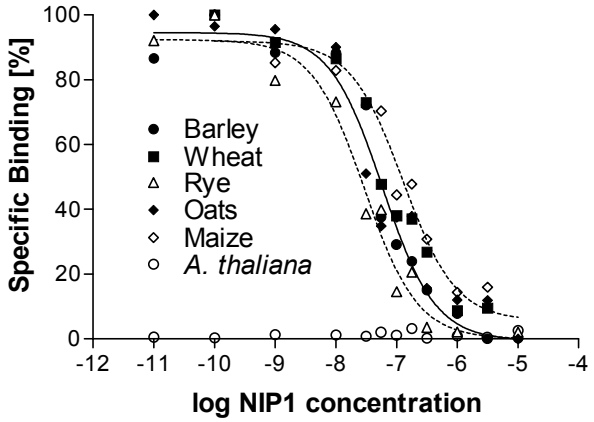

Figure 7: Competitive binding experiments using microsomal membranes from different cereal plant species and *Arabidopsis thaliana*. The dotted lines show the normalized competition curves for rye with the lowest and maize with the highest IC_{50} value, whereas the solid line represents barley. For transparency reasons the error bars are not shown.

Binding of various elicitors to plasma membrane-localized receptors of several plants has been studied. For instance, a 13 amino acid elicitor peptide (Pep-13) released from a 42 kDa glycoprotein of *Phytophthora sojae*, binds to its receptor on parsley plasma membranes with a K_d of 2.4 nM (Nürnberger *et al*., 1994). For elicitins, another class of secreted *Phytophthora* proteins triggering defense reactions in tobacco, K_d values between 2 and 13.5 nM have been reported (Bourque *et al*., 1998; Wendehenne *et al*., 1995). Chitin fragments binding to tomato membranes showed a higher K_d (23 nM, Baureithel *et al*., 1994), whereas a hepta-β-glucoside elicitor from cell walls of *P. sojae* displayed a lower K_d (0.75 nM, Cheong and Hahn, 1991) for binding to soybean membranes. To date the lowest K_d of 0.07 nM was however observed for AVR9 from the tomato pathogen *C. fulvum*, the first *Avr* gene product subjected to binding studies (Kooman-Gersmann *et al*., 1996). The K_d of 5.6 nM calculated for NIP1 is therefore within the range of most elicitor-receptor systems analysed thus far.

The stimulation of PR5 mRNA synthesis upon application of a NIP1 solution to the surface of barley leaves requires a minimum concentration of *ca.* 1 µM (unpublished result), which is 180-fold higher than the calculated K_d. A similar apparent discrepancy between *in vivo* and *in vitro* results has however also been

described for other receptor-ligand interactions. For the fungal phytotoxin fusicoccin a concentration 100-fold higher than the K_d of 1 nM was necessary to stimulate the plant plasma membrane H^+-ATPase (Basel *et al*., 1994). A larger difference (*ca.* 4000-fold) between K_d value and the minimal elicitor concentration required for inducing a hypersensitive response in tomato leaves was described for the AVR9 elicitor from *C. fulvum* (Kooman-Gersmann *et al*., 1996). As also discussed for the latter system, optimal ionic strength and pH may differ at the binding site *in vitro* and in the plant apoplast and could thus contribute to the discrepancy between the concentrations of NIP1 binding and its elicitor activity. In addition, it is to be expected that in the activity assays only a small fraction of NIP1 applied to the leaf surface will diffuse into the leaf apoplast. Consequently, the protein will be significantly diluted and, the effective NIP1 concentration is likely to be lower than the concentration applied to the surface.

A NIP1-binding site is present in barley and other cereal species

The apparent concentration of NIP1-binding sites in barley microsomal preparations of 255 fmol x mg^{-1} of microsomal protein is in the same order of magnitude as described for other elicitor binding sites in plants. For the Pep-13 elicitor from *P. sojae* 88 fmol x mg^{-1} were found in parsley cells (Nürnberger *et al*., 1994), for different elicitins in tobacco 234-403 fmol x mg^{-1} (Bourque *et al*., 1998), for the hepta-β-glucoside from *P. sojae* in soybean cells 1.2 pmol x mg^{-1} (Cheong and Hahn, 1991; Cosio *et al*., 1988), and for chitin fragments in tomato 2.45 pmol x mg^{-1} (Baureithel *et al*., 1994). In comparison, the number of binding sites for the AVR9 elicitor from *C. fulvum* on tomato membranes is 800 fmol x mg^{-1} (Kooman-Gersmann *et al*., 1996).

NIP1 elicits rapid defense reactions exclusively in barley cultivars carrying the *R* gene, *Rrs1*, whereas a NIP1-binding site with identical binding characteristics is present on membranes of both the resistant (*Rrs1*) cultivar Atlas 46 and the near-isogenic susceptible (*rrs1*) cultivar Atlas. In addition, a NIP1-binding site was detected in rye, oats, wheat, and maize, of which the latter two are non-hosts for *R. secalis*. In contrast, no binding site was detectable in *A. thaliana*. These results are similar to data obtained with the AVR9 elicitor from *C. fulvum* for which binding sites are also present in plasma membranes from resistant (Cf-9) and susceptible (Cf-0) tomato genotypes as well as from other solanaceous species (Kooman-Gersmann *et al*., 1996). The presence of NIP1-binding sites in different cereal species correlates with the toxic effect of NIP1 on leaves of these plants (Wevelsiep *et al*., 1991; Wevelsiep *et al*., 1993), while no toxic effects are observed in leaves of *A. thaliana* (K.A.E. van ‘t Slot, unpublished result). In addition, their similar binding characteristics indicate that the binding sites in those crops are highly related.

Loss of elicitor activity of NIP1 on barley is not correlated with loss of binding

Two single amino acid alterations, S23→P in NIP1 type III* and G45→R in NIP1 type IV*, are sufficient to render NIP1 inactive as an elicitor and non-toxic (Rohe *et al*., 1995). However, these inactive mutant proteins still competed efficiently for the NIP1 binding site, displaying IC_{50} values of 14.1 nM (type III*) and 6.9 nM (type IV*) that are close to the K_d found for the active NIP1 type I (5.6 nM). In contrast, NIP1 type II, differing in three amino acid positions from type I, has a much lower affinity for the binding site when compared to NIP1 type I in competitive binding experiment. Its IC_{50} value of 671 nM is more than 100-times higher than the K_d for NIP1 type I, and yet this protein isoform is capable of eliciting the synthesis of PR5 mRNA when applied to *Rrs1* barley leaves, albeit to a somewhat lesser extent (Rohe *et al*., 1995). In addition, races of *R. secalis* carrying the type-II-encoding *NIP1* allele are avirulent on *Rrs1* plants. These data suggest that the affinity of NIP1 isoforms to this binding site is not correlated with its capacity to induce PR protein synthesis.

The lack of correlation between binding and elicitor activity of the different types of NIP1 cannot easily be explained. Recently, the 3D structure of NIP1 has been elucidated by ^{1}H-NMR spectroscopy (Chapter 5). The protein consists of two domains containing two and three anti-parallel β-strands, respectively. Five disulfide bridges render the structure rather rigid except for two flexible loops. The S23→P mutation occurs in one the flexible loops, whereas the G45→R mutation is located in a solvent-exposed β-turn on the same side of the molecule. The elicitor activity may therefore reside in this region of the protein. Two of the type-II-specific amino acids are located in the N-terminal domain of NIP1, the third in the C-terminal domain. At least two of these amino acids appear to be solvent-exposed and may be involved in binding to the primary receptor.

One possible model for the signal transduction mechanism assumes bipartite function of NIP1. The protein interacts through a region containing one or more of the type-II-specific amino acids with a specific domain of the binding protein. This causes a conformational change of the receptor that allows its interaction with the side of NIP1 that contains the type-III- and type-IV-specific amino acids. As the consequence, the transduction of the signal across the membrane is triggered (Knogge, 1996). This model is analogous to the address-message concept proposed for the activation of the flagellin receptor complex in tomato; in a two-step mechanism involving conformational changes of both receptor and ligand, binding of the flagellin N-terminus (address) to the receptor represents the first step that is followed by activation of the response with the C-terminus (message) as the second step (Meindl *et al*., 2000).

An alternative model would be similar to those proposed for the AVR9/Cf-9 interaction (Joosten and de Wit, 1999). Signal transduction may require an additional protein to be involved in the NIP1/NIP1-binding protein complex. This protein may

be prevented from interacting with the receptor-ligand complex by the S23→P and G45→R mutations of NIP1, which however do not affect the affinity for the primary receptor. In contrast, the type-II-specific amino acids affect only the primary binding event, but not the capacity to attract the putative third protein to the receptor complex. In both models, primary binding of NIP1 is not the crucial event (requiring a narrow threshold) distinguishing resistance and susceptibility and the *Rrs1* gene is therefore unlikely to encode the NIP1-binding site (Knogge, 1996). Since the mutations in NIP1 affect both functions of the protein, it remains unclear where the signaling pathways leading to defense gene activation and to H^+-ATPase stimulation and toxicity branch off and, hence, where the *Rrs1* gene product is positioned in the signaling chain. It is however tempting to speculate that in analogy to other systems the Rrs1 protein may "guard" the NIP1 target, which is involved in H^+-ATPase stimulation (Dixon *et al*., 2000; van der Biezen and Jones, 1998). Patch-clamp studies are underway to unravel the very early events that occur at the plant plasma membrane after NIP1 treatment.

MATERIALS AND METHODS

Plant material

The near-isogenic barley (*Hordeum vulgare* L.) cultivars Atlas 46 (*Rrs1*) and Atlas (*rrs1*) were grown as previously described (Lehnackers and Knogge, 1990).

Microsomal fractions and plasma membrane vesicles

Microsomal fractions and plasma membrane vesicles were isolated from 10-day-old barley leaves (Wevelsiep *et al*., 1993; Widell *et al*., 1982). All isolation steps were performed at 4°C. Briefly, barley leaves were shortly infiltrated under vacuum with an ice cold 50 mM HEPES buffer, pH 7.5 (0.5 M sucrose, 5 mM ascorbic acid, 1 mM DTT, 0.6% (w/v) water-insoluble polyvinylpyrrolidon phosphate, 1 mM phenylmethylsulfonyl fluoride). Subsequently, the material was ground in a Waring blender. Microsomal membranes were obtained by filtration through four layers of Miracloth (Calbiochem, La Jolla, CA) and two differential centrifugation steps at 10,000 x g and 100,000 x g, respectively. Plasma membranes were purified from the microsomal membranes by aqueous two-phase partitioning (Larsson *et al*., 1988; Palmgren *et al*., 1990). After isolation, both the microsomal membrane fractions and the plasma membrane-enriched fractions were dissolved in 5 mM potassium phosphate buffer, pH 7.8 (0.33 M sucrose, 3 mM KCl) and subsequently homogenized in a glass potter (Braun, Melsungen, Germany). The membranes were frozen in liquid nitrogen and stored at –80°C.

Preparation of NIP1

NIP1 was expressed in *E. coli* as a histidine-tagged fusion protein (Chapter 3) Briefly, fusion protein was obtained from solubilized inclusion bodies by affinity chromatography using a Ni-NTA affinity chromatography column (Qiagen, Hilden, Germany). Cystine bond shuffling was facilitated using a previously described folding procedure (Beiboer *et al*., 1996) that

involves a cysteine/cystine redox couple. Correctly folded peptide was separated from misfolded peptides by reversed-phase high performance liquid chromatography (RP-HPLC). A factor Xa cleavage site between the mature NIP1 protein and the histidine tag allowed for the preparation of a peptide with identical biochemical properties as the fungus-derived protein. The amount of purified NIP1 was determined by OD_{280} measurement using a molar extinction coefficient of 6440 l x mol^{-1} x cm^{-1}, as determined based on the primary sequence using the Genetics Computer Group (Madison, WI) sequence analysis software package, as well as by using Bradford's reagent (Bio-Rad, Munich, Germany).

Cloning, expression and purification of NIP1 isoforms

The cDNA encoding NIP1, type II, was obtained by RT-PCR on total RNA from *R. secalis*, race AU1, using primer 1 and oligo-dT. The amplified fragment was subjected to a second PCR reaction using primers 1 and 2 (see primer table). Primer 1 is a modification of earlier described primers (Chapter 3). The amino acids specific for isoforms III and IV were introduced into the type I sequence by site-directed mutagenesis to yield the isoforms type III* and type IV*. 3' primers 5 and 6 containing the mutations (underlined) were used along with 5' primer 3 in a first PCR step (see primer table). The products of this PCR were used as mega-primers in combination with 3' primer 4 in a second PCR step. Restriction sites (underlined) for cloning were introduced through primers 1 and 4 (*Sma*I) and primers 2 and 3 (*Bam*HI). The final amplification products were purified from agarose gels, digested with *Bam*HI and *Sma*I and subsequently cloned into the pQE30 expression vector (Qiagen, Hilden, Germany). Expression of the three isoforms in *E. coli* strain DHB4 (Boyd *et al*., 1987) and their purification was performed as described above. MALDI-TOF analysis was used to verify the molecular masses of the peptides. Elicitor activity was analysed as previously described (Hahn *et al*., 1993), routinely using a concentration of 20 ng x μl^{-1} (~ 3 µM).

Primer Table

Primer 1: 5'-GCGGCCCGGGTTAACATTGGCGAAATCCCGTCG-3' (*Sma*I)
Primer 2: 5'-GCGCGGATCCATCGAAGGTAGAGATCGATGCAGATACACCCTTGT TGC-3' (*Bam*HI)
Primer 3 (pQE-30 5'): GCGCGGATCCATCGAAGGTAGAGATCGATGCAGATACACCC TTTGTTGC (*Bam*HI)
Primer 4 (pQE-30 3'): GCGGCCCGGGTTAACATTGGCGGTATCCCGTCG (*Sma*I)
Primer 5 (III*: S→P): GGATTC**TGG**CTCATGTAGGCATG
Primer 6 (IV*: G→R): GCCACCTTC**AC**ATATGAGC

Iodination of NIP1

NIP1 type I was modified with the non-radioactive isotope ^{127}I by using the lactoperoxidase/glucose oxidase system (Sigma, Munich, Germany; (McFarthing, 1992). 100 µl of a 1% D-glucose solution were added to 300 µl PBS buffer, pH 7.0, containing 2 µg NIP1, 10 µl of 100 µM NaI, 1 µg glucose oxidase and 1 µg lactoperoxidase. After 10 min at room temperature, the reaction was stopped by adding 20 µl of a mixture of 100 mg/ml metabisulfate and 10 mg/ml sodium azide. The reaction products were separated on an

analytical RP-HPLC column (Vydac Protein C4, 0.46 x 25 cm, Macherey and Nagel, Dueren, Germany) that was eluted with an acetonitril gradient using a HP chromatograph (Hewlett-Packard, Waldbronn, Germany), and subjected to MALDI-TOF mass analysis. In addition, the elicitor activity of the protein fraction was analysed as described previously (Hahn *et al*., 1993). ^{125}I-NIP1 was obtained from ANAWA Laboratories Inc., Zuerich, Switzerland) with a specific activity of 2,130 Ci/mmol and used in binding assays.

Binding of NIP1 to membrane fractions

Membranes were pre-incubated at room temperature for 15 min in 30 mM HEPES, pH 7.0, 5 mM $MgCl_2$, 1 g/l fatty acid-free BSA (binding buffer) at a membrane protein concentration of 0.5 µg/µl. Binding was initiated by adding different concentrations of ^{125}I-NIP1 in a volume of 10 µl. Non-specific binding was determined in the presence of a 1,000-fold excess of unlabeled NIP1. Glass fibre microfilters (Whatman GF/F) were soaked for several hours in 0.5 % polyethylenimine and transferred to a sampling manifold (Millipore, Bedford, MA). Filters were rinsed with 4 ml of ice-cold washing buffer (binding buffer containing 0.5 M KCl). Filtration of the samples was carried out under vacuum and filters were washed with 2 x 4 ml of binding buffer. The filters were subsequently transferred to 6 ml scintillation vials and 5 ml Ultima Gold scintillation cocktail (Ducheva, Groningen, The Netherlands) were added. After overnight incubation, radioactivity was counted in a scintillation counter (LS1800, Beckman Instruments, Munich, Germany). Analysis of the data was performed using GraphPad Prism® version 3.00 for Windows 98 (GraphPad Software, San Diego, California, USA, www.graphpad.com).

ACKNOWLEDGEMENTS

The authors would like to thank Harrold van den Burg, Laboratory of Biochemistry, Department of Biomolecular Sciences, Wageningen University, for performing the mass spectrometric analyses. In addition, the critical reading of the manuscript by Drs. P. J. G. M. de Wit, D. Scheel, and T. Nürnberger is greatly appreciated. This project was supported by a research grant from the Deutsche Forschungsgemeinschaft (DFG) to W.K.

Chapter 5

Solution structure of the fungal plant disease resistance-triggering protein NIP1 shows a novel β sheet fold

CHAPTER 5

SOLUTION STRUCTURE OF THE FUNGAL PLANT DISEASE RESISTANCE-TRIGGERING PROTEIN NIP1 SHOWS A NOVEL β SHEET FOLD

NIP1 IS A SMALL, SECRETED PROTEIN FROM THE FUNGAL BARLEY PATHOGEN *RHYNCHOSPORIUM SECALIS* THAT HAS DUAL FUNCTIONS; IT IS TOXIC TO LEAF TISSUES OF BARLEY AND OTHER CEREALS AND IT STIMULATES DEFENSE REACTIONS SPECIFICALLY IN BARLEY PLANTS EXPRESSING THE DISEASE RESISTANCE GENE *RRS1*. HERE, WE REPORT THE SOLUTION NMR STRUCTURE OF NIP1. THE NIP1 STRUCTURE IS CHARACTERIZED BY A NOVEL FOLD, CONSISTING OF TWO DOMAINS CONTAINING A β-SHEET OF 2 AND 3 ANTI-PARALLEL STRANDS, RESPECTIVELY. FIVE INTRA-MOLECULAR DISULFIDE BONDS, COMPRISING A NOVEL DISULFIDE BOND PATTERN, STABILIZE THE DOMAINS AND THEIR POSITION WITH RESPECT TO EACH OTHER. A COMPARATIVE ANALYSIS OF THE PROTEIN STRUCTURE WITH THE BIOLOGICAL ACTIVITY OF FOUR NIP1 ISOFORMS SUGGESTS TWO LOOP REGIONS TO BE CRUCIAL FOR THE RESISTANCE-TRIGGERING ACTIVITY OF NIP1.

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To be submitted

INTRODUCTION

Plant resistance to pathogens is frequently controlled by disease resistance (*R*) genes that recognize matching avirulence (*Avr*) genes in the pathogen. Plant defense reactions are activated when the product of an *R* gene directly or indirectly interacts with the product of a microbial *Avr* gene. Sequence analysis of *R* genes from various plant species revealed classes of gene products with common structural motifs such as leucine-rich repeat domains, nucleotide-binding sites, leucine-zipper domains or domains similar to the cytoplasmic Toll/Interleukin-1 receptor (Takken and Joosten, 2000). Furthermore, studies on the signal-transduction components that play a role in plant disease resistance have discovered remarkable similarities with innate immunity pathways in insects and mammals (Cohn *et al*., 2001). In contrast, with the exception of the bacterial *AvrBs3*-homologous genes, such structural similarities have not been found among the *Avr* genes known to date. While the products of *R* genes are putative receptors and part of signal perception and transduction pathways, *Avr* genes encode proteins that are intrinsically virulence factors produced by pathogens to efficiently utilize to the plant's nutrient reservoir. Their secondary role as specific signals in plant resistance triggering therefore appears to be a function of the plant's recognition capability (Chapter 2).

In contrast to more than 40 bacterial *Avr* genes, only very few *Avr* genes have been characterized from plant pathogenic fungi. The *Avr* gene, *Avr-Pita*, from the rice pathogen *Magnaporthe grisea* encodes a putative Zn protease that was recently shown to directly and specifically interact with the product of the *R* gene, *Pi-ta*, within the host cytoplasm (Jia *et al*., 2000). This situation is reminiscent of many bacterial *Avr* gene products that are transferred into host cells via the bacterial type III secretion system (Galán and Collmer, 1999). In contrast, the products of the other characterized fungal *Avr* genes from the tomato pathogen *Cladosporium fulvum* and from the barley pathogen *Rhynchosporium secalis* encode small proteins that function as extracellular elicitors of plant defence reactions when applied to host lines expressing the matching *R* genes (Laugé and de Wit, 1998). In these cases, the mechanism by which the fungal signal is perceived by the plant and transduced into the host cell to launch defence reactions is not known. However, high-affinity binding sites for two of the *Avr* gene products, AVR 9 from *C. fulvum* (Kooman-Gersmann *et al*., 1996) and NIP1 from *R. secalis* (Chapter 4) have been identified on membranes of resistant and susceptible host plants as well as of non-host species of often related plant families.

The *NIP1* gene from *R. secalis* encodes an 82-amino acid protein, which upon cleavage of a signal peptide yields a 60-amino acid mature protein that lacks homology to sequences present in the databases. This protein, NIP1, contains 10 cysteine residues (Rohe *et al*., 1995), all of which are involved in intramolecular disulfide bonds (Chapter 3). Application of NIP1 to leaves of barley lines carrying the

R gene *Rrs1*, but not of a near-isogenic line lacking this gene, induced the rapid accumulation of mRNAs encoding pathogenesis-related proteins (Hahn *et al*., 1993). In contrast, the phytotoxic activity of NIP1 is independent of the plant genotype and appears to be based on an indirect stimulatory effect on the plasma membrane-localized K^+-stimulated, Mg^{2+}-dependent H^+-ATPase (Wevelsiep *et al*., 1993).

All fungal races avirulent on *Rrs1*-barley carry and express the *NIP1* gene. In contrast, virulent races either lack the gene or carry alleles containing point mutations that translate into single amino acid alterations (Rohe *et al*., 1995). So far, four types of NIP1 have been characterized that differ significantly in their biological activities. Understanding the effect of the amino acid differences between the four types of NIP1 on their function is an important step towards the elucidation of resistance triggering. To obtain more insight into the structure-function relationships, the three-dimensional structure of NIP1 was solved using NMR spectroscopy. The resulting structure shows a novel protein fold that consists of two domains comprising β-sheets. By integrating the structural information with the results from binding and biological activity studies using the four NIP1 isoforms, a model is presented for NIP1 perception in the interaction between *R. secalis* and its host plant, barley.

RESULTS

Assignment of disulfide bonds

NIP1 contains five intra-molecular disulfide bridges (Chapter 3). Determination of the disulfide bridge structure of NIP1 involved five steps: (1) partial reduction, (2) cyanylation of the free thiol groups, (3) separation of the different singly reduced peptides, (4) chemical cleavage of the peptide bond between each of the cyanylated cysteines and their preceding amino acid, and finally (5) mass determination of the resulting peptides (Wu and Watson, 1997).

Native NIP1 was partially reduced using the water-soluble reagent TCEP. The reactions were performed in 6 M GuCl to facilitate the accessibility of the five disulfide bridges. In contrast to results reported previously (Wu and Watson, 1997), the disulfide bonds did not appear to be equally accessible to TCEP in 6M GuCl. One disulfide bond opened significantly more easily than the remaining four. After HPLC separation of the reaction mixture (Fig. 1*a*), four singly reduced peptides were identified by MALDI-TOF MS. Mass spectroscopy proved the other peaks to have more than one reduced disulfide bond. The fifth singly reduced NIP1 peptide could not be detected, also not by using the alkylation reagent, NEM as an alternative for the cyanylation reaction (van den Hooven *et al*., 2001). The peptide fragments were analyzed following the protocol described in the Materials and Methods section. Mass determination always resulted in the identification of two of the three expected mass

peaks (Table 1). β-Elimination, a common side-reaction, was less frequently observed than reported by others (Wu and Watson, 1997). Interpretation of the results led to the disulfide pattern presented in Fig. 1*b*.

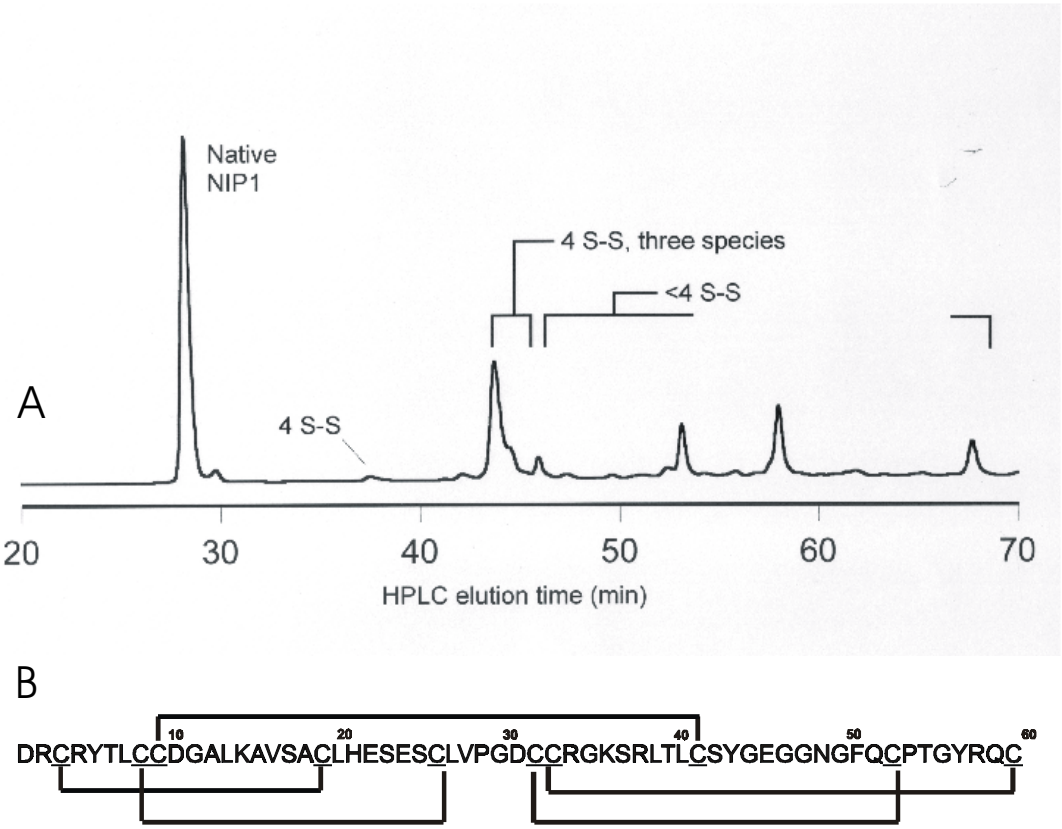


Fig. 1 Assignment of disulfide bonds. ***a***, HPLC separation of NIP1 and its partially reduced/cyanylated isomers at 37 °C. The peaks annotated 4 S-S represent singly reduced/cyanylated isomers, as determined by MALDI-TOF. ***b***, Disulfide bonds in NIP1.

NMR results and structure determination

Spectral assignments and restraints were obtained using the standard strategies on the basis of the suite of experiments mentioned in Materials and Methods. The quality of the data is exemplified in Fig. 2, which presents the $^{15}N,^{1}H$ HSQC spectra of 2 mM NIP1 recorded at pH 6 and 298 K. The spectrum shows a very good dispersion of all backbone amide ^{15}N-H cross peaks, indicative of a well-structured protein. All expected cross peaks (57 backbone amide protons, 11 side chain cross peaks from 2 Asn (i.e., 4 cross peaks), 1 Gln (2 cross peaks) and 5 Arg side chains (5 cross peaks) were identified. The experiments used for assignment (see Materials and Methods) led to the successful identification of all resonance frequencies. The complete proton and nitrogen resonance assignments are deposited in the BMRB database, number 5199. From the NOESY spectra, the H-bonds, the disulfide bridge information and the

torsion angle measurements, 785 constraints were derived, leading to 13.1 constraints per residue. From the NOEs (740), 232 are intra-residual, 241 sequential and 54 medium-range (between two and five residues apart in the sequence). Furthermore, 213 long-range NOEs (five or more residues apart in the sequence) were identified. Fig. 3 shows the distribution of these NOEs over the 60 residues. On the basis of these restraints, 100 structures were calculated using the program DYANA

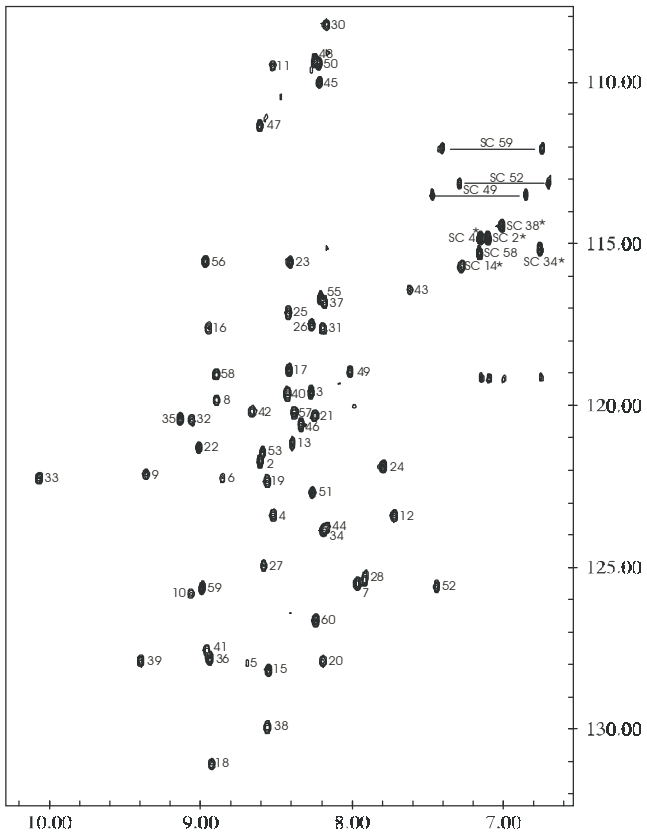


Fig. 2 ^{1}H-^{15}N HSQC spectrum of 2 mM NIP1 at 25 °C, 500 MHz. All cross-peaks are marked by the corresponding residue number. Side-chain cross-peaks have been indicated as well. Folded cross-peaks are marked with an asterisk.

(Guntert *et al*., 1997). The 25 structures with the lowest target function and no distance violations larger than 0.5 Å were selected. Fig. 4*a* shows a superposition of the backbone atoms (N, C_α, C) of residues 3-11, 14-45 and 51-60. The structure of NIP1 reveals two domains. The N-terminal domain consists of two antiparallel β-strands (residues 4-8, β1, 16-20, β2) that are connected by a flexible turn (loop I, residues 10-14) and that end in a type-I β-turn (residues 21-24). The second domain consists of three antiparallel β strands; two longer strands (residues 30-34, β3, 37-41, β4) connected by a β-turn (residues 34-37), and a short β-strand (residues 58-59, β5).

A large flexible loop (loop III, residues 45-51) and a type II β-turn (residues 54-57) connect the long strands with the short one (Fig 4*b*). The NIP1 structure is available from the pdb (1KG1).

Table 1. Calculated m/z values for NIP1 cleavage fragments and observed values (in italics)[1]

Reduction of disulfide	*N-terminus 1st Cys*	*itz-2nd Cys C-terminus*	*β-elimination N-terminus*	*β-elimination C-terminus*	*itz-1st 2nd Cys*
Cys3-Cys19	1946,3	4542,1	1914,3	6167	1695
		4541.4	*1910.4*		*1698.1*
Cys8-Cys26	927,06	3756,2	2700,1	5512	1845,1
		3757.7	*2698.7*		
Cys9-Cys42	1030,2	2053,2	4403,1	5420	3445,9
		2052.2			*3445.2*
Cys32-Cys53	3316,8	953,03	5503,3	3122	2258,5
Cys33-Cys60	3419,9	147,14	6309,2	3019	2962,3
	3415.5				*2961.2*

[1] *Peaks smaller than 1000 m/z were not observed due to the settings of the MALDI-TOF MS mass gate*.

The energetic and geometric statistics of the best 25 structures are shown in Table 2. All structures are in good agreement with the experimental restraints. No structures showed consistent (>60 % of all structures) NOE structure violations larger than 0.2 Å or dihedral angle restraints larger than 3°. The Ramachandran plot of the ensemble showed that 97 % of the residues lie in the allowed regions. From the relaxation measurements, R1 and R2 rates and NOE values were determined and subsequently used to calculate spectral densities, employing the reduced spectral density mapping method (Farrow *et al*., 1994). Fig. 5 shows the spectral densities, which were calculated at zero, ω_N and ω_H (50.7 and 500 MHz, respectively). By comparing the χ^2 values of the 'isotropic' Modelfree analysis with those of the 'anisotropic' one, it was concluded that dynamics of NIP1 could be described with a model in which isotropic tumbling is included. The mean value of R2/R1 ratio was used to assess the overall rotational correlation time. From this ratio a τ_c of 4.8 ns was determined. As can be seen from the reduced spectral density mapping (Fig. 5), the dynamic properties are quite similar for all residues in the protein. Some residues do have different relaxational behaviour, but in the analysis of the Modelfree Approach

these residues could not be fitted well, *i.e.*, the relaxation behaviour of these residues is too complex to be described with the data currently available. Therefore, analysis of the reduced spectral density function provides sufficient information to explain most of the dynamic properties of NIP1.

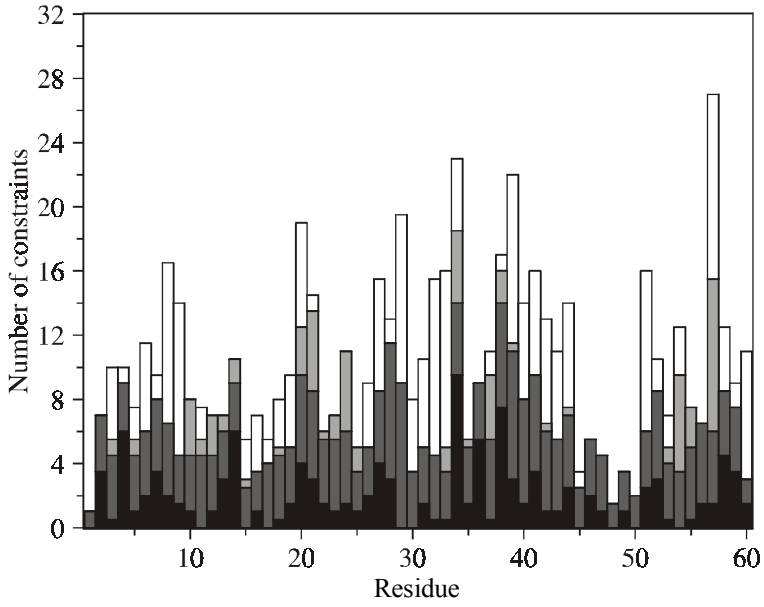


Fig. 3 Histogram showing the number of NOE-derived distance constraints per residue. The bars represent intra-residue restraints (black), sequential (dark gray), medium-range (light gray) and long-range NOEs (white).

DISCUSSION

The NIP1 structure

NIP1 has a well-defined structure that is dominated by β-strands (Fig. 4*b*). It consists of two domains that have a well-defined mutual orientation. The five intra-molecular disulfide bonds play a major role in the folding of NIP1, providing a high level of stability. Circular dichroism measurements showed that most of the secondary structure elements are preserved even up to 100°C (V. Li and W. Knogge, unpublished results). The applied "partial-reduction procedure" to establish the disulfide bond pattern worked well for NIP1, despite the fact that only four singly reduced species were directly identified. The NMR measurements confirmed the positions of the disulfide bonds determined by the biochemical method.

When examined in detail, several interesting features can be identified (Fig. 4*b*). With the exception of the first two amino acid residues at the N-terminus, loop I and in particular loop III, the structure of NIP1 is rather rigid. This is mainly a consequence of the presence of the five disulfide bonds. Starting at the N-terminus,

Table 2. Summary of restraint violations and quality analysis for 25 NIP1 structures

DYANA target function ($Å^2$)	1.52 ± 0.32
upper distance limit violations, average maximum (Å)	0.43 ± 0.10
lower distance limit violations, average maximum (Å)	0.28 ± 0.08
dihedral angle violations, average maximum (°)	0.04 ± 0.02
van der Waals violations, average maximum (°)	0.14± 0.04
Ramachandran plot	
most favoured	67.4 %
additionally favoured	29.5 %
generously allowed	0.9 %
disallowed	2.2 %
RMSDs	
rmsd backbone and heavy side-chain, all residues (Å)	1.753
rmsd backbone,all residues (Å)	1.157
rmsd backbone and heavy side-chain, residues 3-11,14-45,51-60 (Å)	1.273
rmsd backbone, *idem* (Å)	0.660

the first β-sheet is formed by residues 4-8 (β1) and 16-20 (β2). By forming a covalent linkage between the flexible N-terminal part of the protein and β2, thereby crossing the first β-sheet, the disulfide bond between Cys3 and Cys19 provides stability to the first anti-parallel β-sheet. However, the amide protons in this β-sheet exchange faster than those in other parts of the protein (data not shown) indicating that conformational exchange occurs in this β-sheet. Furthermore, residues 5 and 6, in the center of the first β-strand, and residue 16 in β2, have higher J(0) values than their neighboring residues, while their $J(\omega_N)$ and $<J(\omega_H)>$ values are comparable (Fig. 5). Residues 6 and 16 have slowly exchangeable amide protons (data not shown), so this dynamical behavior can be explained by conformational exchange (*vide supra*). The flexible loop

between β1 and β2 (loop I, residues 10(9)-15) is less well defined (Figs. 4*a*, *b*). In addition, residue 13 experiences conformational exchange (Fig. 5). Loop II connects the two domains (between β2 and β3). The first β-sheet ends in a type-I β-turn (residues 21-24). When examining the combination of φ and ψ angles, residues 24-28 in loop II are well defined and can be recognized as β-strand.

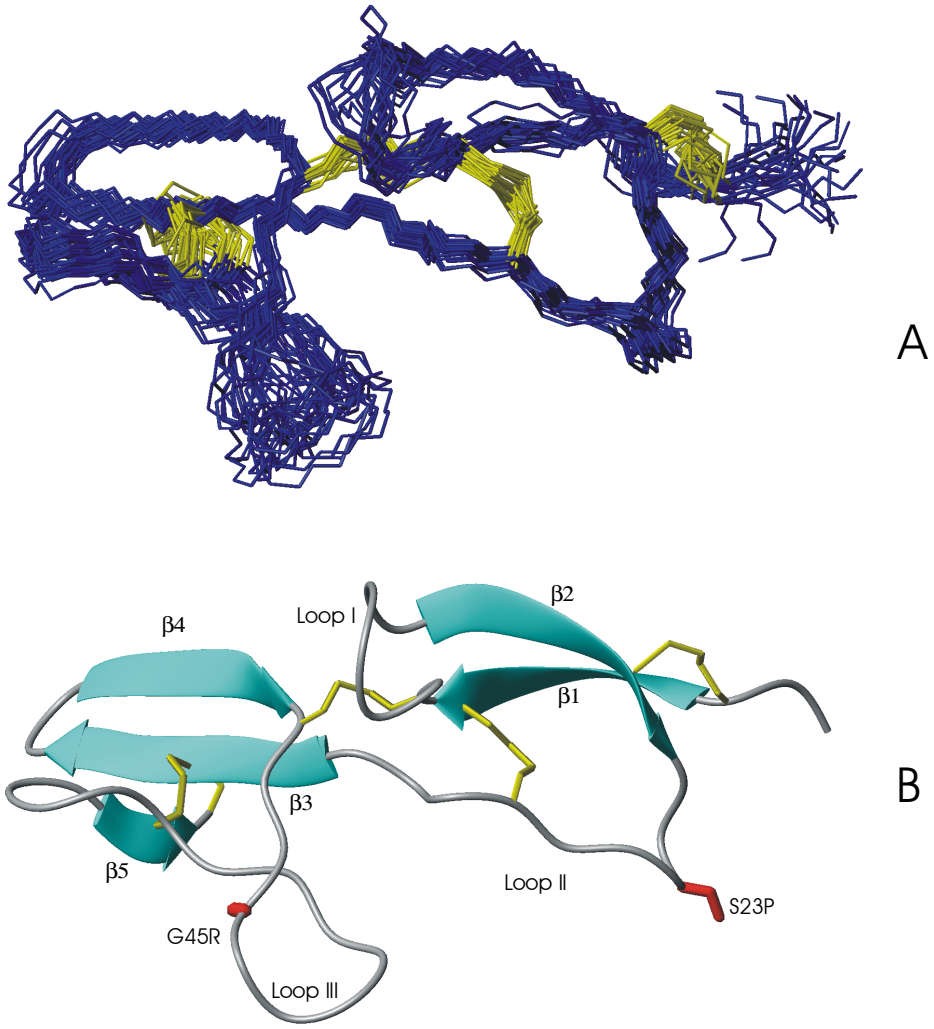


Fig. 4 Solution structure of NIP1. ***a***, View showing best-fit superposition of the backbone atoms (N, C_α, C) of 25 structures of NIP1. The structures were overlaid for residues 3-11, 14-45, 51-60. All cysteine bridges are depicted in yellow. ***b***, Ribbon diagram of the structure of NIP1 type I. The disulfide bridges are depicted in yellow. All secondary structure elements are indicated, as well as the loops and the N- and C-terminus. The side chains specifying NIP1 types III* and IV* have been colored red.

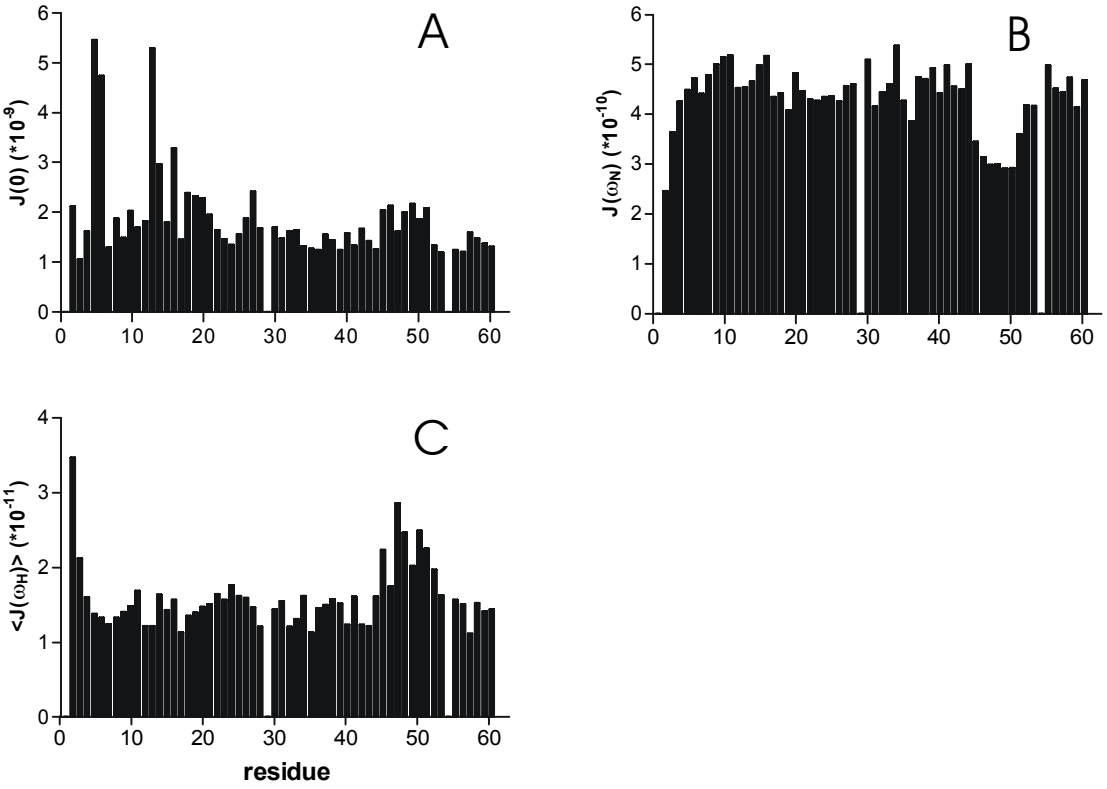


Fig. 5 Values of the spectral density function versus the residue number of NIP1. The spectral density function was determined at the frequencies (A) 0, (B) ω_N and (C) $<J(\omega_H)>$.

However, no hydrogen bond partners are found for the amino acids. The exception is residue 27, the oxygen of which is hydrogen-bonded to the NH of residue 9, thereby forming an isolated β-bridge, which causes a restriction on the possible conformations. In addition, the cystine formed by residues 8 and 26 makes this part of the loop even more stable. Residue 9 is also the cystine partner of residue 42, providing an additional connection between the two domains. The stabilized orientation of the two domains with respect to each other is further illustrated by the presence of multiple NOEs between the domains. Especially, the aromatic residue, Phe51, has many interactions with residues in both domains (not shown).

The β-sheet in the second domain consists of three anti-parallel β-strands; β3 (30-34), β4 (37-41) and β5 (58-59). The sheet is well defined (Figs. 4*a*, *b*) and ends at the C-terminus of the protein, where the cysteine bridge between residues 33 and 60 provides additional stability. The first two strands, β3 and β4, are connected by a β-turn (residues 34-37), while β4 and β5 are connected by loop III. In this loop, residues 43-44 and 52 have well determined φ, ψ angle combinations that fall in the β-strand region. The conformational space in this domain is also restricted by the cystine formed by residues 32 and 53. Residues 45-51 belong to loop III, which can adopt several conformations (Fig. 4*a*). The ends of the loop are restricted by the presence of

a hydrogen bond between residues 44 and 51, forming a second isolated β-bridge. From the relaxation data it is concluded that the loop comprising residues 45-51 is rather flexible. The high $<J(\omega_H)>$ values indicate high-frequency motions for these residues. However, the J(0) values of these residues are also higher, indicating more complex dynamics. These residues are not only subject to high-frequency motions, but experiences slower, millisecond-timescale motions as well. This is probably due to conformational exchange, since for some of these residues small additional exchange peaks are observed in the HSQC-spectrum (data not shown). The loop is rather solvent accessible and glycine-rich; thus this dynamical behaviour might have important implications for the biological function of NIP1. Loop III ends in a type-II β-turn (residue 54-57).

Overall, it can be concluded that the first domain is somewhat 'less stable' than the second. This observation is in agreement with the results from the biochemical determination of the disulfide bonds. The accessible surface of the sulfur atoms, as determined by MOLMOL (Koradi *et al*., 1996) (Table 3), is high for the two sulfurs involved in the bridge between Cys3 and Cys19. This bond could be reduced with significantly more ease than the other bonds. In contrast, the sulfur atoms of Cys32 and Cys53, which are completely buried in the interior of the protein, are inaccessible for TCEP, and no four-disulfide species were detected in which the disulfide bond between Cys32 and Cys53 was reduced. For the AVR9 elicitor protein, the reactivity of the different disulfide bonds with TCEP was reflected in the solvent-accessible surface of the structural homologue carboxypeptidase inhibitor (van de Hooven *et al*., 2001). Concurrently, the presence of 6M GuCl does not facilitate the TCEP reduction of the Cys32-Cys53 bridge, which is positioned between two strands of the anti-parallel β-sheet. The presence of this disulfide bond may stabilize the tertiary structure of this part of the protein, even in 6 M GuCl. This has been reported for lysozyme (Chao and Cheng, 1992). Even the Cys33-Cys60 is hardly reduced by TCEP although Cys60 has quite an extensive solvent accessible surface. If we consider that the tertiary structure remains even at 6 M GuCl than it is not surprising to see that also this bond is almost unaffected by the reductans. Additionally, the negative charge at the carboxyl-group at the C-terminus (Cys60 is the C-terminus) may be unfavorable for the negatively charged phosphine-group in TCEP to attack this bond at the more solvent exposed sulfur. Conclusively, we see that calculated tertiary structure of NIP1 gives a more than satisfactory explanation for the different ratios observed for the different singly reduced species.

The NIP1 elicitor has a novel fold

The structure of NIP1 shows no homology to structures in the protein data bank, as was verified using the DALI server (http://www2.ebi.ac.uk/dali/). In addition, proteins with a comparable biological function, all show different structural features. The

global fold of the AVR9 elicitor from the fungal tomato pathogen *Cladosporium fulvum* has been determined (Vervoort *et al*., 1997), and, although the tertiary structure of the protein has not yet been completely elucidated, the presence of a cystine knot motif could be revealed. The preliminary three-dimensional structure of AVR9 shows homology to carboxypeptidase inhibitor, of which the X-ray structure is known (Joosten *et al*., 1997; Rees and Libscom, 1982; Vervoort *et al*., 1997). This structural motif was not found in NIP1. The only additional structure available from a microbial elicitor protein, the elicitin cryptogein (Gooley *et al*., 1998) from *Phytophthora cryptogea*, consists mainly of α-helical elements. Therefore, the NIP1 structure can be regarded as a novel fold and it may be the first representative of an evolutonary superfamily.

Table 3. Solvent-accessible surface of the sulfur atoms in the final NMR-structure of NIP1

	Sulfur area (Å^2) per Cys	*Total area (Å^2) per Cys*
Cys3-Cys19	3.0 (4.5 %) - 26.4 (38.7 %)	66.3 - 68.3
Cys8-Cys26	0.5 (0.7 %) - 2.0 (2.9 %)	73.3 - 68.3
Cys9-Cys42	0.0 - 8.5 (11.9 %)	75.8 - 71.3
Cys32-Cys53	0.0 - 0.0	65.3 - 74.8
Cys33-Cys60	28.4 (42.9 %)-1.0 (1.3 %)	75.3 - 75.3

Structure-function relationship

Binding of NIP1 to a binding site on *Rrs1*-barley membranes is regarded as the initiating event in a signal transduction pathway that ultimately leads to resistance. The mechanism by which this and other elicitors trigger the plant defense response remains under intensive analysis. An equally intriguing subject is the basis for differences in the activity of NIP1 isoforms as elicitor. Minor variations in the sequence influence the biological activity of NIP1 in a profound way. NIP1 type I displays the highest elicitor activity, while three amino acids alterations in NIP1 type II result in a reduced activity (Rohe *et al*., 1995). NIP1 type III and NIP1 type IV differ in single amino acid positions from NIP1 type II (Rohe *et al*., 1995). Integration of these amino acid alterations into the NIP1 type I sequence yielded the biologically inactive proteins type III* and type IV* (Chapter 2). The solution NMR studies of NIP1 reported here are an important step toward an understanding of the relationships between structure and the observed biological effects.

The NIP1 types III* and IV* show neither elicitor nor toxic activity, implying that the residues that are altered are important for the biological function of NIP1. The

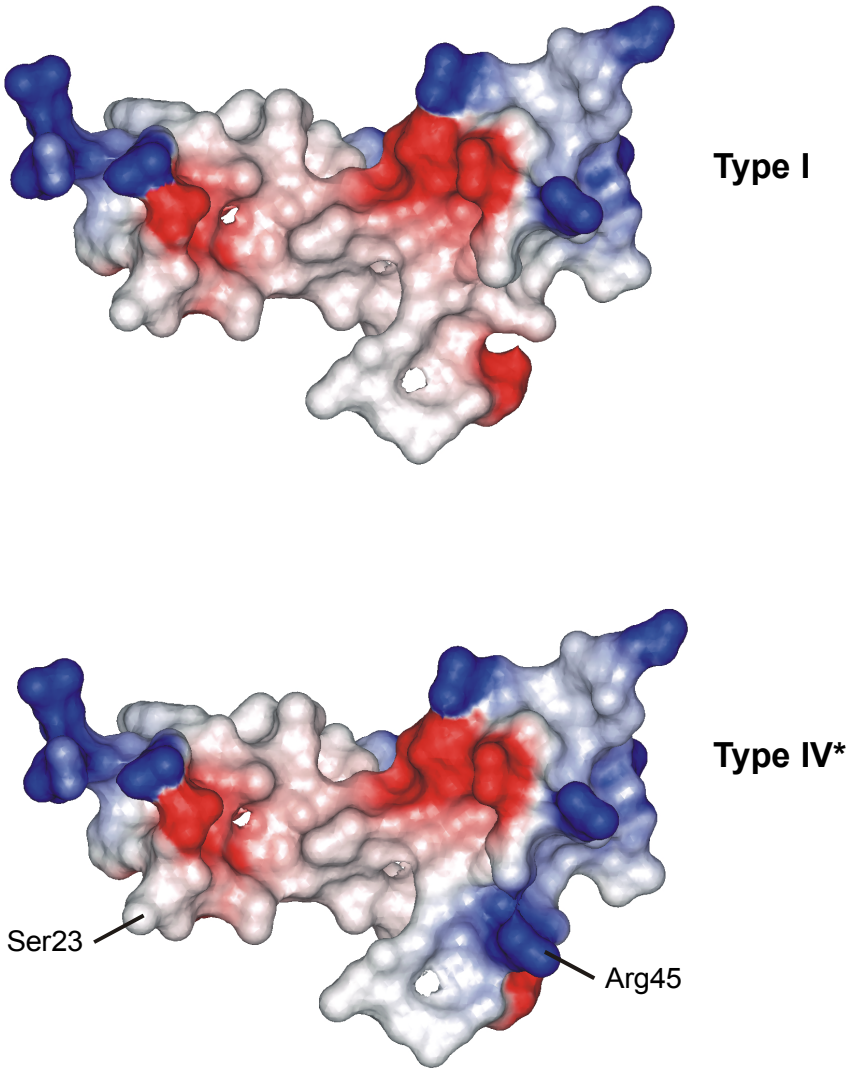


Fig. 6 Electrostatic surface potential of NIP1, type I and type IV*. Surface color reflects the sign of electrostatic potential: red, negative; blue, positive; white, neutral. The structure of NIP1 type IV* was generated by exchanging Gly45 by Arg in the program MOLMOL (Koradi *et al*., 1996). Compared to Fig. 4, the structure of the protein was rotated by 180° around the y-axis.

amino acid alteration in NIP1 type III*, S23P, occurs at the third residue in a type-I β-turn. It is well known that type-I β-turns are compatible with any amino acid residue at its four positions, except a Pro cannot occur at position 3 (Creighton, 1983). Therefore, in NIP1 type III* the β-turn cannot form, and the introduction of a Pro at

this position most probably has a strong impact on the orientation of loop II. The relative position of the two domains may thereby be altered.

NIP1 type IV* carries the mutation G45R. It is positioned in the part of loop III that undergoes high-frequency motions and slower, millisecond-timescale motions. Changing a glycine into an arginine has huge structural consequences. Not only the size difference, but also the introduction of a positive charge in a region, which consists of two negatively charged glutamine residues, has dramatic consequences (Fig. 6). Surprisingly, G45 is also flanked, like S23 (E24), by a negatively charged glutamic acid residue, E46. This indicates that for these two mutations, electrostatic interactions may play an important role in the transmission of the NIP1 signal. Additionally, the introduction of an arginine at position 45 most probably changes the dynamics of the loop. Thus the mobility in this region seems important for the protein function.

Recently, a high-affinity binding site for NIP1 type I was identified on barley plasma membranes (Chapter 4). Interestingly, NIP1 types III* and IV* appear to be efficient competitors for this binding site. Possibly, residues S23 and G45 are important for the interaction with a third component, which is required in the signal transduction pathway that leads to the plant resistance response.

MATERIALS AND METHODS

Sample preparation

For ^{15}N labeling, *E. coli* strain BL21 carrying the pQE30-*NIP1* vector was grown in 5 l minimal medium (Sambrook *et al*., 1989) consisting of M9 salts, 20% glucose, Fe^{2+} (5 ng l^{-1}) and thiamine (500 ng l^{-1}). $^{15}NH_4Cl$ (ARL, Groningen, the Netherlands) was used as the sole nitrogen source. When bacterial cultures reached an OD_{600} of 0.5, IPTG was added to a final concentration of 2 mM, and cells were harvested after incubation for 6 h at 37 °C. Following the protein isolation protocol previously (Chapter 3), 7.2 mg of ^{15}N-NIP1 were obtained from inclusion bodies. The ^{15}N-incorporation was shown to be 97 % with MALDI-TOF MS. The elicitor activity of the labeled protein did not differ from unlabeled NIP1 (data not shown).

Disulfide bond assignments

All solvents used were HPLC grade. Reagents were from Sigma. TCEP stock solutions were stored as previously described (Gray, 1993). Native NIP1 type I (80 µg; M_r = 6433.3 Da) was dissolved in 10 µl of 0.1 M citrate buffer (pH 3) containing 6 M GuCl to facilitate the accessibility of the five disulfide bridges (Wu and Watson, 1997). Partial reduction was performed with a 5 molar excess of TCEP, a reagent, which has proven to be an excellent reducing agent for disulfides at acidic pH (Wu and Watson, 1997). This reaction mixture was incubated at various temperatures for 15 min, directly followed by the addition of 4-5 µl of 0.1 M CDAP, which resulted in cyanylation of the free sulfhydryl groups. An alternative

alkylation reagent, NEM, was used to achieve a better HPLC separation between singly reduced peptides. The partially reduced NIP1 mixture was separated by analytical RP-HPLC, using 0.1 % (v/v) TFA in acetonitrile as eluting solvent. A 150 x 3.9 mm Delta Pak C_{18} column (300 Å, 5 μm, Waters Corporation, Milford, USA) was used. After purification of the singly reduced peptides the masses of the corresponding peptides were determined by MALDI-TOF MS. The singly reduced peptides were freeze-dried, and subjected to chemical cleavage by adding 16 μl 1M aqueous NH_4OH (pH 10) containing 1.5 M GuCl. After 1 h at ambient temperature the samples were air dried in a Speed Vac. The remaining cystines were completely reduced by adding 4 μl TCEP at pH 3. The resulting peptide fragments were further analyzed by mass spectrometry to assign the disulfide bonds (Wu and Watson, 1997; Wu *et al*., 1996; Wu and Watson, 1998).

Molecular masses were determined by MALDI-TOF MS on a Perseptive Biosystems Voyager DE-RP. A saturated matrix solution (α-cyano-4-hydroxycinnnamic acid, Aldrich) was freshly prepared in acetonitrile/water/TFA (50/50/1, v/v/v). One μl of each peptide sample was mixed with 1 μl of matrix solution on the MALDI target plate. External calibration was performed with a tryptic digest of the C116S mutant of 4-hydroxybenzoate 3-monooxygenase (EC 1.14.13.2) using fragments with calculated $[M+H]^+$ of 1099.6 Da and 2086.2 Da, bovine insulin (5734.6 Da) and bovine cytochrome C (12230.9 Da).

NMR experiments

The NMR experiments were performed on Bruker DMX 600 and Varian Inova 500 and 600 spectrometers equipped with a pulsed-field gradient unit and triple resonance probe. The concentration of the NIP1 sample was 2 mM. The spectra were acquired at 25 ^oC and pH 6.0 (pH meter reading). 3D ^{15}N-edited NOESY (80 and 150 ms mixing time), ^{15}N-edited TOCSY (30 ms mixing time), ^{15}N HMQC-NOESY-GHSQC (150 ms mixing time), 2D ^1H-^{15}N HSQC, ^1H COSY (H_2O and D_2O), ^1H NOESY (H_2O and D_2O, 80 and 150 ms mixing time) and ^1H TOCSY (H_2O and D_2O, 25 ms mixing time) spectra were used for the assignments. The spectra were processed using the NMRPipe program (Delaglio *et al*., 1995) running on Silicon Graphics workstations. The data were interpreted using the program XEASY (Bartels *et al*., 1995). Employing the 2D and 3D-NMR spectra using the standard strategy (Wütrich, 1986) successfully performed the resonance assignments of NIP1. Spectra were calibrated relative to TMS (Wishart *et al*., 1992). $^3J_{NH\text{-}H\alpha}$ coupling constants were determined in 3D HNHA (Vuister and Bax, 1992), 2D HMQC-J (Kay and Bax, 1992) and three 2D MJ-HMQC (Xia *et al*., 2000) spectra. $^3J_{N\text{-}H\alpha}$ coupling constants were determined from a 3D HNHB experiment. 2D NOESY and TOCSY experiments were analyzed as well. In the HMQC-J and the MJ-HMQC spectra the coupling constants were determined from the in-phase doublets in the ^{15}N dimension using the fitting procedure INFIT (Szyperski *et al*., 1992).

Steady-state ^1H-^{15}N NOE values, R1 and R2 ^{15}N relaxation times were determined using gradient enhanced sensitivity pulse sequences (Farrow *et al*., 1994). Spectra were acquired on a 500 MHz Varian Inova spectrometer. Two pairs of NOE experiments were recorded with (NOE) and without (NONOE) the use of ^1H saturation applied before the start of the experiment, respectively. Series of R1, with time delays of 15, 45, 105, 200, 400, 600, 800, 1000, 1300, 2000 ms, and R2, with time delays of 8.2, 24.6, 41, 57.5, 73.9, 90.3, 121.3,

172.4, 238, 320 ms, experiments were collected. NOE spectra were acquired 2048 × 170 complex points. The R1 and R2 spectra were acquired using 2048×300 complex points. The relaxation parameters were determined from the peak heights. Data were analyzed using the Modelfree software (Palmer *et al*., 1991) and reduced spectral density mapping (Farrow *et al*., 1994).

Slowly exchanging amide protons were determined from the NH resonances from the fingerprint region cross peaks present in a TOCSY, COSY and NOESY, recorded at 298 K of a NIP1 sample which was fully protonated and lyophilized. The spectra were recorded after dissolving the sample in D_2O within the first 12 h of exchange. These slow exchanging amides are thought to arise from strong hydrogen bonds within the structure.

Structure Calculation

Quantitative distance constraints were obtained from the 3D ^{15}N- NOESY HSQC (80 ms mixing time), HMQC-NOESY-GHSQC (150 ms mixing time) and the 2D NOESY (D_2O, 80 ms mixing time). Distances (upper limits) were calibrated using the program DYANA (Guntert *et al*., 1997). Since DYANA only calibrates upper limits, lower limits were introduced in a later stage. Inter-strand d_{NN} and sequential $d_{\alpha N}$ and $d_{\alpha\alpha}$ distances (taken from the thus far calculated structures) were used for the calibration of the HMQC-NOESY-GHSQC, 3D NOESY HSQC and NOESY spectrum, respectively. Lower limits were set by shortening the distances r by 20 %. Stereospecific assignments and angle restraints for NIP1 were obtained from a quantitative analysis of the various J-coupling spectra. Structures were calculated using 34 ϕ and 11 φ angles constraints, and 740 distance constraints.

The slowly exchanging amide protons resulted in 28 H-bonds. Constraints for the disulfide bridges were added according to the experiment described above. During the structure calculations, 24 lower limits of 4 Å were included, corresponding to NOEs not present in the spectra.

ACKNOWLEDGEMENTS

W. K. was supported by a grant from the Deutsche Forschungsgemeinschaft, C. H. M. P. by a grant from the Swedish Natural Science Research Council. H. A. B. was funded by the Dutch Foundation of Chemical Research (SON) and Life Sciences Foundation (SLW) with financial aid from the Dutch Organization for Scientific Research (NWO).
The NMR measurements were performed at the Dutch National NMR Facility and at the Swedish NMR Center. Jos Joordens and Charlotta Damberg are acknowledged for their help. Dr. Alexey G. Murzin, MRC Laboratory of Molecular Biology (Cambridge, UK), is acknowledged for critically 'looking at the structure'

Chapter 6

Concluding remarks

Plants constantly need to be prepared for defensive battle against putatively hostile microbes. During co-evolution plants have acquired efficient passive, preformed barriers that provide protection against the majority of microbes. This passive resistance is often supplemented by active resistance responses, such as wound-induced impregnations and cell wall appositions to seal these favorite entry sites against trespassing microorganisms. In addition, plants possess an array of active mechanisms to defend themselves at or after pathogen penetration (Kombrink and Somssich; 1995). Pivotal prerequisite for any active defense is recognition of the intruder. Therefore, similar to the animal immune system, plant defense mechanisms must not only be composed of an element capable of responding to pathogen attack, but also of an efficient recognition system to initiate the response. This recognition system should provide the plant with the ability to detect an intruder as early as possible to be able to effectively launch counter-measures. From a number of fungi, molecules have been isolated that trigger plant defense reactions in the plant. These compounds are called elicitors and several review articles have covered various aspects related to their function (e.g., Chapter 2, Knogge; 1996). In the plant, resistance is only activated if the pathogen expresses the elicitor-encoding gene. Interactions at or above the species level are not amenable to genetic analysis. Below the species level, however, single genes frequently control resistance in individual genotypes of otherwise susceptible host species to a limited number of fungal isolates. In these gene-for-gene interactions (Flor; 1955, 1971) the expression of a particular resistance gene allows a plant cultivar to recognize a fungal strain containing the complementary gene for avirulence. Worldwide, a large number of plant genes conferring resistance to different types of pathogens are being studied, as are a few fungal avirulence genes.

This thesis presents a study on the NIP1 elicitor from the barley (*Hordeum vulgare* L.) pathogen *Rhynchosporium secalis*. Besides a detailed biochemical and structural characterization of the protein, the interaction between NIP1 and a plant plasma membrane-localized binding site has been studied. This pathosystem is one of the few model systems amenable to study the molecular basis of the gene-for-gene hypothesis in plant – fungus interactions. The characterization and understanding of the processes, which discriminate between compatible and incompatible interactions between plant and pathogen, could contribute to the development of novel strategies for crop protection. The research described in this thesis focuses on unraveling the complex interaction between barley and *Rhynchosporium secalis*, with emphasis on the role of NIP1 herein.

NIP1 – *the protein*

Chapter 3 describes the heterologous expression of NIP1 in *E. coli*. Crucial in the expression procedure is an unfolding / refolding step, which is required to obtain correctly folded NIP1. NIP1 cannot adopt its native fold in the *E. coli* cytosol, whose reductive environment is not conducive for disulfide bonds to be formed. A cysteine-rich protein such as NIP1 commonly forms protein complexes. Aggregation of recombinant proteins in *E. coli* is a well-known phenomenon. Often, this aggregation results from unspecific association of completely unfolded peptide chains due to hydrophobic interactions. However, Speed *et al.* (1996) showed in studies with recombinant proteins from the P22-phage in *E. coli* that aggregation resulted from specific interactions between certain folding intermediates. Protein folding *in vitro* is a spontaneous process that is dictated exclusively by the amino acid sequence and the medium (Anfinsen, 1973). Two stages in the protein folding pathway have been characterized, and both are apparently governed by thermodynamic laws (Anfinsen; 1973, Seckler and Jaenicke; 1992). The first stage of folding is packing of the polypeptide chain, and the driving force is most likely "hydrophobic collapse" (Dill; 1990), which can be regarded as a rapid clustering of hydrophobic residues. This process is accompanied by a near-random pairing of cysteines. Intermediates in the folding pathway of cysteine-rich proteins can be trapped in intermediate stages by quickly lowering the pH and folding can be resumed by increasing the pH (Weissman and Kim; 1991). These intermediates can be isolated by RP-HPLC. The optimum conditions for *in vitro* folding of disulfide-rich proteins are achieved by mimicking the conditions in the lumen of the endoplasmic reticulum (ER), where extracellular proteins normally fold. In the ER, disulfide-rich proteins are believed to fold in the presence of a redox couple, reduced (GSH) and oxidized (GSSG) glutathion, and the enzyme protein disulfide isomerase (PDI). Although five-disulfide bridge-containing proteins have never been tested in comparable assays, NIP1 most likely behaves in a similar manner. Starting with HPLC-purified, completely reduced NIP1, folding progresses most efficiently when the redox couple cysteine / cystine is used in the optimum ratio of 2:1 at 8°C, pH 9.0 (Van 't Slot, unpublished results). The folding reached equilibrium after 180 minutes. Addition of PDI to a concentration of 5 μM increased the speed of the reaction (equilibrium was reached after 60 minutes), but not the yield of the correctly folded cystine isomer, a result that was also observed for the *in vitro* folding of AVR9 (van den Hooven et al; 1999). The *in vitro* folding of NIP1 is a key step in obtaining elicitor-active protein from in the heterologous expression system described in chapter 3. However, the folding efficiency of NIP1 starting from Ni-NTA agarose-purified NIP1 isoforms is far less efficient as compared to folding starting with completely reduced NIP1. Folding was most efficient when a cysteine**:**cystine ratio of 1:8 was used in the presence of 2 M urea (Van 't Slot;

unpublished results). The folding reaction reached equilibrium after incubation for at least 8 h at 4°C and the reaction rate could not be increased by addition of PDI.

The amino acid sequence of NIP1 does not show homology to the sequences of other proteins in the databases. Moreover, the three-dimensional structure of NIP1 presented in chapter 5 does not show any homology to structures in the databases. The mature NIP1 protein contains 10 cysteines. The disulfide bond pattern was not reported before. However, the spacing pattern of the first 8 cysteines in NIP1 (-C-CC-C-C-CC-C-) is also found in a different class of fungal proteins, the hydrophobins. These have been characterized from a number of fungi including plant pathogens (Wessels *et al*; 1991, Carpenter *et al*; 1992, St. Leger *et al*; 1992, Talbot *et al*; 1993). Hydrophobins are cell surface constituents involved in the formation of fungal aerial structures (Wessels *et al*; 1991). They self-assemble at hydrophobic-hydrophilic interfaces into an amphipathic membrane, and show several remarkable biochemical features (for review see Wösten & de Vocht; 2000). To date, only very limited structural data are available for the hydrophobin family. Using infrared spectroscopy and circular dichroism measurements it could be established that the SC3 hydrophobin from *Schizophyllum commune* in solution is rich in β-sheet structure. Upon self-assembly, however, hydrophobins change their structure and α-helical segments are observed in intermediate stages of folding. The disulfide bridge pattern was partially resolved for the hydrophobin cerato-ulmin (CU) from the Dutch elm disease pathogen, *Ophiostoma ulmi* (Yaguchi *et al*; 1993). Interestingly, CU also acts as a virulence factor. In addition, the hydropathy profiles of NIP1 and CU are strikingly similar (V. Li and W. Knogge, unpublished results). However, the disulfide bridge pattern in NIP1 differs from that of CU suggesting that the fold of CU markedly different from NIP1. Hence, it is unlikely that NIP1 is a functional hydrophobin.

NIP1 ***as virulence factor***

During the early stages of the interaction between *R. secalis* and barley, mycelium is confined to grow beneath the cuticle, and no severe degradation of plant cell walls is visible. (Lehnackers and Knogge; 1990). Therefore, in order to release nutrients from host cells, the fungus appears to produce and secrete toxic compounds that are capable of moving across plant cell walls. NIP1 was originally identified as an unspecific toxin causing necrotic lesions upon injection into barley leaves independent of their resistance genotype. Relatively high concentrations of the protein occurred at the onset of lesion formation during pathogenesis on susceptible plants indicating a possible involvement of NIP1 in symptom development (Wevelsiep *et al*; 1991). Further evidence for a role in virulence comes from a fungal *NIP1* gene replacement mutant that displays reduced virulence compared to the parental $NIP1^{+}$ strain on

susceptible barley cultivars (W. Knogge, unpublished data). In addition, NIP1 type I stimulates the Mg^{2+}-dependent, K^+-stimulated H^+-ATPase in plasma membrane vesicles from both susceptible and resistant barley cultivars (Wevelsiep *et al*; 1993). This stimulatory activity of NIP1 is most likely associated with its toxicity and, hence, its contribution to fungal virulence.

The physiological role, structure and regulation of H^+-ATPases have been studied extensively (Palmgren; 2001, Portillo; 2000). Several lines of evidence rule out a direct interaction between NIP1 and the H^+-ATPase. Firstly, H^+-ATPase purified using a glycerol gradient could no longer be stimulated by NIP1 indicating that a mediator component had been lost during this purification step (Wevelsiep *et al.*, 1993). Secondly, NIP1 acts extracellularly, whereas the regulatory domain at the C terminus of the H^+-ATPase is located intracellularly (Palmgren *et al* 1991; Wevelsiep *et al.*, 1993). This regulatory domain was shown to be involved in the mechanism by which fusicoccin, a phytotoxic H^+-ATPase-stimulating metabolite from the fungus *Fusicoccum amygdali*, activates the enzyme (Olsson et al; 1995) in a strictly concentration-dependent manner (De Michaelis et al; 1996). However, fusicoccin itself does not interact with the H^+-ATPase (Fullone et al; 1997, Jahn et al; 1997). Instead it binds to a member of a class of regulatory proteins, collectively called 14-3-3 proteins, that are capable of binding to a large number of target proteins in eukaryotic cells (Chung et al; 1999).

14-3-3 proteins are involved in the current model explaining the regulation of the H^+-ATPase. Binding of a 14-3-3 protein to the regulatory domain of the enzyme results in increased activity (Baunsgaard *et al*; 1998, Svennelid *et al*; 1999). For 14-3-3 binding to occur at the regulatory domain the phosphorylation state of the enzyme is essential (Rosenquist *et al*; 2000). Several positions of the plant plasma membrane H^+-ATPase are phosphorylated *in vivo*. A phospho-threonine residue that is protected from dephosphorylation has been identified in purified spinach H^+-ATPase at the penultimate position of the regulatory domain (Olsson *et al*; 1998). Blue light activation of mung bean H^+-ATPase involved protein kinase-mediated phosphorylation (Kinoshita and Shimazaki; 1999). This phosphorylation targets Ser and Thr residues at the regulatory domain and is accompanied by 14-3-3 binding. Oat root plasma membrane H^+-ATPase is likewise phosphorylated at both Ser and Thr residues (Schaller and Sussman; 1988), but whether this creates 14-3-3 binding sites is not known. To reverse the activation of the H^+-ATPase that is induced by the protein kinase-mediated generation of the 14-3-3 binding site, the regulatory domain needs to become dephosphorylated by a protein phosphatase. An enzyme that may serve this role has been purified from maize membranes (Camoni *et al*; 2000).

In the current model explaining fusicoccin toxicity, the fungal metabolite inhibits the dissociation of the 14-3-3 protein from the regulatory domain of the H^+-ATPase. As the consequence, an almost irreversible complex is formed (Fuglsang *et*

al; 1999) that causes the constitutive stimulation of the H^+-ATPase. A prolonged activation of the H^+-ATPase leads however to a severe disruption of the intracellular ion balance and pH control, to impairment of the water balance and finally to cell death. It is tempting to speculate that NIP1 may act in a similar way.

NIP1 ***as an avirulence factor***

NIP1 induces a rapid and transient induction of PR5 biosynthesis in plants carrying the *Rrs1* gene, but not in plants of the *rrs1* genotype, a plant response that can be seen as a marker for resistance (Hahn et al; 1993). Further evidence for a role of NIP1 as a race-specific elicitor came from physiological and genetic complementation experiments (Rohe *et al*., 1995). Addition of the purified protein to suspensions of the virulent strain AU2 of *R. secalis* prior to inoculation resulted in an incompatible interaction with *Rrs1*-barley. Likewise, transformation of strain AU2 with the *NIP1* gene yielded mutants avirulent on *Rrs1*-barley. In both cases, the interaction with *rrs1*-barley remained unaffected. Final proof of the avirulence function of NIP1 was provided by replacement of the *NIP1* gene in the avirulent strain UK7 by a non-functional gene through homologous recombination. The resulting mutant gained virulence on *Rrs*1 plants (W. Knogge, unpublished data).

Inoculation of *Rrs1*-barley with *R. secalis* strains UK7 and AU1 carrying the type I- and type II-encoding *NIP1* allele, respectively, induced a massive PR5 mRNA accumulation in *Rrs1*-barley. In contrast, strains AU3 and AU2 carrying the type III- and type IV-encoding allele, respectively, failed to induce PR5 biosynthesis, and both are virulent on *Rrs1*-plants (Rohe *et al*; 1995). The data described in chapter 4 show that the single amino acid alterations specifying NIP1 type III (S23P) and type IV (G45R) suffice to compromise the elicitor activity of the protein.

Since the initial growth of the fungus is strictly confined to the subcuticular space of host leaves, the epidermis can be expected to play a decisive role in pathogen recognition by the plant. Binding experiments provided no evidence for a significantly higher number of NIP1 binding sites in microsomal fractions obtained from epidermal strips of *Rrs1*-barley leaves in comparison with microsomes obtained from total leaves (Van ‘t Slot and Knogge, unpublished results). An analysis of defense-related gene expression in epidermis and mesophyll upon inoculation with fungal strain UK7 carrying the type I-encoding *NIP1* allele revealed tissue-specific responses and upon treatment with NIP1 type I (Steiner-Lange *et al.*, manuscript in preparation). Upon fungal infection of resistant plants the biosynthesis of PR1, PR5, PR9 and other proteins was induced in the mesophyll, whereas PR10 is expressed in the epidermis. Interestingly, NIP1 induced the tissue-specific activation only of the PR subset of the genes analyzed upon fungal infection. However, NIP1 treatment of mesophyll tissue after enzymatic removal of the epidermis did not result in PR5 mRNA accumulation

(K. Toth-Sagi and W. Knogge, unpublished results). This indicates that NIP1 does not directly induce PR5 mRNA accumulation in this tissue and that a different so far unknown signal is responsible for defense-related gene expression in the leaf mesophyll.

***Perception of* NIP1**

Prerequisite for a successful plant defense response upon challenge by a pathogen is the ability to recognize the intruder. To date, biochemical and genetic information on recognition of race-specific elicitors by plants is accumulating rapidly. According to the "specific elicitor / receptor" interpretation of the genetic gene-for-gene model, the elicitor is the product of an avirulence gene that binds to a complementary binding site on the plant, which is encoded by the resistance gene (Keen; 1982). The most straightforward perception of the elicitor by a resistant host would be a direct interaction with a matching resistance gene product. Such an interaction between elicitor and plant receptor subsequently leads to the activation of defense responses (Hammond-Kosack and Jones; 1996). The *Pto* gene of tomato, conferring resistance to *Pseudomonas syringae* pv. *tomato*, was the first resistance gene cloned that complied with this elicitor-receptor model. The product of the *Pto* gene is a serine/threonine-specific protein kinase that phosphorylates a second serine/threonine-specific protein kinase, Pti1 (Zhou *et al*; 1995). Pto was shown to directly interact with AvrPto. When the avirulence gene *AvrPto* from *P. syringae* pv. *tomato* and the *Pto* resistance gene were introduced into a single yeast cell in the yeast two-hybrid system (Field and Song; 1989), a strong interaction was observed (Tang *et al*; 1996, Scofield *et al*; 1996). However, Van der Biezen and Jones (1998) questioned the role of *Pto* as a resistance gene and proposed instead that *Pto* encodes the virulence target of AvrPto. A second example, in which direct physical contact between an avirulence gene product and the corresponding *R* gene product was found, is the interaction between AVR-Pita of *M. grisea* and the rice resistance protein Pi-ta (Jia *et al*; 2000). Pi-ta is predicted to be a cytoplasmic protein containing a nucleotide binding site and a C-terminal leucine-rich domain region (Bryan *et al*; 2000). AVR-Pita is a putative Zn^{2+} protease that is inactive as elicitor of resistance when applied to the host apoplast. Surprisingly, therefore, this protein needs to get into the host cytoplasm to exert its activity. To date it remains unknown how AVR-Pita is translocated into the host cell and whether it is proteolytic cleavage of Pi-Ta may be involved in the activation process.

In other plant-pathogen interactions, the classical elicitor-receptor model fails to describe the perception of race-specific elicitors by plant factors. In the pathosystem *Cladosporium fulvum* / tomato it was shown that a high-affinity binding site for the fungal AVR9 elicitor exists on host plasma membranes. However, the

presence of this putative receptor does not correlate with resistance to the pathogen, since the presence of the binding site could be detected in both resistant and susceptible tomato leaves (Kooman-Gersmann *et al*; 1996). In addition, a number of different binding assays was performed, all failing to show binding between AVR9 and the Cf-9 protein (Luderer *et al*; 2001).

Binding of NIP1 to plasma membrane factors of barley is in several respects comparable to the AVR9 – Cf-9 system in the *C. fulvum* – tomato interaction (Kooman-Gersmann et al; 1996). Firstly, like the AVR9 binding site, NIP1 binding activity resides in the plasma membrane, indicating that signaling occurs from the extracellular space inwards. A membrane translocation system, like the type III secretion system for bacterial avirulence factors, does not appear to occur in both interactions. Secondly, in both the AVR9 – tomato and the NIP1 – barley system, binding does not correlate with the presence of the resistance gene in the host. Susceptible cultivars possess elicitor binding sites with binding characteristics and amounts very similar to those of resistant cultivars. Thirdly, binding sites with similar physical characteristics were detected in several related species, but are not ubiquitous in the plant kingdom. Finally, it is unlikely that either *Cf-9* or *Rrs1* encode the primary elicitor binding site, but the *R* gene products may play a role early in the signaling pathway leading to defense responses.

However, differences exist between the consequences of AVR9 and NIP1 perception. In contrast to *Cf-9*, resistance based on the *Rrs1* gene is not mediated by a hypersensitive response (HR). A number of AVR9 mutants were generated and a correlation between their affinity to the membrane-localized binding site and their HR-inducing activity in *Cf-9* carrying tomato was found, indicating that binding is a critical and limiting step. For the NIP1 isoforms type II, III and IV, a correlation between affinity for the binding site and their PR5-mRNA inducing activity was not observed. The binding data presented in chapter 4 indicate that recruitment of the putative additional component, the signal transducer protein, may be the limiting step in the NIP1 signal transduction pathway.

The structure of NIP1 isoform type I was solved by NMR spectroscopy techniques. In comparison with type I NIP1, type II NIP1 shows only a slight reduction in biological activity. However, type II NIP1 competes with far less efficiency for the binding site than type I NIP1. It is unclear which amino acid, or combination of amino acids, is responsible for this reduction in binding. The introduction of a negative charge due to the residue alteration A18E may affect the β-sheet structure in domain 1 and creates additional charge in this motif. It is situated in the center of domain 1, and rather buried in the interior of the protein. The other two mutations (H21Q and T55K) are more exposed on the surface of the molecule. This might explain the effect of the mutation on binding. Furthermore, an additional

positive charge is introduced in the T55K mutation, which may repulse the binding site.

NIP1 *signaling*

An interesting emerging hypothesis in this context is the so-called 'guard hypothesis', which assumes the resistance gene product to guard the target of a virulence factor (van der Biezen and Jones, 1998). The model implies an indirect perception of the elicitor by the *R* gene product, thus explaining the limited number of host-pathogen systems in which direct interaction between the products of *R* and Avr genes could be proven. Also, since the avirulence factor functions by interacting with its virulence target, as the guard model suggests, the virulence target and the elicitor binding protein represent the same molecule. As the consequence of this model, both functions of the elicitor would be mediated through the same binding site. In an incompatible plant – pathogen interaction, physical contact between the elicitor and the virulence target would lead, via the action of the *R* gene product, to a defense response, whereas the virulence target will be affected in the absence of the *R* gene, possibly leading to enhanced virulence. This model implies the cooperation of at least three components for the induction of the defense response: the elicitor, the primary elicitor binding site and the resistance gene product. Binding of the elicitor to the binding site is perceived by the third component, possibly the *R* gene product. In such a scenario, the resistance gene product effectively guards the virulence target. The perception of the NIP1 signal may comply with the guard hypothesis.

The research described in this thesis has led to new insights into the recognition of NIP1 and the initiation of defense responses in barley plants carrying the *Rrs1* resistance gene. The three alternative models presented in Fig 1 are based on the current data on NIP1 perception and signaling, and on other signaling pathways. In panel A, a model is presented in which binding of NIP1 to a NIP1 binding protein induces transmembrane signaling, for example by inducing conformational changes in the putative receptor. In susceptible plants, this activation leads to an indirect stimulation of the H^+-ATPase, while in resistant plants a defense response in initiated via the action of the Rrs1 protein. Type II NIP1 binds with reduced affinity to the NIP1 binding site, but this binding is still sufficient to activate the downstream processes to infection or resistance. In this model, the S23P and G45R mutations in NIP1 both suffice to inactivate NIP1 by their inability to induce transmembrane signaling.

A second model (panel B) is based on the finding that many signal transduction pathways are triggered upon ligand-induced receptor dimerization. The NIP1-binding site complex attracts and activates a second NIP1 binding protein of the same type. Upon dimerization of the two NIP1 binding proteins the NIP1 signal is

transmitted across the plant plasma membrane. Type II NIP1 binds less efficient to the NIP1 binding site, but is capable of inducing receptor dimerization and transmitting of the signal. The S23P and G45R mutant proteins bind efficiently to the NIP1 binding site, but fail to induce dimerization or activation of the complex. However, no evidence for receptor dimerization has been obtained.

A third model is presented in panel C. Upon binding of type I NIP1 to the binding site, a third component is recruited which is activated by the NIP1 – binding site complex. In susceptible barley cultivars, this activation triggers indirectly the activation of the H^+-ATPase, whereas in plants with the *Rrs1*-genotype defense genes are activated that arrest fungal growth. Type II NIP1 shows a reduced affinity for the NIP1 binding site. However, based on the findings that fungal isolates carrying the type II-encoding NIP1 allele are strictly avirulent on *Rrs1* cultivars, and that NIP1 type II is a potent inducer of PR5-mRNA, we conclude that this reduced binding affinity is sufficient to associate with the putative third component in the signaling pathway. Fungal strains carrying the type III or IV isoforms are virulent on *Rrs1* barley. In addition, the types III* and IV* mutations abolish the ability of NIP1 to induce PR5 mRNA production. However, both types III* and IV* efficiently compete for the NIP1-binding site with type I ^{125}I-NIP1. Thus, type III or IV NIP1 – binding site complexes fail to activate the signal transducer protein and the NIP1 signal is not transmitted. This third model complies with the guard hypothesis. The guard hypothesis (van der Biezen and Jones, 1998) proposes that a second component in the plant is required for the perception of an AVR protein, and that this additional component is the virulence target of the AVR protein. In the presence of the R protein, binding of the corresponding elicitor to the virulence target, will result in the onset of defense responses, whereas in susceptible plants binding will result in transmission of its virulence function. Based on the guard model it was proposed by Luderer and Joosten (2001) that a correlation exists between the binding affinity of the elicitor to the putative signal transducer protein and its efficiency to induce a defense response. However, the specificity of NIP1 perception appears to be based on the recruitment and activation of additional molecules, rather than on association with the binding site. The interaction between barley and *Rhynchosporium secalis* therefore provides an excellent model system to prove or adapt the guard hypothesis.

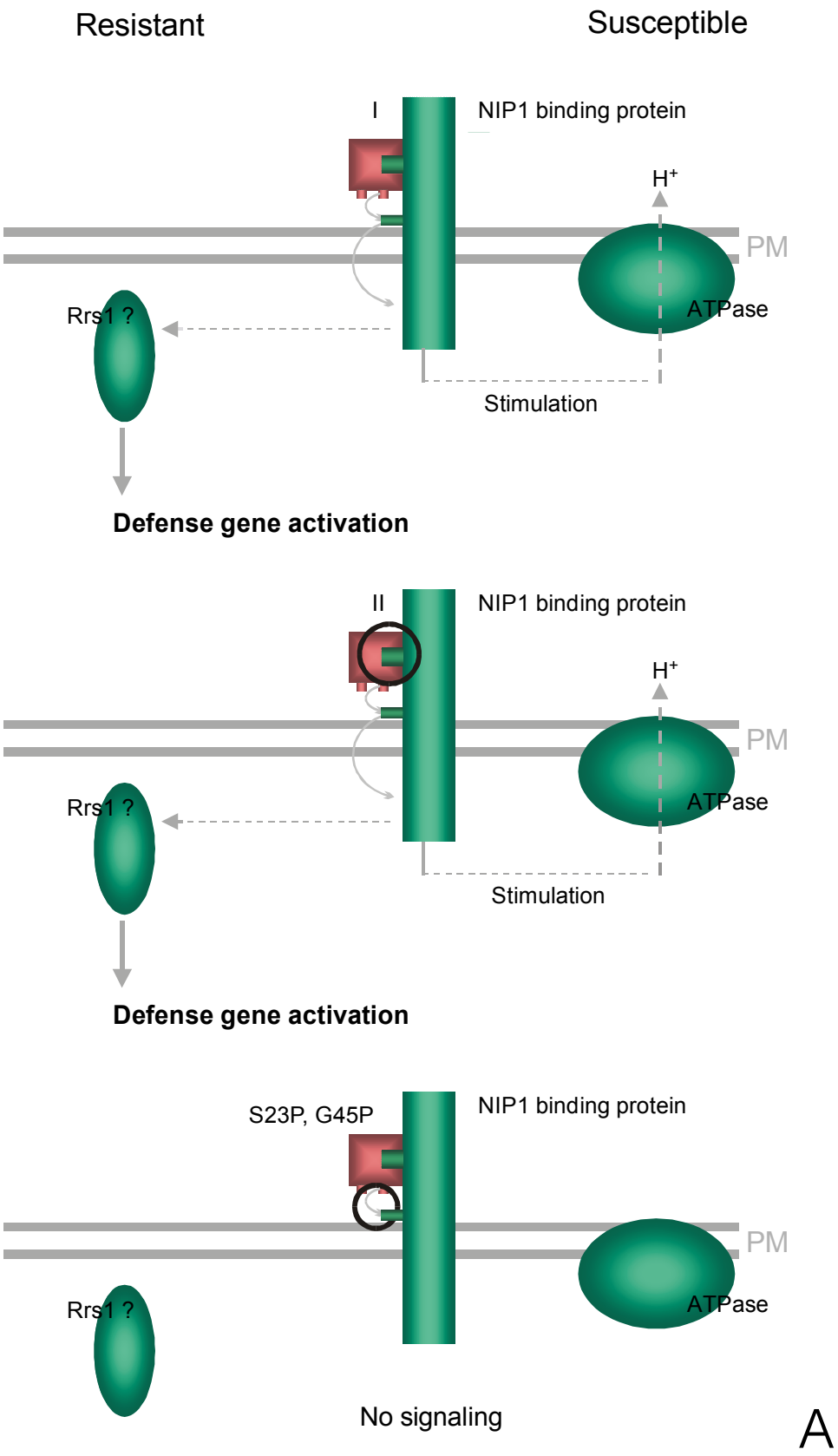
Figure 1 (following pages). Three models that could explain the interaction of NIP1 with its receptor and further signaling towards resistance or susceptibility.

The avirulence (elicitor) function is depicted on the left and the virulence (toxin) function of NIP1 is depicted on the right of each panel.

(A) Binding of type I NIP1 (upper panel) to the binding site triggers a defense response in resistant barley plants through a conformational change in the NIP1 receptor and the activation of the Rrs1 protein, whereas toxic activity results from an indirect stimulation of the H^+-ATPase. Binding of type II NIP1 (middle panel) to the NIP1 binding site is less efficient, however, a conformational change similar to that of type I NIP1 is induced, leading to the defense response on resistant, or H^+-ATPase-stimulation on susceptible plants. Type III* (G45R) and IV* (S23P, lower panel) efficiently bind to the binding site, yet fail to trigger resistance or H^+-ATPase activation.

(B) *Idem*. Both avirulence function and toxicity of NIP1 are mediated through the same receptor, which dimerizes upon NIP1 binding.

(C) *Idem*. Both avirulence function and toxicity of NIP1 are mediated through the same receptor, which recruits and activates a signal transducer molecule.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Stimulation
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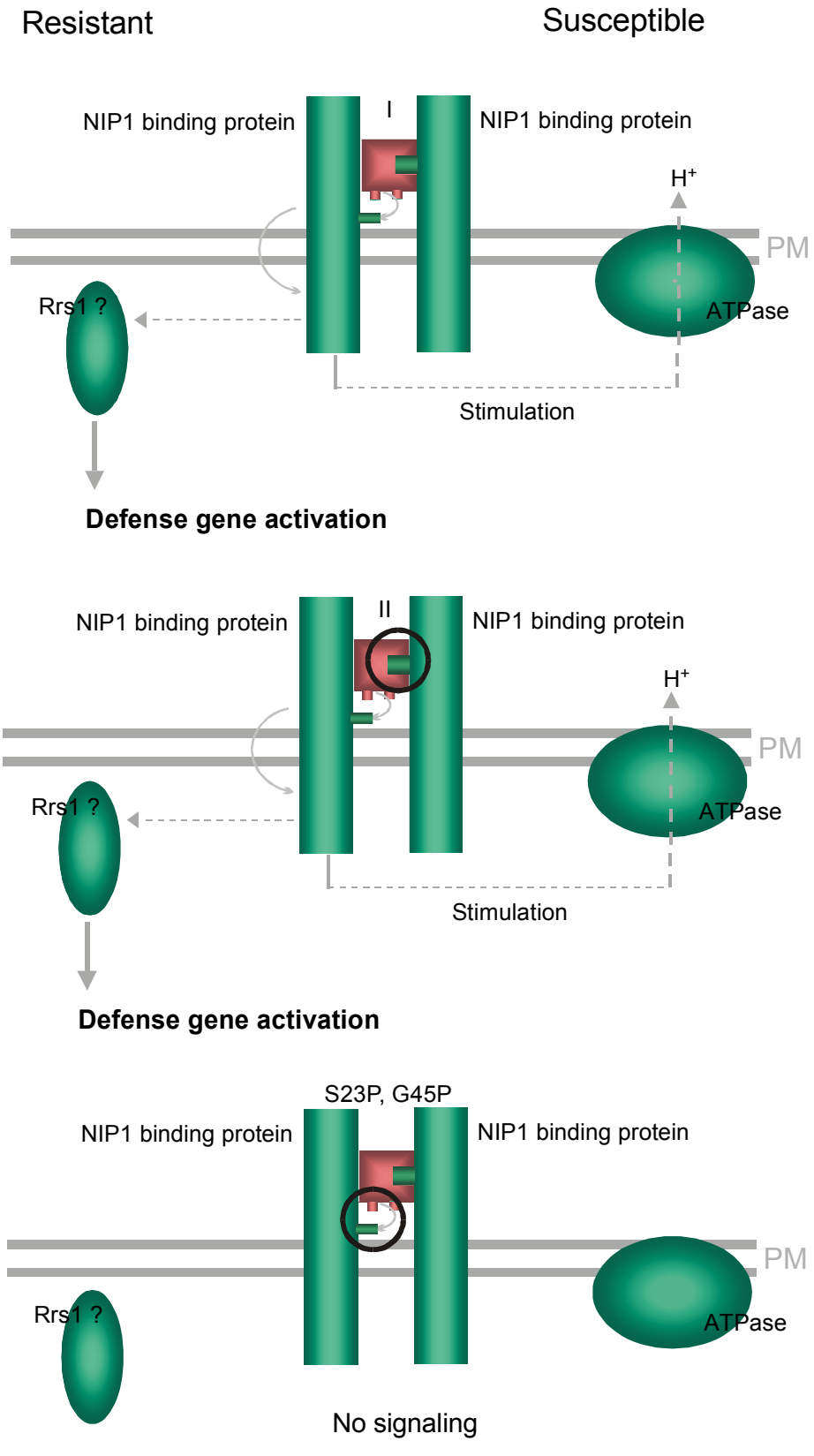
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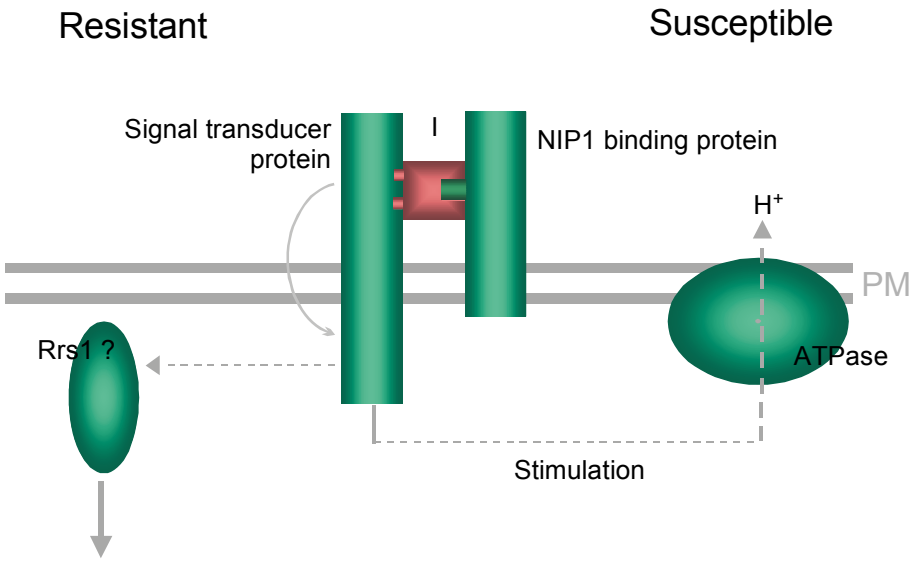
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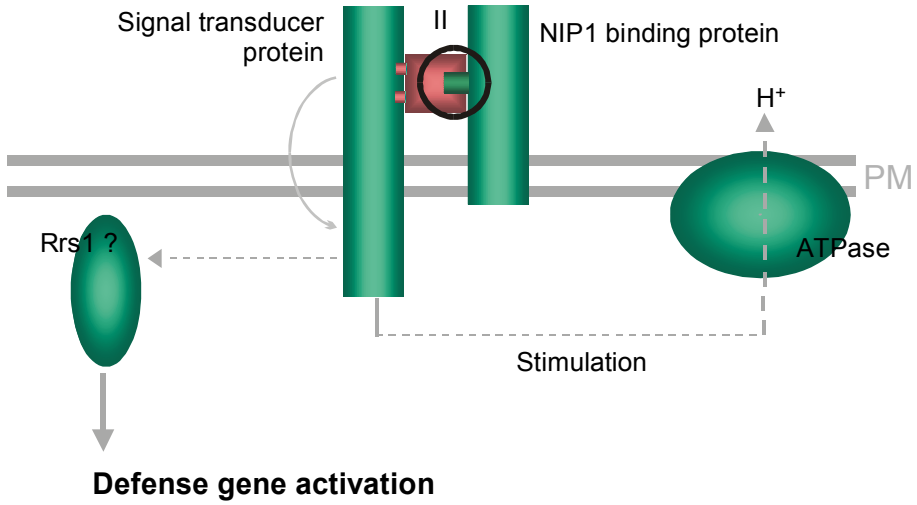
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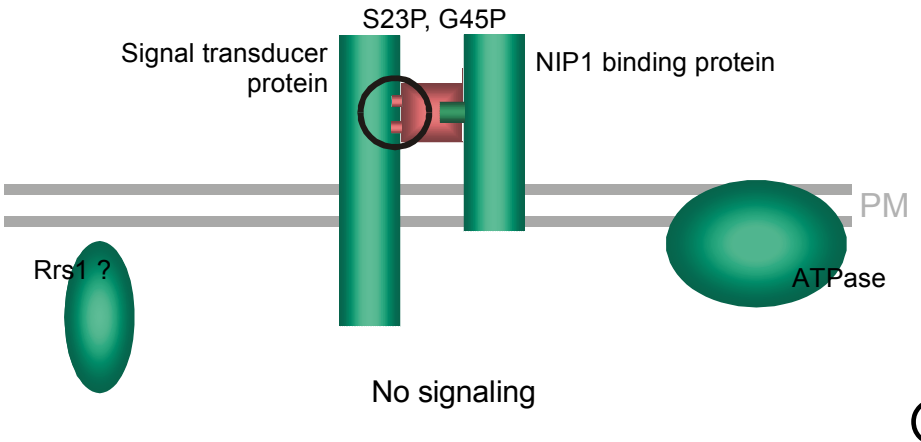
S23P, G45P
Signal transducer protein
NIP1 binding protein
PM
Rrs1 ?
ATPase
No signaling

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Summary

Rhynchosporium secalis is the causal agent of leaf scald on barley, rye and several other grasses. The fungus belongs to the class of *Deuteromycetes*, also referred to as *Fungi Imperfecti*, indicating that a sexual stage has not been observed in nature. Germinating fungal spores are capable of penetrating the cuticle. Then the fungal hypha ramifies as mycelium beneath the cuticle causing the collapse of epidermal cells. In a later stage of the infection, mesophyll cells start to collapse and disease symptoms become visible. Only at this stage, a few fungal hyphae can be detected between dead mesophyll cells. In resistant barley cultivars the infection is halted after the collapse of a few epidermal cells. This is caused by the onset of a defense mechanism by the plant, which involves the massive production of pathogenesis-related (PR) proteins. The response of the plant is triggered upon recognition of the fungus. It was established that *R. secalis* strains carrying the avirulence gene *AvrRrs1*, which codes for the NIP1 protein, are unable to infect barley plants carrying the resistance gene *Rrs1*. In this thesis, we describe the biochemical and biological properties of the NIP1 protein, its tertiary structure and its interaction with the plant plasma membrane.

NIP1 consists of 60 amino acid (a.a.) residues, including 10 cysteines, all of which are involved in intramolecular disulfide bonds. The a.a. sequence of NIP1 shows no similarity to sequences in the databases. By determining the DNA sequence of *AvrRrs1* alleles of a limited set of field isolates, four NIP1 isoforms were deduced displaying only a limited number of a.a. alterations. Two of these, types I and II, although differing in three amino acids, are able to induce PR protein biosynthesis in *Rrs1* barley as well as leaf necrosis independent of the resistance genotype. This leaf necrosis in caused by a stimulation of the plasma membrane H^+-ATPase. Types III and IV both differ from type II in the single a.a. alterations G45R and S23P, respectively. We engineered type I NIP1 protein into type III and IV-like proteins (types III* and IV*) by exchanging the activity-blocking type III and IV amino acids. These mutated proteins are inactive as elicitor.

Application of NIP1 to leaves of *Rrs1*-plants is sufficient to elicit the production of PR proteins in a manner comparable to amounts induced after infection by the fungus. In addition to a function as elicitor, NIP1 is able to cause necrosis on barley plants independent of their resistance genotypes, and a role for NIP1 in virulence was therefore proposed. For several virulence factors of other plant-pathogens an additional role as elicitor of defense responses has been shown. In viruses, all proteins encoded by the few genes in the genome are expected to be important for viral replication and multiplication. Several plants have developed defense responses that are initiated upon recognition of specific viral gene products. Bacteria, fungi, oomycetes and nematodes have specialized genes for pathogenicity and virulence, some of which can induce resistance in plants as they recognize

inflicted activities of the intruding pathogen. The number of factors for which dual functions in virulence and avirulence is described is accumulating rapidly. The emerging picture is that plant recognition systems targeted against important pathogen virulence factors may possess common characteristics. This opens up multiple possibilities to utilize natural mechanisms in engineering more durable resistance against a larger number of pathogens.

A heterologous expression system was set up in *E. coli*, allowing the production of milligram quantities of NIP1. In this system, NIP1 is produced as a fusion protein, where a histidine tag is fused to the N-terminus of NIP1. In order to obtain NIP1 containing the correct disulfide bond pattern, an unfolding / refolding procedure, involving the use of a cysteine / cystine redox couple was applied. A protease Xa cleavage site between the histidine tag and NIP1 allowed the removal of the His-tag. The NIP1 protein produced by this heterologous expression procedure has elicitor activity similar to the native NIP1 protein purified from *R. secalis*. In addition, the expression system allowed the expression and purification of mutated NIP1 proteins.

To identify putative NIP1 binding sites, NIP1 was labeled with radioactive iodine. Binding studies using ^{125}I-NIP1 and barley membrane fractions revealed the presence of a single class of high-affinity NIP1 binding sites on barley plasma membranes. Binding of NIP1 to membrane fractions was specific, reversible, and saturable. The equilibrium dissociation constant, K_d, was determined at 5.6 nM and the concentration of binding sites was calculated to be 255 fmol per mg of microsomal membrane protein. The binding site was detected in both resistant (*Rrs1*) and susceptible (*rrs1*) barley plants, suggesting that the *Rrs1* gene does not encode the NIP1 binding site. In addition, a binding site with very similar affinity for NIP1 was detected in microsomes from the related plant species wheat, rye, oats and maize, but not from *Arabidopsis thaliana*. These results correlate with the toxic effect of NIP1 on leaves of the cereal plant species, while no toxic effect was observed in leaves of *Arabidopsis thaliana*. Two NIP1 isoforms type III* and type IV* both competed successfully for the binding site, although they are not active as elicitors of PR5 biosynthesis. We conclude from these data that these mutant proteins bind efficiently to the binding site, but are unable to activate downstream signaling. In contrast, NIP1 type II is characterized by a drastically increased K_d value. An only slight decrease in elicitor activity and H^+-ATPase-stimulating activity of this isoform indicates that binding of NIP1 is not the limiting step in the signal transduction pathway.

The three-dimensional structure of NIP1 was determined by NMR spectroscopy. The disulfide bond pattern of NIP1 was determined with biochemical techniques, based on partial reduction of individual disulfide bridges and subsequent analysis of the released fragments after cleavage by mass spectroscopy. The structure of NIP1 can be regarded as a protein consisting of two motifs. The orientation of these

motifs with respect to each other is well defined due to the stabilizing effect of the disulfide bond connecting the two motifs. The N-terminal motif contains an anti-parallel β-sheet comprised of 2 β-strands, whereas the C-terminal motif consists of three antiparallel β strands and a relatively large flexible region. NIP1 has a novel fold; no proteins with homologous structures were described so far. The activity-blocking mutations S23P and G45R both have a high impact on the structure.

The perception of NIP1 by barley plants may comply with the guard hypothesis, which proposes a second component in the plant to be required for perception of an elicitor, and that this additional component is the virulence target of the AVR protein. In the presence of the R protein, binding of the corresponding elicitor to the virulence target, will result in the onset of defense responses, whereas in plants lacking the *R* gene binding will result in transmission of its virulence function.

Samenvatting

Rhynchosporium secalis is de veroorzaker van de bladvlekkenziekte op gerst, rogge en een aantal andere grassoorten. De schimmel behoort tot de klasse der *Deuteromyceten*, ook wel *Fungi Imperfecti* genaamd, hetgeen inhoudt dat een sexueel stadium in de levenscyclus van deze schimmel nog niet bekend is. Kiemende sporen van *R. secalis* zijn in staat de cuticula van de waardplant te penetreren. De infectiehyfe vertakt zich als mycelium vlak onder de cuticula, hetgeen het afsterven van enige epidermiscellen teweegbrengt. In een later stadium sterven tevens mesofielcellen af en worden de eerste symptomen zichtbaar. Slechts in dit vergevorderde stadium van de infectie kunnen enkele hyfen tussen de dode mesofielcellen worden gedetecteerd. In resistente gerstecultivars stopt de infectie nadat enkele epidermiscellen zijn gestorven. Dit is het gevolg van het aanschakelen van een afweermechanisme in de plant, waarbij grote hoeveelheden pathogenese-gerelateerde (PR) eiwitten worden geproduceerd. De afweer van de plant begint met het herkennen van de schimmel. Voorgaand onderzoek heeft aangetoond dat *R. secalis* stammen die drager zijn van het avirulentiegen *AvrRrs1*, dat codeert voor het NIP1 eiwit, niet in staat zijn gersteplanten te infecteren die drager zijn van het resistentiegen *Rrs1*. In dit proefschrift beschrijven we de biochemische en biologische eigenschappen van NIP1, de tertiaire structuur van NIP1 en de binding van NIP1 aan membraanfrancties van de plant

NIP1 bestaat uit 60 aminozuren; tien hiervan zijn cysteïnes, die alle betrokken zijn bij de vorming van intramoleculaire disulfide bruggen. De aminozuursequentie van NIP1 vertoont geen gelijkenis met sequenties van andere bekende eiwitten. Door de DNA sequenties te bepalen van *AvrRrs1* in een beperkte set van veldisolaten zijn vier NIP1 isoformen geïdentificeerd, die slechts in enkele aminozuren van elkaar verschillen. Twee van deze isoformen, type I en II NIP1, zijn beide in staat zowel PR-eiwit biosynthese in *Rrs1* gerst planten alsook cultivar-onafhankelijke bladnecrose te induceren, hoewel ze verschillen op drie aminozuurposities. De bladnecrose is het gevolg van het stimuleren van de H^+-ATPase in membranen van gerst door NIP1. Type III en IV NIP1 verschillen elk van type II in slechts één aminozuur (te weten G45R en S23P, respectievelijk) en zijn biologisch inactief. We hebben type I gemuteerd in type III en IV-achtige eiwitten (type III* en IV*) door de aminozuren die de activiteit blokkeren te introduceren. Deze mutante eiwitten zijn biologisch inactief.

Toedienen van NIP1 aan bladeren van *Rrs1*-planten is afdoende om de productie van PR eiwitten te induceren ("eliciteren") in hoeveelheden die vergelijkbaar zijn met de hoeveelheden die worden geïnduceerd tijdens infectie door de schimmel. Naast een functie als elicitor is NIP1 in staat necrose op gersteplanten te induceren, ongeacht het resistentiegenotype. Om deze reden werd een rol voor NIP1 in virulentie verwacht. Voor verschillende virulentiefactoren van andere plant-pathogenen kon ook een elicitorfunctie worden aangetoond. In virussen kan van alle

eiwitten, slechts gecodeerd door de enkele genen die het virale genoom bevat, worden verwacht dat ze van belang voor virale ontwikkeling in de waardplant. Een aantal planten heeft afweerresponsen ontwikkeld die worden geïnitieerd door herkenning van specifieke virale genproducten. Bacteriën, schimmels, oömyceten en nematoden hebben gespecialiseerde genen die coderen voor pathogeniteits- en virulentiefactoren. Een aantal van deze factoren kan tevens resistentie induceren in planten die de schade-veroorzakende activiteiten van het binnendringende pathogeen kunnen herkennen. Het aantal factoren waarvan zo'n dubbele functie in virulentie en avirulentie bekend is neemt snel toe. Het beeld dat onstaat is dat de herkenningssystemen van de plant die gericht zijn tegen belangrijke virulentiefactoren gemeenschappelijke karakteristieken kunnen hebben. Dit geeft meerdere mogelijkheden om deze natuurlijke afweermechanismen in te zetten in het ontwikkelen van meer duurzame resistentie tegen een groot aantal verschillende pathogenen.

Door de ontwikkeling van een heteroloog expressiesysteem voor NIP1 in *E. coli* werd het mogelijk milligram hoeveelheden van het NIP1 eiwit te verkrijgen. In dit systeem wordt een fusiegen tot expressie gebracht dat codeert voor een eiwit waarin een histidine staart gefuseerd is aan de N-terminus van NIP1. Om het juiste cysteïnebrug patroon te verkrijgen werd een ontvouwings / hervouwings protocol toegepast, dat gebaseerd is op het cysteïne / cystine redoxkoppel. Het NIP1 eiwit dat via dit heterologe expressie protocol is geproduceerd heeft een elicitoractiviteit die vergelijkbaar is met die van het NIP1 eiwit gezuiverd uit cultuurfiltraten van *R. secalis*. Met behulp van dit expressiesysteem waren we tevens in staat om op een efficiënte manier gemuteerde NIP1 eiwitten te produceren en op te zuiveren.

Om mogelijke NIP1 bindingsplaatsen te identificeren werd type I NIP1 gelabeled met radioactief jodium. Door middel van bindingsstudies met dit ^{125}I-NIP1 en membraanfracties uit gerst werd de aanwezigheid van een enkele klasse van NIP1 bindingplaatsen met een hoge affiniteit aangetoond. Binding van NIP1 aan membraanfracties was specifiek, reversibel en verzadigbaar. De evenwichts dissociatie constante K_d is bepaald op 5.6 nM en uit verdere berekeningen volgt dat de concentratie van bindingsplaatsen 255 fmol per mg microsomaal membraaneiwit is. De bindingplaats werd aangetoond in membranen van zowel resistente (*Rrs1*) als vatbare (*rrs1*) gerstecultivars, hetgeen suggereert, dat het *Rrs1* gen niet codeert voor de NIP1 bindingsplaats. Tevens werden bindingsplaatsen met vergelijkbare affiniteit voor NIP1 gedetecteerd in membraanfracties van gerelateerde plantensoorten, te weten tarwe, rogge, haver en mais, maar niet in membranen van *Arabidopsis thaliana*. Deze resultaten zijn in overeenstemming met het toxische effect van NIP1 op bladeren van graansoorten, terwijl geen toxisch effect werd gevonden in bladeren van *Arabidopsis thaliana*. Twee NIP1 isoformen, type III* en IV* NIP1, competeren efficiënt voor de bindingsplaats, terwijl deze niet actief zijn als elicitor van PR

genexpressie. We concluderen uit deze gegevens, dat deze isoformen kunnen binden aan de receptor, maar niet in staat zijn om het NIP1 signaal door te geven. In tegenstelling tot type III* en IV* laat type II NIP1 een dramatische toename van de K_d waarde zien. Slechts een kleine reductie in elicitor activiteit en H^+-ATPase-stimulerende activiteit van deze isoform suggereert dat binding van NIP1 niet de limiterende stap is in de signaaltransductie keten.

De drie-dimensionale structuur van NIP1 is bepaald door middel van kernspin resonantie (NMR) spectroscopie. Het disulfide bruggen patroon van NIP1 is bepaald met behulp van biochemische technieken, gebaseerd op partiële reductie van individuele disulfide bruggen en verdere analyse van de fragmenten na reductie door massaspectroscopie. De structuur van NIP1 kan worden beschouwd als bestaande uit twee motieven. De oriëntatie van deze motieven ten opzichte van elkaar is gefixeerd als gevolg van het stabiliserende effect van een disulfide brug die deze motieven met elkaar verbindt. Het N-terminale motief bevat een anti-parallele β-sheet die bestaat uit twee β-strands, terwijl het C-terminale motief bestaat uit een β-sheet met 3 β-strands en een relatief groot flexibel deel. NIP1 heeft een unieke vouwing die tot nu toe niet gevonden is in andere eiwitten. De activiteits-blokkerende mutaties S23P en G45R hebben een groot effect op de structuur.

De perceptie van NIP1 door gersteplanten is mogelijk in overeenstemming met de zogenaamde “guard” hypothese, die veronderstelt dat een tweede component in de plant noodzakelijk is voor de perceptie van een elicitor en dat deze component een virulentiedoelwit is. In aanwezigheid van het resistentie eiwit zal binding van het corresponderende elicitor eiwit aan het virulentiedoelwit leiden tot het aanschakelen van afweerresponsen, terwijl in afwezigheid van *Rrs1* binding resulteert in het overbrengen van de virulentiefunctie.

NAWOORD

Geen van de hoofdstukken in dit proefschrift had tot stand kunnen komen zonder een verscheidenheid aan bijdragen van velen. Dit nawoord geeft mij eindelijk de gelegenheid hen te bedanken.

Firstly, I would like to thank Wolfgang, my supervisor and co-promoter. It was a great pleasure for me to work in your group in Cologne. I greatly appreciated the general guidance. You let me benefit from a large liberty concerning the orientation of my work, especially at times when NIP1 refused to co-operate. In the end, however, we managed to unravel some of the mysteries of this weird protein. Also, I enjoyed our biannual excursions to Wageningen. It always motivated me and in addition you taught me all I ever need to know about the Bundesliga and American football. I'm proud that, as a return, I could teach you at least one important lesson in life: Roken brengt de gezondheid ernstige schade toe....

Pierre, mijn promoter, jou wil ik bedanken voor je steun op zowel het wetenschappelijke als organisatorische vlak. Onze discussies hebben me altijd bijzonder gemotiveerd en je adviezen zijn op een aantal belangrijke momenten van doorslaggevend belang geweest.

Professor Hahlbrock, you were always interested in my work; thanks for the discussions we had. Also, I would like to thank Elmond, Erich, Imre and Berndt for their suggestions concerning this project.

Een deel van het project werd uitgevoerd aan de afdeling Biofysische Chemie van de Universiteit van Nijmegen. Professor Hilbers: ik wil u bedanken voor de mogelijkheden en vooral de steun die ik kreeg om dit werk af te maken. Daarnaast natuurlijk Tineke Papavoine, die veruit de grootste bijdrage heeft geleverd aan het tot stand komen van de structuur. Zonder jou, Tineke, was de structuur waarschijnlijk nog steeds verborgen in een groot woud van pieken. Ook toen je naar Gotenburg verhuisde heb je nog erg veel tijd geïnvesteerd in NIP1. Cathelijne wil ik bedanken voor het maken van vele aanschouwelijke schetsen met hoog artistiek gehalte waaruit steeds duidelijker de definitieve structuur van NIP1 naar voren kwam. Jan Aelen ben ik erkentelijk voor zijn steun bij het labelen van NIP1.

Ook de Wageningen Universiteit was betrokken bij de opheldering van de structuur van NIP1. Harrold en Jacques: bedankt voor de opheldering van het disulfide patroon van NIP1. "Een eiwit met vijf bruggen is de ultieme test voor mijn procedure om zwavelbruggen toe te kennen", zei je, Harrold, maar helaas duurde het toch flink wat langer dan je had verwacht. Bedankt voor jullie hulp, en jullie meedenken!

Most of the work, however, was done in the lab at the Max-Planck-Institut für Züchtungsforschung in Köln. My colleagues Angela, Annette, Corinne, Daniela,

Hiltrud, Magdalena, Marion, Ralph, Sabine, Sylvie and Volkhart: danke für die schöne Zeit!

Een aantal mensen heeft de tijd in Keulen tot een bijzonder aangename tijd gemaakt. Allereerst Lars. Zonder jou was het maar saai geweest in Keulen. We hebben aardig wat liter *pleur* met z'n tweeën gedronken. Van slap tot bijzonder sterk. Also, I want to thank the people I lived with in the Samenhaus, by far the most remarkable place I ever had the privilige to live in.... Emma, Sarah, Jessica, Lu, Martin *"Marguarita*" Klebsattel, Lars and so many of whom I never knew the name. It was always nice to come "home" after a hard day's work.

Na mijn werk in Keulen ben ik een jaar werkzaam geweest bij de Plantenziektenkundige Dienst. Hoewel ik toch aardig ingewijd dacht te zijn in de wereld van de plantenziekten, ging er bij jullie een hele andere wereld voor me open. Marie-Josée, Nicoline, Sietske, Nico, Jos, Bram, en Marc: bedankt voor jullie medeleven en voor de interessante projecten waar ik een steentje aan mocht bijdragen. Ook wil ik mijn nieuwe collega's Francine, Peter, Lars, Wubei, Wilco, Maita, Rays en Rob van Fytopathologie danken voor hun warme belangstelling van de afgelopen tijd.

Naast alle dankwoorden aan mijn collega's is er natuurlijk ook mijn familie die mij altijd enorm gesteund heeft. Mijn ouders, schoonouders, zus en zwagers wekten steeds de indruk erin te geloven dat ik dit boekje zou voltooien. Ik ben dan ook trots en blij dat ik jullie eindelijk kan laten zien waar ik mij de afgelopen jaren mee heb bezig gehouden. Pa en ma: jullie belangstelling voor het onderzoek en jullie vertrouwen in mij hebben me altijd erg goed geholpen. Tenslotte maar bovenal: dank je Tienke voor je geduld en liefde, ook toen je te horen kreeg dat ik WEER een verlenging in Duitsland kreeg. Je had het niet altijd even makkelijk met een echtgenoot-op-afstand, ook al waren de weekendjes *Engelsburg* altijd erg leuk. Zonder jou had ik nu niet kunnen schrijven:

HET IS AF!

Hoogste tijd om te gaan genieten van ons nieuwe leven in Elst.....

CURRICULUM VITAE

Klaas Adriaan Evert (André) van 't Slot werd 14 juni 1970 geboren te Velp (gemeente Rheden). Na het behalen van het HAVO en VWO diploma aan de Rijksscholengemeenschap "*Simon Vestdijk*" te Harlingen begon hij in 1989 aan de studie Moleculaire Wetenschappen aan de Landbouwuniversiteit Wageningen. De doctoraalfase omvatte afstudeervakken bij de vakgroepen Plantenfysiologie, Moleculaire Biologie en Fytopathologie van de toenmalige Landbouwuniversiteit, en een praktische stageperiode aan het International Potato Centre (CIP) te Quito, Ecuador. In augustus 1995 sloot hij zijn studie met goed gevolg af. Van februari 1996 tot mei 2000 verrichtte hij als Ph. D. student onderzoek aan de afdeling Biochemie van het Max-Planck-Institut für Züchtungsforschung te Keulen, Duitsland, gedetacheerd vanuit de leerstoelgroep Fytopathologie van Wageningen Universiteit. Hij bestudeerde de thema's beschreven in deze thesis. Tussen juni 2000 en juni 2001 was hij werkzaam bij de afdeling voor Fytosanitaire Aangelegenheden van de Plantenziektenkundige Dienst te Wageningen. Sinds juni 2001 is hij werkzaam als post-doctoraal onderzoeker in de onderzoeksgroep van mw. dr. ir. F. Govers, aan de leerstoelgroep Fytopathologie van Wageningen Universiteit.