Regulation of the avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum*

Sandor S. Snoeijers

Promotor: dr. ir. P.J.G.M. de Wit
Hoogleraar in de Fytopathologie

Copromotoren: dr. T. Goosen
Universitair docent Laboratorium voor Genetica
dr. ir. M.H.A.J. Joosten
Universitair docent Laboratorium voor Fytopathologie

Regulation of the avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum*

Sandor S. Snoeijers

Proefschrift

ter verkrijging van de graad van doctor

op gezag van de rector magnificus,

van Wageningen Universiteit,

dr. ir. L. Speelman,

in het openbaar te verdedigen op dinsdag 10 oktober 2000

des namiddags te half twee in de Aula.

The research described in this thesis was carried out at the Laboratories of Genetics and Phytopathology, Wageningen University, the Netherlands. The research was supported by the the Dutch Earth and Life Science Foundation, which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Regulation of the avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum*/ Sandor S. Snoeijers.- [S.l. :s.n.].
Thesis Wageningen with summary in Dutch.
ISBN 90-5808-287-3

Stellingen

1) De niet fytopathogene schimmel *Aspergillus nidulans* lijkt een geschikt model om de expressie van het avirulentiegen *Avr*9 van de tomatenpathogene schimmel *Cladosporium fulvum* te bestuderen (*dit proefschrift*).

2) De in de *Avr*9 promoter aanwezige TAGATA-sequenties zijn cruciaal voor de inductie van deze promoter in *Aspergillus nidulans* (*dit proefschrift*).

3) De belangrijkste regulator, *Nrf*1, van het *Cladosporium fulvum* avirulentiegen *Avr*9 is tevens betrokken bij de regulatie van het stikstofmetabolisme van deze schimmel (*dit proefschrift*).

4) Onderzoek naar de fysiologische aspecten van een plant-pathogeen interactie is sterk onderbelicht in vergelijking met de moleculaire aspecten.

5) Het toegenomen aantal geïsoleerde avirulentiegenen heeft er nog niet toe geleid dat het inzicht in de intrinsieke functie van deze genen is vergroot.

6) Een recombinant DNA-laboratorium is een speelplaats voor grote mensen, het onderzoek laat zich dan ook omschrijven als knippen en plakken met DNA.

7) Op de fiets doet de Wageningse berg soms zijn naam eer aan.

8) Het druk hebben is een luxeprobleem.

9) Een goedgelovig wetenschapper is de waarheid vaak ontrouw.

10) Normaal zijn alleen de mensen die je niet kent.

11) Soms is het resultaat teleurstellend, dit betekent echter nog niet altijd dat het een teleurstellend resultaat is.

12) Dit proefschrift is een grote stap voor mij maar een kleine stap voor de wetenschap (*vrij vertaald naar Neil Armstrongs eerste stap op de maan, 20 juli 1969*).

Stellingen behorende bij het proefschrift van Sandor Snoeijers: Regulation of the avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum*. Wageningen, 10 oktober 2000.

Voorwoord

Ik heb me lang afgevraagd hoe het zou zijn om het voorwoord te schrijven en nu is het dan zover. Het schrijven van het voorwoord betekent in principe dat het proefschrift af is. Aan de ene kant ben ik dan ook opgetogen en aan de andere kant heeft het iets melancholisch omdat ik hiermee toch een hele fijne periode afsluit.

Mijn onderzoeksproject was een samenwerking tussen de Laboratoria van Moleculaire Genetica en Fytopathologie. Hierdoor was ik in de gelukkige positie om van beide labs kennis en technieken te absorberen.

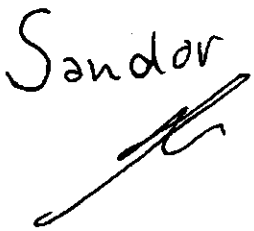
Dit proefschrift had natuurlijk nooit tot stand kunnen komen zonder de hulp van een groot aantal mensen. Allereerst ben ik dank verschuldigd aan de staf van mijn thuisbasis, het lab van Moleculaire Genetica. De technische adviezen van Theo, het nooit moeilijk doen als ik weer iets duurs wilde bestellen van Bert en de wijze woorden van Henk zal ik missen.
Het was heel prettig om met jullie samen te werken en in de toekomst zal ik dan ook zeker proberen om het contact warm te houden. Verder waren de jaarlijkse zeiltripjes naar het Veluwemeer o.l.v. Bert altijd weer een enorm succes en iets om weer vele weken op te kunnen teren. Daarnaast wil ik Diana en Hans bedanken die mij als verse OIO de eerste stapjes op het lab hebben bijgebracht.

Ik werd tijdens mijn onderzoeksperiode bijgestaan door een flink aantal studenten. Luc, Serkan, Bart, Roy, Asha, Philipp, Marcus en Clara bedankt voor jullie hulp en het doorzettingsvermogen dat jullie aan de dag wisten te brengen als er weer een proefje niet was gegaan zoals we gewild hadden. Gelukkig hebben jullie je er niet door laten afschrikken en hebben de meeste van jullie zelfs gekozen voor een vervolgcarrière binnen de wereld die wetenschap heet. Special was the collaboration with our Spanish post-doc Alejandro. Alejandro, many thanks for your contribution to this thesis and the good times we had also besides the work. Especially, I will never forget the great performance you gave on my birthdayparty a couple of years ago. When you think about quitting science one day there would be a brilliant career for you in the future as a singer, for sure.

Natuurlijk wil ik ook de gehele populatie van de vakgroep Genetica bedanken voor de goede tijd die ik aldaar heb doorgebracht. De discussies tijdens de koffiepauzes waren van allerhande niveau maar desalniettemin buitengewoon gezellig. Als ik weer eens te laat was voor de koffie was daar altijd Henny die iets voor me had achtergehouden zodat ik alsnog van m'n "caffeïneshot" kon genieten. In het bijzonder wil ik buurman Wim bedanken met wie ik

naast het lableven ook vele (sportieve) uren heb doorgebracht en met wie ik de emotionele pieken en dalen van het OIO-schap van dichtbij heb gedeeld.

Twee mensen van het Fytolab waren ontzettend belangrijk voor de totstandkoming van dit proefschrift. Matthieu, bedankt voor je enorme steun tijdens vooral de schrijffase. Je wist door je precieze manier van nakijken, soms tot grote frustratie van mij want meestal had je gelijk, net iets beter de puntjes op de i te zetten. Pierre, ondanks het feit dat ik toch een beetje een vreemde eend in de bijt was van het Fytolab heb ik dat nooit zo gevoeld en kon ik wat betreft begeleiding altijd voor de volle honderd procent op je rekenen.



-Contents-

Chapter one

General introduction

S. S. Snoeijers, A. Pérez-García, M. H. A. J. Joosten and P. J. G. M. De Wit

Adapted from European Journal of Plant Pathology 106: 493-506

Plants acquire nitrogen from two major pools: soil and the atmosphere. Nitrogen from the soil is usually taken up as nitrate, whereas atmospheric molecular nitrogen is incorporated through symbiotic fixation by micro-organisms (Mylona et al., 1995). Irrespective of the source, in higher plants inorganic nitrogen must eventually be reduced to ammonia before it can be assimilated (Lea, 1992). Ammonia is incorporated into glutamine, glutamate, asparagine and aspartate, which are the predominant nitrogen-carrying molecules in plants (Lam et al., 1996). They provide building blocks for synthesis of additional amino acids, proteins, nucleotides, hormones, chlorophyll and a variety of other essential plant constituents.

Successful colonisation of plants by a pathogen requires utilisation of nutrient resources present in host tissues. Although little is known about how plant pathogens assimilate nitrogen after their entry into the host, it is tempting to speculate that the nutritional status of the plant affects transcription of specifically *in planta*-induced genes of pathogens. The nitrogen sources available for a pathogen in the host plant are dependent on the tissue that is being colonised. Nitrogen sources used by a root pathogen might be different from those used by a leaf pathogen. Similarly, a necrotrophic pathogen which kills tissues, is probably able to use a broader spectrum of nitrogen sources than a biotrophic pathogen which feeds on living host tissue and only has access to nitrogen sources available in the apoplast and/or the haustorial matrix. Knowledge of nitrogen metabolism of phytopathogenic bacteria and fungi is limited. However, extensive studies on nitrogen metabolism and its regulation have been conducted with model organisms like the enteric bacteria *Escherichia coli* and *Salmonella* spp. (Magasanik, 1996), and the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* (Marzluf, 1997).

Nutritional limitation of various types, in particular of nitrogen, appears to affect pathogenesis. The observation that bacterial and fungal genes (Talbot et al., 1997) are both induced during pathogenesis and under nitrogen-limiting conditions in artificial media, suggests that during growth *in planta* there is limited nitrogen available for pathogens. Here we describe the effect of nitrogen and nitrogen-regulated genes on development of bacterial and fungal pathogens in the host. *In planta*-induced pathogenicity, virulence and avirulence genes, that are also induced *in vitro* under nitrogen- or nutrient-limiting conditions, are also discussed.

Effect of the availability of nitrogen on plant disease development

Nitrogen supply can affect disease development. High concentrations of nitrogen often increase susceptibility of plants to diseases (Agrios, 1997). Pathogens and diseases that are stimulated by nitrogen supply to the host are presented in Table 1.

Table 1. Pathogens, hosts and diseases stimulated by increased nitrogen supply to the host.

Pathogen	Host	Disease	References
Corynebacterium sepedonicum	potato	ring rot	Gallegly and Walker, 1949
Erwinia amylovora	pear	fire blight	Agrios, 1997
Erwinia stewartii	corn	Stewart's wilt	McNew and Spencer, 1939
Pseudomonas syringae subsp. savastanoi	olive	olive knot	Balestra and Varvaro, 1997
Streptomyces scabies	potato	scab	Lapwood and Dyson, 1966
Xanthomonas campestris pv. vesicatoria	tomato	bacterial spot	McGuire et al., 1991
Botrytis cinerea	grape	botrytis bunch rot	Chérif and Boubaker, 1997
Colletotrichum gloeosporioides	tomato	fruit and root rot	Williams, 1965
Erysiphe graminis	wheat	powdery mildew	Last, 1953
Magnaporthe grisea	rice	rice blast	Teng, 1994
Puccinia graminis	wheat	stem rust	Daly, 1949
Verticillium albo-atrum	potato, tomato	wilt	Wilhelm, 1950

In general, nitrogen is needed to provide plants with building blocks required for growth and to resist or recover from disease injury. Plants suffering from a lack of nitrogen are weaker, grow slower and age faster. Such plants become more susceptible to pathogens that are specialised in infecting weak, slowly-growing plants. It has been reported that reduced availability of nitrogen increases the susceptibility of tomato to wilt caused by *Fusarium oxysporum* f.sp. *lycopersici*, early blight of many solanaceous plants caused by *Alternaria solani* and damping-off of seedlings, resulting from *Pythium* spp. infections (Agrios, 1997). The form of nitrogen available to plants and pathogens also affects the severity of the disease

(Huber and Watson, 1974). For example, ammonia stimulates diseases caused by *Fusarium*, *Rhizoctonia* and *Sclerotium* on citrus, wheat, cotton, tomato and sugar beet. Alternatively, corn and pea root rots, cotton root rot and tobacco and tomato wilts, diseases caused by *Pythium*, *Phymatotrichum* and *Pseudomonas*, respectively, are favoured by nitrate. Contradictory results have been reported by McElhaney et al. (1998) who studied the interaction between cabbage and *Xanthomonas campestris* pv. *campestris*. Irrespective of the source of nitrogen used, high levels of nitrogen dramatically reduced the level of systemic colonisation of the xylem by the bacterium as well as the development of black rot lesions.

Modification of plant nitrogen metabolism by pathogens

Nitrate, which is the major source of inorganic nitrogen available for plants is, after uptake from the soil, either stored in the vacuole or converted into nitrite by nitrate reductase (NR). After conversion, nitrite enters the chloroplast (or plastid in the root) and is reduced by nitrite reductase (NiR) to ammonia, which is then converted to various amino acids by glutamine synthetase (GS) (Crawford, 1995). Pérez-García et al. (1995) found that during infection of tomato by *P. syringae* pv. *tomato* a novel GS isoform accumulated in infected leaves. This isoform might be involved in reassimilation and transport of nitrogen released during protein degradation in infected tissues to healthy parts of the plant. However, the amino acid levels detected in infected leaves indicate that asparagine, rather than glutamine, the main precursor for the synthesis of all other amino acids, is involved in nitrogen transport (Pérez-García et al., 1998). Interestingly, asparagine is also the major amino acid involved in remobilization of nitrogen during leaf senescence, while the most important route for asparagine biosynthesis in plants is glutamine-dependent. This suggests that, both during natural senescence and during pathogenesis similar mechanisms are induced to save nitrogen.

Although increased synthesis of amino acids and other nitrogen-carrying compounds is necessary for active plant defence, knowledge of nitrogen metabolism and amino acid synthesis during host plant colonisation by pathogens is very limited. It is known that phenylalanine and hydroxyproline are amino acids important in active plant defence. Following infection, phenylalanine is converted, by phenylalanine ammonia lyase (PAL), into trans-cinnamic acid, an important precursor for biosynthesis of phenylpropanoid compounds. These include phytoalexins, as well as precursors of structural defence molecules such as lignin (Dixon and Harrison, 1990). Hydroxyproline is the most abundant amino acid present

in hydroxyproline-rich glycoproteins, which strongly increase in concentration during active defence and are deposited in cell walls. There they may contribute to resistance by trapping the pathogen, or act as structural barriers and sites for lignin deposition (Showalter, 1993).

Some pathogens have acquired specialised virulence factors (mainly toxins), that interfere with nitrogen metabolism of the host. Among the wide variety of toxins produced by bacterial and fungal pathogens, some inhibit biosynthesis of amino acids (antimetabolites), resulting in amino acid deficiency. The best known antimetabolite toxins produced by phytopathogenic bacteria are tabtoxin and phaseolotoxin, both produced by pathovars of *Pseudomonas syringae* (Bender et al., 1999). Tabtoxin is a monocyclic ß-lactam that is not toxic by itself, but after hydrolysis by host aminopeptidases releases the toxic tabtoxinine (Durbin and Uchytil, 1984). Tabtoxinine irreversibly inhibits GS, resulting in ammonia accumulation, causing disruption of the thylakoid membrane of the chloroplast and the uncoupling of photosynthesis and photorespiration, leading to chlorosis (Turner and Debbage, 1982).

Phaseolotoxin competitively inhibits ornithine carbamoyltransferase (OCTase), which converts ornithine and carbamoyl phosphate to citrulline, a precursor of arginine (Mitchell, 1976; Moore et al., 1984). The toxin is hydrolysed in plants by peptidases to produce octicidine, a more potent, irreversible inhibitor of OCTase and apparently the active form of the toxin in plants. Inhibition of OCTase causes accumulation of ornithine and deficiency in intracellular pools of arginine, leading to chlorosis (Mitchell and Bielski, 1977).

One obvious advantage for a pathogen to produce an antimetabolite toxin is the induction of metabolic deficiency in host cells and the concomitant accumulation of intermediates that can be metabolised by the pathogen itself. Most of the antimetabolite toxins secreted by pathogens possess antimicrobial activity with a different spectrum and efficiency (Völksch and Weingart, 1998). Thus, the antagonistic activity of antimetabolite toxins could be an advantage for the toxin-producing bacteria to adapt to different habitats in competition with other micro-organisms. This is supported by the observation that in *P. syringae*, genes for toxin production seem to be conserved among most pathovars, suggesting that they are important for competitive ability of the bacteria in plants. Thus, toxins interfering with amino acid biosynthesis appear to be pathogenicity factors, facilitating pathogens to colonise host tissues.

Bacterial and fungal genes that are induced *in planta* and under conditions of nitrogen limitation, *in vitro*

Whether a plant is susceptible or resistant to an attacking pathogen depends in most cases on the presence of specific proteins produced by both the plant and the pathogen. Proteins from the pathogen that are recognised by the host are called elicitors and are encoded by avirulence (*Avr*) genes. After recognition of the pathogen (through its elicitors) by the host, carrying the matching resistance (*R*) gene, the plant often mounts a hypersensitive response (HR) which is considered to be the most versatile plant resistance response to viruses, bacteria, fungi, nematodes and insects (Keen et al., 1990; Joosten and De Wit, 1999). During HR, a cascade of defence responses is activated. These responses often include early irreversible membrane damage, generation of reactive oxygen species and induction of genes coding for enzymes involved in synthesis of phytoalexins, hydroxy proline-rich cell wall glycoproteins and pathogenesis-related proteins (PRs) (Lucas, 1998).

Pathogenicity (*Path*), virulence (*Vir*) and *Avr* genes are usually highly expressed during growth of the pathogen in the host tissue. In the remaining part of this review examples of bacterial and fungal genes which are highly expressed *in planta*, but which are also induced under conditions of nitrogen- or general nutrient limitation *in vitro*, are discussed.

Expression of bacterial pathogenicity, (a)virulence and regulatory genes *in planta* and *in vitro*

The Gram-negative phytopathogenic bacteria of the four major genera *Pseudomonas*, *Xanthomonas*, *Ralstonia* and *Erwinia*, contain *hrp* (for hypersensitive response and pathogenicity) genes, which are essential for the interaction with both susceptible and resistant plants (Bonas, 1994; Lindgren, 1997). Hrp proteins are homologous to the proteins of the mammalian bacterial pathogen type III secretion system and are thought to be involved in transfer of (a)virulence and pathogenicity factors to host cells (Long and Staskawicz, 1993; Bonas and Van den Ackerveken, 1997; Rossier et al., 1999). This hypothesis is supported by the observations that bacterial *Avr* genes only function in the presence of a complete set of *hrp* genes (Dangl, 1994) and that injection of bacterial AVR proteins into the intercellular

spaces of leaves of plants containing the matching resistance genes, does not result in the induction of a HR (Knoop et al., 1991).

In general, *hrp* genes are highly expressed in minimal media, whereas they are usually not expressed in rich media (Rhame et al., 1992; Wei et al., 1992; Bonas, 1994). Transcriptional activation of *hrp* genes of *P. syringae* during co-culture with tobacco cells did not occur, but could be achieved by incubating the bacteria in nitrogen-deficient media (Yucel et al., 1989). Also in *Erwinia amylovora*, high levels of expression of the *hrp* loci, comparable to those obtained during the development of a HR in tobacco, were detected in nitrogen-limiting media (Wei et al., 1992).

Regulation of *hrp* genes has been studied extensively in the phytopathogenic bacterium *P. syringae* pv. *syringae*. In this strain, the *hrp* genes *hrp*R, *hrp*S and *hrp*L are part of a multicomponent regulatory system that controls the expression of certain *hrp* and *Avr* genes. The HrpR and HrpS proteins are related to the bacterial NtrC class of nitrogen regulators (Xiao et al., 1994). NtrC is a member of a two-component regulatory system consisting of an environmental sensor (NtrB) and a response regulator (NtrC) (Albright et al., 1989; Lindgren, 1997). The NtrB/NtrC pair regulates transcriptional activation of various genes involved in nitrogen assimilation. The amino-terminal domain of NtrC acts as the regulatory domain. Under conditions of nitrogen limitation phosphorylated NtrB interacts with this domain to activate NtrC by phosphorylation (Figure 1A; Merrick and Edwards, 1995). A characteristic feature of genes activated by the NtrC class of proteins is the requirement for sigma factor 54 (encoded by the *rpo*N gene) as coactivator. HrpR and HrpS differ from most members of the NtrC family as they lack the amino-terminal, regulatory domain. However, they do contain the conserved carboxy-terminal domain of NtrC, which is a helix-turn-helix motif that enables NtrC to recognise specific enhancer sequences.

In general, sigma factors control a large array of bacterial genes that are expressed during nutrient limitation. Conserved sigma factor 54 motifs have been found in promoters of a number of *P. syringae hrp* and *Avr* genes (Innes et al., 1993; Shen and Keen, 1993; Xiao and Hutcheson, 1994). An important sigma factor in bacteria is the RpoS protein which regulates a set of genes that serves to maintain viability during periods of starvation and environmental stress (O'Neal et al., 1994). Though highly sensitive to a number of environmental stresses, an *E. amylovora rpo*S mutant was not compromised in its ability to grow or cause disease on apple seedlings (Anderson et al., 1998). Similarly, the *rpo*N gene of *Xanthomonas campestris* pv. *vesicatoria* is not the only regulatory gene required for pathogenicity (Horns and Bonas, 1996).

Avirulence gene D (*Avr*D) from *P. syringae* pv. *tomato*, of which the encoded product directs the synthesis of syringolide elicitors inducing a genotype-specific HR, contains a typical sigma factor 54-dependent promoter (Keen et al., 1990; Midland et al. 1993; Shen and Keen, 1993). The *Avr*D gene is highly induced upon colonisation of host tissues or when the bacteria are growing *in vitro* at low pH or in media containing low concentrations of carbon or nitrogen (Shen et al., 1992; Shen and Keen, 1993). The *Avr*B and *Avr*E genes from *P. syringae* pv. *glycinea* and pv. *tomato*, respectively, show comparable expression patterns when grown under similar conditions (Huynh et al., 1989; Lorang and Keen, 1995). This is also the case for the *Avr* genes *Dsp*A and *Dsp*E from *E. amylovora* (Gaudriault et al., 1997; Bogdanove et al., 1998).

Research on bacterial infectious diseases of mammals has a long history. The "nutrition-inhibition" hypothesis, stating that facultative intracellular pathogens encounter both an inhibitory and a nutrient-limited environment during infection, was proposed more than forty years ago (Groisman and Ochman, 1994). A direct role for RpoS in regulation of expression of pathogenicity factors has been confirmed for a number of mammalian pathogens, such as *Salmonella* and *Yersinia* species (Heiskanen et al., 1994; Iriarte et al., 1995). A stress response of *Salmonella typhimurium* occurs when this bacterium is starved for essential nutrients, such as phosphate, carbon or nitrogen. The physiological changes that *S. typhimurium* undergoes in response to starvation stress are referred to as the starvation stress response (SSR). The genetic loci whose expression increases in response to starvation-stress, together form the SSR stimulon. Loci of the SSR stimulon encode transport systems, enzymes involved in carbon catabolism, protective enzymes, respiratory enzyme systems, regulatory proteins, virulence factors and unclassified products. The majority of these loci are under positive control of RpoS. Furthermore, there might be a link between SSR and virulence, since RpoS is required for full virulence of *Salmonella*. Moreover, the *spv* (*Salmonella* plasmid-associated virulence) genes, required for *Salmonella* to cause systemic disease, are N (and P- and C-)-starvation-inducible (Nickerson and Curtiss, 1997; Spector, 1998). However, a direct link between starvation-stress and virulence has not been conclusively established yet.

The infectious gram-positive bacteria *Listeria monocytogenes* and *L. ivanovii* carry a special set of *Vir* genes that are switched on when the bacterium encounters a host. In *Listeria*, the PrfA protein regulates *Vir* gene expression during pathogenesis. A peak in *PrfA* expression during growth in liquid media coincides with the onset of the stationary phase when nutrients become limiting, suggesting that nutrient starvation contributes to

upregulation of *Listeria Vir* genes (Mengaud et al., 1991). Table 2A gives an overview of the nitrogen-induced or repressed *Path, Avr* and *Vir* genes and their regulators in bacteria.

Table 2A. Bacterial genes induced during infection of the host and during nitrogen starvation *in vitro*.

Bacterial pathogen	Host	Induced gene(s)	Possible function(s)	Regulatory genes	References
Pseudomonas syringae	tomato, soybean	*hrp* genes	secretion of avirulence factors		Rhame et al., 1992; Xiao et al., 1994
	tomato, soybean	*Avr*B, *Avr*D and *Avr*E	(a)virulence	*hrp*L, *hrp*R, *hrp*S, *rpo*N	Huynh et al., 1989; Shen et al., 1992; Lorang and Keen, 1995
Erwinia amylovora	pear	*Dsp*A, *Dsp*E	(a)virulence	*rpo*S	Gaudriault et al., 1997; Bogdanove et al., 1998
	pear	*hrp*	secretion of avirulence factors		Wei et al., 1992
Erwinia spp, *Salmonella typhimurium*	several species mammals	*hrp* and *Avr*	secretion of avirulence factors and avirulence	*rpo*S	O'Neal et al., 1994
Listeria monocytogenes, L. ivanovii	mammals	*Vir*	virulence	*Prf*A	Mengaud et al., 1991

Figure 1A shows a model of the bacterial nitrogen-catabolic pathway. The proposed role of NtrC for induction of nitrogen-dependent *Path* and *Avr/Vir* genes in bacterial pathogens is also shown in this figure.

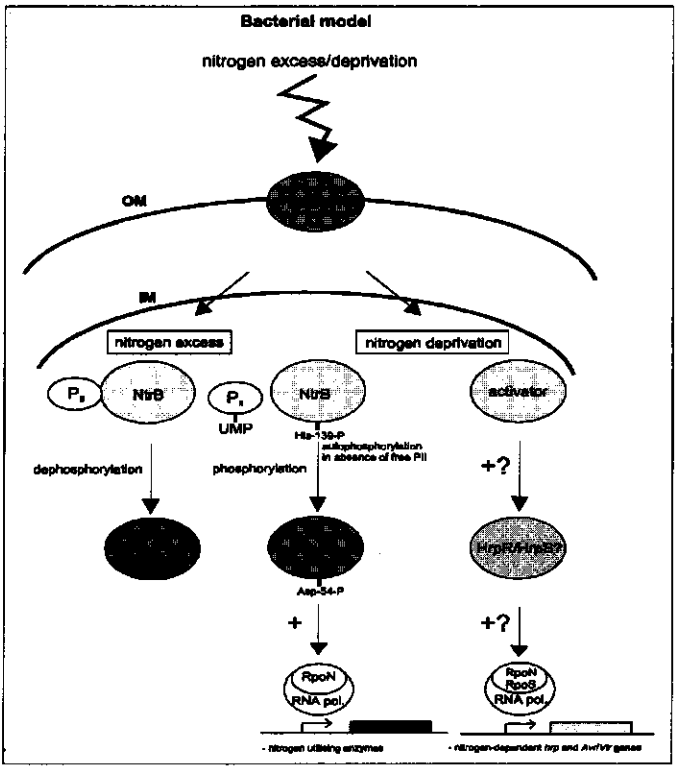


Figure 1A. Model for nitrogen-sensing and induction of nitrogen-dependent *hrp* and *AvrlVir* genes of bacterial pathogens. Under conditions of nitrogen excess the P_{II} protein binds to NtrB to activate its phosphatase activity. When this occurs, NtrB dephosphorylates NtrC so it can not bind to enhancer sequences to increase transcription. However, P_{II}-UMP, which is present during nitrogen deprivation, can not bind to NtrB. In this situation, NtrB is autophosphorylated on His-139. When NtrB is in its phosphorylated state, it catalyses the phosphorylation and activation of NtrC. NtrC has an amino-terminal that acts as the regulatory receiver domain. Phosphorylated NtrB interacts with this domain to phosphorylate Asp-54 of the NtrC protein. The NtrC central domain contains a conserved nucleoside-binding site and is believed to be the domain responsible for interacting with sigma factor RpoN to activate transcription. The carboxy-terminus contains a helix-turn-helix motif which facilitates interaction of NtrC with specific enhancer sequences. The bacterial HrpR and HrpS proteins are related to the NtrC class of proteins, although they lack the amino-terminal domain of NtrC, an interaction with RpoN/RpoS, that induce several *AvrlVir* and hypersensitive response and pathogenicity (*hrp*) genes is hypothesised (for further details see text).

OM: outer membrane; **IM:** inner membrane; **P:** phospate; **UMP:** 2p-deoxyuridine 5p-monophosphate

Expression of fungal pathogenicity, (a)virulence and regulatory genes *in planta* and *in vitro*

To be a successful pathogen, a fungus must be able to adjust its metabolism to utilise nutrients available within the host tissue. Little is known about metabolic control circuits in phytopathogenic fungi and the role of regulation of metabolism and/or nutritional signals in disease development. This is in contrast to the non-pathogenic filamentous fungi *Aspergillus nidulans* and *Neurospora crassa* of which nitrogen metabolism has been studied extensively (Marzluf, 1997). In the latter fungi the major, positively-acting regulatory genes *are*A (*A. nidulans*) and *nit*-2 (*N. crassa*) mediate global nitrogen repression and derepression. The regulatory proteins that these genes encode, possess both a DNA-binding domain which consists of a single Cys2/Cys2-type zinc finger motif (Scazzocchio, 2000). Both AREA and NIT2 are members of the GATA-family of transcription factors that bind to promoter domains containing a GATA sequence (Fu and Marzluf, 1990; Punt et al., 1995). AREA and NIT2 activate the expression of many genes whose products are required for the utilisation of nitrogen from various secondary sources (e.g. nitrate- and nitrite reductase) or when nitrogen is limited (Marzluf, 1997).

In pathogenic fungi, the loss of such a major, wide-domain nitrogen regulator might repress the expression of genes that are necessary for pathogenicity and could affect the ability of the pathogen to grow and proliferate within the host. AREA-like proteins with similar structure and function have been described in *Penicillium* and *Aspergillus* (Haas et al., 1995; Ellis, 1996; Christensen et al., 1998; Hensel et al., 1998; McCabe et al., 1998; Gente et al., 1999), in the phytopathogens *Magnaporthe grisea* (Froeliger and Carpenter, 1996), *Gibberella fujikuroi* (Tudzynski et al., 1999) and *Cladosporium fulvum* (A. Pérez-García et al., unpublished), and in the insect pathogen *Metarhizium anisopliae* (Screen et al., 1998).

Avirulence gene *Avr*9 of the biotrophic fungal pathogen *C. fulvum* (Van Kan et al., 1991; Van den Ackerveken et al., 1992) is both induced *in planta* and during nitrogen starvation *in vitro* (Van den Ackerveken et al., 1994; Snoeijers et al., 1999). Although the intrinsic function of the AVR9 peptide is unknown, it triggers a HR in tomato plants carrying the matching *Cf-9* resistance gene (De Wit et al., 1995; Joosten and De Wit, 1999). The *Avr*9 promoter, which contains twelve putative AREA-binding sites, was found to be also active in *A. nidulans*. In an *A. nidulans are*A null mutant, however, the promoter was not induced upon nitrogen starvation, suggesting that an AREA-like transcription factor is involved in the induction of *Avr*9 expression in *C. fulvum* (Van den Ackerveken et al., 1994; Snoeijers et al.,

1999). The *C. fulvum areA*-homologous gene (*Nrf*1) has been cloned (Pérez-García et al., unpublished.) and gene disruption experiments will reveal whether the NRF1 protein is involved in regulation of *Avr*9 expression in *C. fulvum*. From this fungus five unique, differentially expressed cDNAs have been isolated after screening a cDNA library, obtained from nitrogen- and carbon-starved mycelium, with cDNA probes prepared from infected tomato leaf tissue (Coleman et al., 1997). Northern hybridisation confirmed that all five cDNAs were both starvation- and *in planta*-induced. Two of the clones were found to encode an alcohol- and aldehyde dehydrogenase, respectively (Coleman et al., 1997). In addition, six different hydrophobin-encoding genes have recently been cloned from *C. fulvum* (Segers et al., 1999; Spanu et al., 2000). Two of those, *HCf*-4 and *HCf*-5, showed clear induction under nitrogen-limiting conditions. Fungal hydrophobins have been shown to play an important role in many morphogenetic processes including sporulation, fruiting body development and infection structure formation (Wessels, 1997; Kershaw and Talbot, 1998).

For the rice blast fungus *M. grisea*, a pathogen of various cereals and grasses (Valent and Chumly, 1991; Talbot, 1995), the role of the *areA*-like gene, *nut*1, in pathogenesis was studied by generating *nut*1 null mutants (Froeliger and Carpenter, 1996). Under standard assay conditions, colonisation of susceptible plants by *nut*1 null mutants was similar to that of wild-type *M. grisea* strains. Although only a small number of host plants has been tested, the major nitrogen regulator NUT1 appears to only partly affect pathogenicity of this fungus, causing smaller lesions on plants infected by the *nut*1 null mutants when compared to the wild-type strain. It was suggested that in these transformants, which can not utilise secondary nitrogen sources, nitrogen starvation and inhibition of fungal growth might occur sooner than in wild-type strains. Two additional nitrogen-regulatory genes, non-allelic to *nut*1, designated *npr*1 and *npr*2 (for nitrogen pathogenicity regulation genes 1 and 2), were identified and mutation of either of these genes resulted in an *areA*-like mutant phenotype and a dramatic loss of pathogenicity. It appeared that NPR1 and NPR2, in addition to their involvement in nitrogen regulation, are required for starvation-related gene expression in *M. grisea* (Lau and Hamer, 1996). NPR1 and NPR2 are likely to be alternative global nitrogen regulators of a wider control mechanism, that regulates genes involved in pathogenesis. Furthermore, Talbot et al. (1997) found that under nitrogen starvation *M. grisea* also secretes products that cause senescence of rice leaves, reminiscent of the symptoms caused by the fungus itself. Strains defective in *nut*1, *npr*1 or *npr*2, produced only residual senescence-inducing activity.

The *mpg*1 gene from *M. grisea* was identified in a differential cDNA screen for fungal genes expressed during growth *in planta*. The *mpg*1 gene encodes a small hydrophobic

protein that is highly expressed during appressorium formation, which is required for successful penetration of this fungus into host cells (Talbot et al., 1993). Examination of the regulation of *mpg*1, revealed that the gene is induced during nitrogen- and carbon limitation *in vitro*. The *mpg*1 promoter also contains typical GATA-sequences (Talbot et al., 1993). Although NUT1 is required for high-level expression of *mpg*1 (Lau and Hamer, 1996), a direct role for these GATA-sequences in regulation of *mpg*1 expression has not yet been demonstrated.

The most striking example of genes of which expression is induced under nitrogen-limiting conditions *in vitro*, and *in planta* comes from the genus *Colletotrichum*, which includes pathogens that infect a wide range of tropical crop plants. A cDNA clone (p*CgGS*) that preferably hybridised to a cDNA probe prepared from leaves of the forage legume *Stylosanthes guianensis* infected by *C. gloeosporoides*, has been isolated by differential screening of a cDNA library from a nitrogen-starved axenic culture of this fungus (Stephenson et al., 1997). The sequence of p*CgGS* is highly homologous to genes encoding glutamine synthetase (GS). Expression studies indicated that in *C. gloeosporoides* induction of GS occurred during early infection and also under nitrogen-limiting conditions *in vitro* (Stephenson et al., 1997). In addition, an essential *Path* gene, called *CgDN*3 has been isolated from this fungus. It was suggested to be a suppressor of plant defence, since its disruption led to loss of pathogenicity and a strong induction of defence responses in the host. *CgDN*3 is expressed at early stages of infection and is also induced in axenic culture by nitrogen starvation. The *CgDN*3 promoter also contains GATA sequences, potentially interacting with AREA-like transcription factors (Stephenson et al., 1998).

The production of extracellular proteases seems particularly important for insect and nematode-infecting fungi. In the entomopathogenic fungus *Metarhizium anisopliae*, the products encoded by the genes *pr*1A and *pr*2 show protease activity. Both genes are major determinants of pathogenicity and their expression is subject to both carbon and nitrogen repression (St. Leger et al., 1992; St. Leger, 1995; Smithson et al., 1995). This has also been observed for the extracellular serine protease PII of the nematode-trapping fungus *Arthrobotrys oligospora* (Ahman et al., 1996). Both *pr*1A and *pr*2 genes contain GATA sequences in their promoters, suggesting that they are under control of the *M. anisopliae* AREA-like protein, designated NRR1 (Screen et al., 1998).

For the fungus *Aspergillus fumigatus*, pathogenic on mammals and the major agent of invasive aspergillosis, two observations support the importance of an *are*A-like gene (*afare*A) for growth in lung tissue. First, in neutropenic mice, which have a strong reduction of

resistance against pathogens, inoculated with an *afare*A-deletion mutant, the onset of symptoms of aspergillosis was delayed compared to mice inoculated with the *afare*A wild-type parent strain. Secondly, among fungal colonies rescued from lung tissue inoculated with an *afare*A disruptant, the percentage of revertants was approximately 40%, compared to approximately 5% among colonies that had been growing on artificial medium with ammonium as nitrogen source. These results indicate that the AFAREA regulator protein is beneficial for growth in lung tissue, an environment where the fungus encounters different nitrogen sources that require the induction of several nitrogen-catabolic genes (Hensel et al., 1995; Hensel et al., 1998).

In *N. crassa*, mutation of the *nmr* (for nitrogen metabolic regulation) gene results in derepression of nitrate reductase and other nitrogen-controlled genes, in the presence of ammonia or glutamine concentrations that completely repress expression of these genes in *nmr* wild-type strains (Tomsett et al., 1981). The *nmr* genes of *N. crassa*, *A. nidulans* and *G. fujikuroi* have been cloned (Young et al., 1990; Andrianopoulos et al., 1998; Tudzynski et al., unpublished). The encoded proteins have no distinctive characteristics, such as DNA-binding or protein kinase motifs. Most likely the *N. crassa* NMR protein functions as a negative regulator by binding to the NIT2 protein. Direct interaction between NMR and NIT2 has been shown to occur in the yeast two-hybrid system (Xiao et al., 1995) and in *in vitro* binding assays (Xiao and Marzluf, 1993). *In vitro* mobility shift assays suggested that NMR inhibits binding of NIT2 to DNA (Xiao et al., 1995). Most probably the NMR protein binds directly to the NIT2 protein, thereby blocking trans-activation of NIT2 when sufficient concentrations of primary nitrogen sources (e.g. glutamine or ammonia) are available. Isolation and characterisation of *nmr* homologues from pathogenic fungi should give more insight into the role of this gene during pathogenesis. Table 2B gives an overview of nitrogen-induced *Path* and *Avr/Vir* genes, and their regulators in fungi.

Table 2B. Fungal genes induced during infection of the host and during nitrogen starvation *in vitro*.

Fungal pathogen	Host	Induced gene(s)	Possible function(s)	Regulatory genes	References
Cladosporium fulvum	tomato	*Avr9*	avirulence factor	*Nrf1**	Snoeijers et al., 1999
		p*SI-9*	aldehyde dehydrogenase		Coleman et al., 1997
		p*SI-10*	alcohol dehydrogenase		Coleman et al., 1997
Magnaporthe grisea	rice and several grasses	*mpg*1	hydrophobin	*nut*1, *npr*1, *npr*2	Talbot et al., 1993; Froeliger and Carpenter., 1996; Lau and Hamer., 1996
Colletotrichum gloeosporioides	tropical legumes	p*CgGS*	glutamine synthetase	n.i.	Stephenson et al., 1997
		*CgDN*3	suppressor of plant defences		Stephenson et al., 1998
Metarhizium anisopliae	insects	*pr1*A	protease	*nrr*1	St. Leger, 1995; Smithson et al. 1995
		*pr*2	protease		
Arthrobotrys oligodpora	nematodes	*p*II	protease	n.i.	Ahman et al., 1996

* Isolation of the *Nrf*1 gene from *C. fulvum* is published in Chapter five.
n.i. = not isolated

In Figure 1B a model for the fungal nitrogen-catabolic pathway and the proposed role of the AREA-like protein for induction of nitrogen-dependent *Avr/Vir* genes in fungal pathogens are shown.

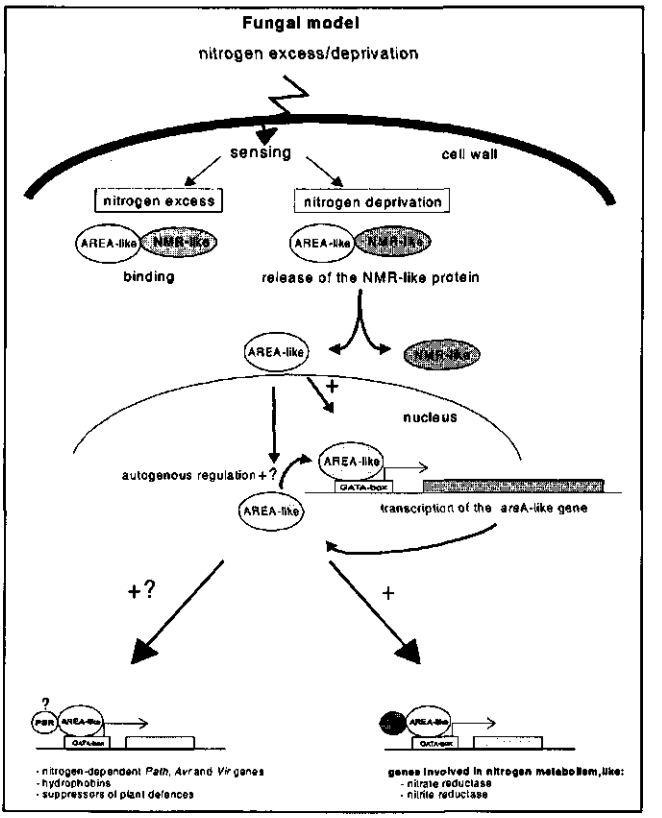


Figure 1B. Model for nitrogen-sensing and induction of nitrogen-dependent pathogenicity (*Path*) and (a)virulence (*Avr/Vir*) genes of fungal pathogens. The encoding genes are depicted as grey boxes, GATA sequences are depicted as white boxes in the promoter regions. When primary nitrogen sources are available, the negative regulatory (NMR-like) protein binds to the major positive-acting (AREA-like) protein preventing activation of genes involved in nitrogen utilisation. Under conditions of nitrogen limitation the NMR-like protein dissociates from the AREA-like protein. The released AREA-like protein induces the expression of a broad range of nitrogen metabolism genes (like nitrate- and nitrite reductase; for further details see text). It is assumed that the AREA-like proteins co-operate with multiple positive-acting, pathway-specific regulatory (PSR) proteins to turn on specific sets of nitrogen-catabolic genes, depending upon the availability of substrates and need for nitrogen. Whether the PSR proteins bind the AREA-like regulators is speculative. Several *are*A-like genes contain potential AREA-binding sites in their promoters, suggesting autogenous regulation (here depicted as a loop). A proposed role for the AREA-like proteins on expression of nitrogen-dependent *Avr/Vir* genes in fungal pathogens is shown.

Concluding remark

The general introduction consists of an overview concerning bacterial and fungal genes which are both specifically induced *in planta* during pathogenesis and during nitrogen (nutrient) starvation *in vitro*. Based on the data described in the introduction, we hypothesise that most of the *in planta*-induced genes are probably nutrient-survival genes, necessary to supply the pathogen with the suitable type of nutrients during growth in a nutrient-limiting micro-environment.

Acknowledgements: S. S. Snoeijers was supported by a grant from the Dutch Earth and Life Science Foundation (ALW; Projectnumber: 805.45.006), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Outline of the thesis

The aim of the research presented in this thesis is to obtain better insight into the regulation of avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum*. Expression of the *Avr*9 gene is strongly induced *in planta* and is also induced *in vitro* under conditions of nitrogen starvation.

As mentioned in the general introduction, the *Avr*9 promoter contains (TA)GATA sequences which are known as specific binding sites for a global positive nitrogen regulator in *Aspergillus nidulans* and *Neurospora crassa*, designated AREA and NIT2, respectively. Van den Ackerveken et al. (1994) hypothesised that the *Avr*9 gene is regulated by a protein similar to AREA and NIT2, which can bind to these specific sequences and induce *Avr*9 gene expression. As there are *A. nidulans are*A mutants available in our laboratory and gene-targeting systems have been developed for *A. nidulans*, initially this fungus was used as a model system to study *Avr*9 regulation.

Chapter two describes the induction of *Avr*9 promoter activities in different *A. nidulans are*A backgrounds, following nitrogen starvation. The observation that no *Avr*9 promoter activity was obtained in *A. nidulans are*A null mutants indicates that the *A. nidulans* AREA protein is able to induce *Avr*9 expression solely.

Chapter three emphasises the importance of the (TA)GATA sequences for *Avr*9 promoter activity in *A. nidulans*. Interestingly, the overlapping TAGATA sequences which are most proximal to the start codon were found to be crucial for promoter activity. However,

C. fulvum transformants containing *Avr9* constructs with various mutations in the promoter region that resulted in abolishment of inducibility in *A. nidulans*, were still avirulent on tomato plants containing the *Cf-9* gene, indicating that AVR9 is produced by these transformants. Subsequent *in vitro* expression studies revealed that in the *C. fulvum* transformants *Avr9* is also expressed in nutrient-rich liquid media, indicating that probably due to multiple integrations of the *Avr9* construct throughout the genome, *Avr9* is expressed independently from any nitrogen-regulatory element, and is under control of additional factors. The latter result emphasises the importance of a gene-targeting system for *C. fulvum*, allowing insertion of a construct of interest to a specific locus in the genome, by site-directed integration. Using such a targeting-system, promoter activities can be compared, without the influence of the chromosomal environment on expression. The development of such a *C. fulvum* gene-targeting system, based on the *C. fulvum pyr*1 marker gene, is described in **Chapter four**.

The isolation of the *C. fulvum areA/nit*-2-like gene, designated *Nrf*1 (for nitrogen response factor 1), and the role of the NRF1 protein in induction of *Avr9* expression *in vitro* and *in planta* is described in **Chapter five**. The data described in this chapter suggest that NRF1 is a major regulator of the *Avr9* gene, but that *in planta* at least one additional positive regulator of *Avr9* is active.

Chapter six deals with the question whether nitrogen starvation is the signal for *Avr9* expression *in planta*. Tomato plants containing both the functional *Cf-9* gene and elevated levels of nitrate in their apoplast, where the fungus resides, were more susceptible to strains of *C. fulvum* containing the *Avr9* gene, than wild-type *Cf9*-containing plants. This indicates that the *Avr9* gene is repressed in these plants and suggests that *in planta*, nitrogen-limiting conditions induce expression of the *Avr9* gene.

Finally, the implications of the results are discussed in **Chapter seven** and embedded in a broader perspective concerning communication between plants and their pathogens.

Chapter two

Transcription of the avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* is regulated by a GATA-type transcription factor in *Aspergillus nidulans*

S. S. Snoeijers, P. Vossen, T. Goosen, H. W. J. Van den Broek and P. J. G. M. De Wit

Adapted from Molecular and General Genetics 261: 653-659

Summary

The avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* is highly induced during infection of tomato plants. Expression of the *Avr9* gene can also be induced *in vitro* when grown on synthetic liquid medium containing little or no nitrogen. The *Avr9* promoter contains six copies of the sequence TAGATA and six additional copies of the core sequence GATA in the region immediately 0.4 kb upstream of the translation start site. In the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, these promoter sequences have been identified as the binding sites for a wide-domain GATA-type regulator (AREA in *A. nidulans* and NIT2 in *N. crassa*) involved in nitrogen utilisation. Quantification of GUS activity of *A. nidulans* transformants, containing a single copy of the fully active *Avr9* promoter-*uid*A (GUS) reporter gene fusion in different *are*A backgrounds, following nitrogen starvation, showed that induction of the *Avr9* promoter is similarly regulated in *A. nidulans* and *C. fulvum*. This suggests that AREA can regulate the *Avr9* promoter and that *C. fulvum* contains an AREA-like regulator that can bind to these specific sequence motifs. Comparison of induction of *Avr9* and *nia*D showed that *Avr9* expression is independent of NIRA, as is *nia*D expression upon nitrogen- starvation. Studies with *Avr9* promoter-*uid*A fusions in which all or most of these sequences had been deleted, showed that *Avr9* promoter activity is dependent

on the presence of these specific *cis* regulatory elements, suggesting that they are functional in transcriptional regulation of the *Avr9* gene.

Introduction

The interaction between the biotrophic, imperfect fungus *Cladosporium fulvum* Cooke (syn. *Fulvia fulva*) the causal agent of tomato leaf mold and its only host tomato (*Lycopersicon esculentum*), is an established model system to study plant-fungus gene-for-gene relationships (Oliver, 1992; De Wit, 1995; Xing et al., 1997; Hammond-Kosack and Jones., 1997). In incompatible *C. fulvum* tomato-interactions, avirulent races of the pathogen are recognized by resistant plants. Recognition is mediated by perception of extracellular race-specific elicitors, the products of *avir*ulence (*Avr*) genes of the fungus, by tomato genotypes carrying the matching resistance genes, resulting in the induction of a hypersensitive response (HR) and other defence responses arresting fungal growth immediately after penetration of the leaf through the stomata (De Wit and Spikman., 1982; De Wit et al., 1986; Scholtens-Toma and De Wit, 1988; Van Kan et al., 1992; Wubben et al., 1994; Joosten et al., 1997).

The avirulence gene *Avr9* of *C. fulvum* encodes a pre-pro-protein that is processed into a mature extracellular cysteine-rich peptide of 28 amino acids (Van den Ackerveken et al., 1993). The AVR9 peptide shows structural homology to cystine-knotted peptides which include ion-channel blockers, protease inhibitors and growth factors (Pallaghy et al., 1994; Isaacs, 1995; Vervoort et al., 1997) but it is not yet known whether these structural homologies have biological relevance.

Northern blot analysis indicated that expression of the *Avr9* gene is highly induced *in planta* during pathogenesis in a compatible interaction (Van Kan et al., 1991). Expression of the *Avr9* gene could also be induced *in vitro* when grown on synthetic liquid medium containing little or no nitrogen (Van den Ackerveken et al., 1994). This raises the question whether limitation of nitrogen, which is the inductive condition for *Avr9* expression *in vitro* reflects the growth conditions *in planta*. In contrast to *C. fulvum*, the regulation of nitrogen metabolism in filamentous fungi such as *Aspergillus nidulans* and *Neurospora crassa* has been studied extensively. In both fungi, positive-acting global regulatory genes, designated *are*A in *A. nidulans* (Caddick et al., 1994) and *nit-2* in *N. crassa* (Marzluf et al., 1992), encoding AREA and NIT2, respectively, specify GATA-type transcription factors which activate the expression of nitrogen metabolic genes (e.g. nitrate- and nitrite reductase) when

primary nitrogen sources (e.g. ammonium or glutamine) are not available (Marzluf, 1997). Wide-domain regulatory proteins with a similar structure and function are also present in fungi such as *Penicillium chrysogenum* (Haas et al., 1995) and *Magnaporthe grisea* (Froeliger and Carpenter, 1996). Both AREA and NIT2 bind to promoter sequences containing at least two copies of the sequence (TA)GATA (Fu and Marzluf, 1990).

Previous studies have shown that the 0.6 kb promoter fragment immediately upstream of the translation start fused to the *E. coli uid*A (GUS) reporter gene (Jefferson et al., 1987) is expressed *in planta* (Van den Ackerveken et al., 1994). In this promoter region six copies of the TAGATA sequence and six additional copies of the core sequence GATA are present. We will describe the construction of *Avr*9 promoter deletions and their GUS activity when introduced in *C. fulvum*. The results of this study suggested that in *C. fulvum*, induction of the *Avr*9 promoter *in vitro* and *in planta* is dependent on the presence of these sequence motifs.

If the nitrogen control circuit is sufficiently conserved in filamentous fungi, a detailed promoter study could be performed in *A. nidulans*, for which both efficient gene-targeting systems and a variety of *are*A mutants are available. Here we describe experiments, using *Avr*9 promoter-*uid*A fusion constructs, which show that regulation in *A. nidulans* involves the AREA protein and is similar to the regulation in *C. fulvum*.

Results

Deletion of (TA)GATA sequences in the *Avr*9 promoter affects its expression both *in vitro* and *in planta*

In order to get more information on the sequences that are necessary for *Avr*9 expression constructs pCF211, -241, -251, -261,-271, -281, -291 (Figure 1) and pCF004 were introduced at ectopic locations into a *pyr*$^-$ strain of *Cladosporium fulvum*. GUS activity was determined both after 48 hours of nitrogen starvation *in vitro* and *in planta* during infection of tomato genotype MM-Cf0.

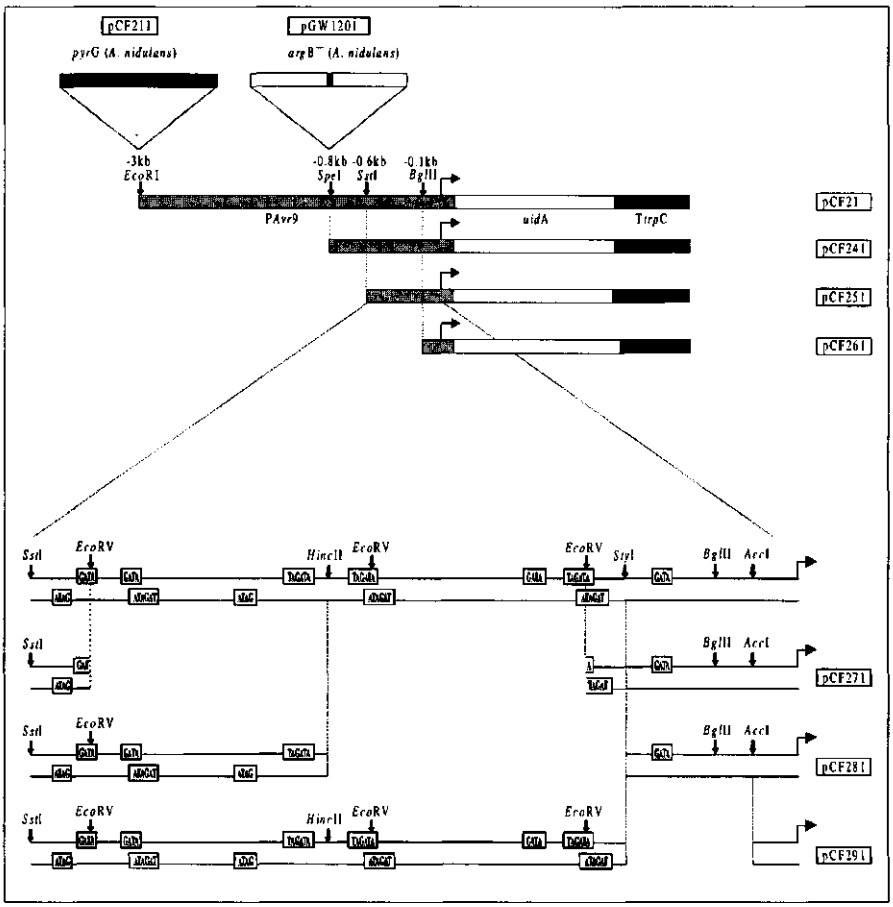


Figure 1. Overview of the constructs used in this study. Only relevant parts of the constructs are shown. pCF21 contains a 3 kb *Avr*9 promoter (P*Avr*9) fragment fused to the *uid*A gene and the terminator of the *A. nidulans trp*C (T*trp*C) gene (Roberts et al., 1989, Van den Ackerveken et al., 1994). A blunt *Hind*III-*Sst*I fragment, containing the *A. nidulans pyr*G gene was introduced into the filled-in *Eco*RI site to form pCF211. Deletions were made from pCF211 by removing the *Eco*RI-*Spe*I fragment (pCF241, 0.8 kb of the promoter left), the *Eco*RI-*Sst*I fragment (pCF251, 0.6 kb of the promoter left) and *Eco*RI-*Bgl*II fragment (pCF261, 0.1 kb of the promoter left). *Eco*RV, *Hinc*II and *Acc*I and *Sty*I and *Acc*I were used for making internal deletions in the promoter fragment of pCF251, resulting in plasmids pCF271, pCF281 and pCF291, respectively. A 3.4 kb *Xba*I fragment containing a mutant *A. nidulans arg*B allele (Punt et al., 1990) was inserted into the *Spe*I site of pCF21 to form pGW1201.

The results (Table 1) show that dissection of the *Avr9* promoter from 3 kb to 0.6 kb from the translation start, does not affect GUS activity both *in planta* and *in vitro*. These data confirm previously described results showing that 0.6 kb of the promoter is sufficient for *Avr9* expression *in planta* (Van den Ackerveken et al., 1994). Further deletion removing all (pCF261) or several (pCF271, pCF281, pCF291) putative AREA/NIT2 binding sites showed strong reduction or complete loss of GUS activity. These results suggest that both *in vitro* and *in planta*, *Avr9* promoter activity is dependent on the presence of these specific sequence motifs.

Table 1. GUS activity *in vitro* and *in planta* of *C. fulvum* transformants containing various lengths of the *Avr9* promoter upstream of the translation start.

construct[a]	promoter size (kb)[b]	PCR[c]	GUS-expression (*in vitro*)[d]	GUS-expression (*in planta*)[d]
pCF211	3	+	+	+
		+	+	+
		+	+	+
pCF241	0.8	+	+	+
		+	+	++
		+	+	++
pCF251	0.6	+	+	+
		+	+	++
		+	+	+
pCF261	0.1	+	-	-
		+	-	-
		+	-	-
pCF004	0	-	-	-
		-	-	-
		n.d.	-	-
pCF271	0.4	+	-	-
	*Eco*RV deletion	+	-	-
		+	-	-
pCF281	0.4	+	+/-	+/-
	*Acc*I-*Hinc*II	+	+/-	+/-
	deletion	+	-	-
pCF291	0.5	+	+/-	+/-
	*Sty*I-*Acc*I	+	-	+/-
	deletion	n.d.	+	+

[a] Three independent transformants were analysed for each construct
[b] Size of the *Avr9* promoter upstream of the start codon
[c] PCR using primers P1 and P2 was performed on spores to verify the presence of the constructs
[d] Mycelium was stained with X-gluc. Very strongly stained mycelium was scored as ++, strongly stained mycelium as +, weakly stained mycelium as +/- and no staining as -. Each score is the average of two independent determinations. n.d. = not determined

The *Avr9* promoter is active and regulated in *A. nidulans*

A. nidulans was transformed with pGW1201 which contains the 0.8 kb *Avr9* promoter fused to the *uid*A gene and the mutant *arg*B allele (Figure 1). Prototrophic *arg*B$^+$ transformants were selected and one (WG802) with a single copy integrated at the *arg*B locus was picked. When WG802 was grown in liquid medium containing proline as sole nitrogen source, no GUS activity could be detected. In *A. nidulans*, nitrate utilisation is dependent on the presence of active AREA a condition that can be met by growth on nitrate. Upon growth of WG802 on medium containing nitrate as sole nitrogen source no *Avr9* promoter activity could be detected. However, upon growing the transformant under nitrogen starvation conditions GUS activity was induced 30 minutes after the medium shift, reaching maximum levels after 3 hours (data not shown). Depletion of carbon, sulphate or phosphate did not result in induction of *Avr9* promoter activity. Thus, the *Avr9* promoter is induced under nitrogen limiting conditions in *A. nidulans* in a similar way as in *C. fulvum*. As in *C. fulvum*, deletion construct pCF261 did not exhibit any promoter activity in *A. nidulans*.

In *A. nidulans* the *Avr9* promoter is regulated by the AREA protein

WG800 and WG801 were constructed by transforming the *A. nidulans* strains SAA244 (containing the *xpr*D1 mutation, a derepressed *are*A allele) and SAA236 (containing the *are*A19 mutation, a loss-of-function mutation in the *are*A gene) (Kudla et al., 1990) with pGW1201, respectively, and selecting *arg*B$^+$ transformants with a single copy of the plasmid integrated at the *arg*B locus. When the transformants were grown for 17 hours on liquid medium with proline as nitrogen source, no GUS activity was observed with WG801 and WG802, but high activity was observed in the *are*A constitutive mutant WG800 (Figure 2A, white bars). In response to nitrogen starvation, expression in the *are*A wild-type WG802 is induced (Figure 2A, grey bars).

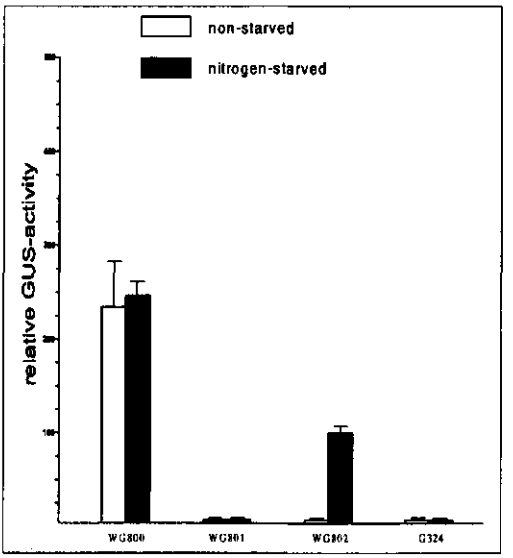


Figure 2A. GUS activity of transformants containing one copy of the construct of interest at the *arg*B locus in different *A. nidulans* backgrounds. Each bar represents the average GUS activity of three independent determinations. The GUS activity of the *are*A $^{+}$ transformant (WG802) after starvation is assigned 100% and GUS activity present in each transformant is shown as a percentage of WG802. G324 is the untransformed *A. nidulans* strain. WG800 and WG801 are transformants containing the *Avr*9 promoter-*uid*A fusion construct in an *are*A constitutive and *are*A^{-} background, respectively.

The *are*A loss-of-function mutant WG801 shows no expression, whereas expression in WG800 is at the same level as on proline. These results indicate that under these conditions the *A. nidulans* transcription factor AREA is (both) necessary (and sufficient) to mediate induction of expression of the *Avr*9 promoter.

Induction of both *Avr*9 and *nia*D expression on starvation is independent of NIRA

Induction of expression of the nitrate assimilatory genes is regarded to be dependent on the pathway-specific regulatory gene *nir*A (Burger et al., 1991a, b). To investigate a possible role of NIRA in *Avr*9 expression, WG803 was constructed as a targeted single copy transformant of pGW1201 in *A. nidulans* strain SAA9003 (containing a loss-of-function mutation in the *nir*A gene). GUS activity of this transformant was compared to that of WG802 and to G324[pTRAN3-1A] and SAA9003[pTRAN3-1A]. The latter two contain a fusion of the promoter of the *nia*D gene to the *uid*A reporter gene in the *are*A^{+} and *nir*A^{-} background, respectively. The results (Figure 2B) show that induction of *Avr*9 expression is not dependent on functional NIRA. Surprisingly, starvation induction of the expression of *nia*D is also independent of NIRA, in contrast to induction by nitrate which is absolutely NIRA dependent (Punt et al., 1995).

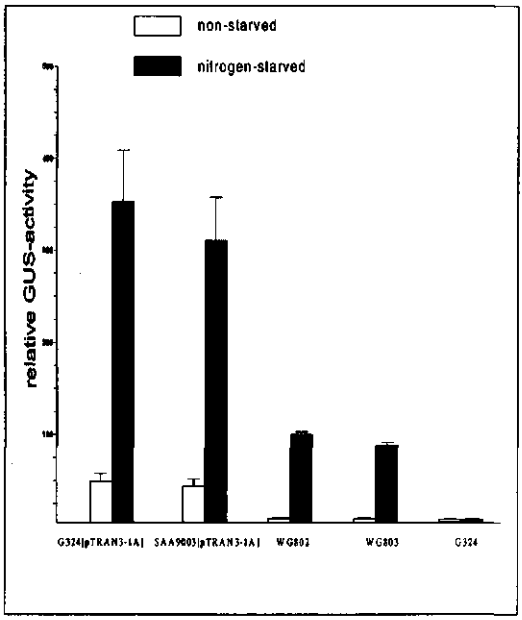


Figure 2B. GUS activity of transformants containing one copy of the construct of interest at the *arg*B locus in different *A. nidulans* backgrounds. Each bar represents the average GUS activity of three independent determinations. The GUS activity of the *are*A $^{+}$ transformant (WG802) after starvation is assigned 100% and GUS activity present in each transformant is shown as a percentage of WG802. G324 is the untransformed *A. nidulans* strain. WG803 contains the *Avr*9 promoter-*uid*A fusion construct in an *are*A^{+}, *nir*A^{-} background. G324[pTRAN3-1A] and SAA9003[pTRAN3-1A] contain a fusion of the promoter of the *nia*D gene to the *uid*A reporter gene in the *are*A^{+}, *nir*A^{+} and *are*A^{+}, *nir*A^{-} background, respectively.

Discussion

The observations that *Avr*9 expression is induced *in vitro* under nitrogen-limiting conditions, together with the fact that the *Avr*9 promoter contains several (TA)GATA sequence motifs, which are canonical nitrogen regulatory elements, suggest that expression of the *Avr*9 gene in *C. fulvum* might be regulated by a nitrogen response protein binding to one or more of these consensus sequences.

Previous experiments have shown that sequences required for expression *in planta* are contained in a small region region of the *Avr*9 promoter (0.6 kb) upstream of the translation start, in which all the putative binding sites are located (Van den Ackerveken et al., 1994). However, detailed gene expression studies in *C. fulvum* are hampered by a general lack of genetic information and specifically by a lack of mutants affecting the regulation of nitrogen metabolism. Furthermore, no gene-targeting system is available for *C. fulvum*, which results in the majority of cases in integration of the constructs of interest at random locations in the genome. Since the expression of an introduced gene-construct can be highly influenced by the chromosomal environment (Timberlake and Marshall, 1989), comparison of different constructs is greatly complicated. In *A. nidulans*, on the other hand, both an efficient gene-targeting system and a variety of *are*A mutants are available. For these reasons we have

chosen *A. nidulans* as a host for initial studies on the regulation of the *Avr9* promoter. An additional justification for this choice is the observation that functional interchange of GATA-transcription factors between fungi is possible (Davis and Hynes, 1987; Haas et al., 1995; Froeliger and Carpenter, 1996).

Monitoring GUS activity during nitrogen starvation of a single copy *A. nidulans* transformant, containing the *Avr9* promoter *uid*A fusion construct, showed promoter activity 30 minutes after the medium shift, reaching maximum levels after 3 hours. Maximal induction *in vitro* in *C. fulvum* takes 20-24 hours of starvation as measured by Northern blot analysis (Van den Ackerveken et al., 1994). Probably, the large difference in time required to deplete the intracellular pool of nitrogen reflects the difference in timing of *Avr9* expression. The *A. nidulans* transformant showed no *Avr9* promoter activity when grown under conditions where carbon, sulphate or phosphate was limiting, suggesting that *in vitro* the *Avr9* promoter is regulated in a similar way, both in *A. nidulans* and *C. fulvum*.

A comparison of GUS activity in *C. fulvum* and *A. nidulans* transformants containing constructs in which stretches of the *Avr9* promoter region had been deleted, showed the essence of this region for induction of expression in both organisms. This is compatible with the suggestion that *A. nidulans* contains a transcription factor(s), homologous to those of *C. fulvum*, which regulate(s) the *Avr9* promoter. Therefore, *A. nidulans* can probably be used as a model system to perform detailed *Avr9* promoter analyses.

Results obtained in different *are*A backgrounds showed high *Avr9* promoter activity in the constitutively expressed *are*A *A. nidulans* transformant (WG800) both during growth in normal, nitrogen-containing medium and after nitrogen starvation. The *are*A gene of this transformant is under control of a strong promoter which results in high levels of active AREA. This can explain the difference in *Avr9* promoter activity of transformant WG800 and the *are*A wild-type transformant (WG802) on nitrogen- starvation media. In contrast, in the transformant with a non-functional *are*A gene (WG801), no expression occurs under either condition. These observations strongly suggest that the AREA transcription factor is mediating the expression of the *Avr9* promoter, and by extrapolation that a similar factor is involved in the regulation of expression of the *Avr9* gene in *C. fulvum*.

Expression of the extensively studied nitrate reductase gene *nia*D is also subject to *are*A regulation, but in addition this gene is also regulated by the product of the pathway-specific *nir*A gene. Classically, expression of the *nia*D gene is induced by changing the nitrogen source to nitrate (Cove, 1979). This is thought to increase the concentration/activity of both active AREA and NIRA. No expression of *nia*D in the absence of active NIRA has

ever been reported. In our experiments we have shown that upon nitrogen starvation AREA alone is sufficient to induce *nia*D expression. In *Aspergillus niger* it has been shown that *are*A expression is more strongly induced by starvation than induction on nitrate (MacCabe et al., 1998). We conclude that the level of active AREA is probably (much) higher under starvation conditions, or a slightly different AREA product is formed. The potency of the *are*A gene to encode long and short versions of mRNA has been reported before (Langdon et al., 1995). The NIRA product is not involved in the regulation of *Avr*9 expression, nor is it induced by nitrate. This suggests that only one of the alternative forms of AREA is able to stimulate expression of *Avr*9.

Recently, cDNA clones from *C. fulvum* mycelium starved for nitrogen and carbon were isolated which are derived from genes that are also induced during pathogenesis (Coleman et al., 1997). Nitrogen deprivation might be linked to pathogenesis and the loss of a major nitrogen-regulatory factor can possibly reduce the virulence of pathogenic fungi (Lau and Hamer. 1996, Talbot and Hamer. 1997). An *are*A loss-of-function mutant of the mammalian lung pathogen *A. fumigatus* shows significantly reduced virulence, when compared to an isogenic *are*A wild-type strain (Hensel et al., 1995). Also strains of the rice blast fungus *Magnaporte grisea*, carrying mutations in the genes *npr*1 and *npr*2, which appear to act as wide-domain nitrogen regulatory genes, showed a dramatic reduction of pathogenicity (Lau and Hamer. 1996). On the other hand, inactivation of the functional *M. grisea are*A equivalent gene (*nut*1) did not result in reduction of virulence on susceptible rice plants (Froeliger and Carpenter. 1996). A better understanding of nitrogen metabolism in *C. fulvum* is needed to unravel the mechanism of regulation of the *Avr*9 gene and to provide further insight in the biological function of the *Avr*9 gene for the pathogen. If *Avr*9 expression *in planta* requires a functional GATA-transcription factor, disruption of the gene encoding this factor should change the phenotype of a *C. fulvum Avr*9$^+$ strain on tomato genotype MM-Cf9 from avirulent to virulent. Currently, isolation of the gene encoding this regulatory protein from *C. fulvum* is underway.

Experimental procedures

Strains and growth conditions

The following *Aspergillus nidulans* strains were used: SAA236 (yA2, *met*H2, *arg*B2, *are*A19), SAA244 (*si*A1, *met*H2, *arg*B2, *xpr*D1), SAA9003 (yA1, *pyro*A4, *arg*B2, *nir*A1) and G324 (*w*A3, yA2,

*met*H2, *arg*B2, *gal*A1, *sC*12, *ivo*A4). For mycelial growth approximately $2x10^8$ conidiospores were transferred to 100ml supplemented minimal medium (1.5g/l KH_2PO_4 (pH 6.0), 0.5g/l $MgSO_4.7H_2O$, 0.5g/l KCl and 10g/l D-glucose, 75mg/l methionine, 4mg/l biotin, 0.1mg/l pyridoxine, and 1g/l L-proline). Cultures were incubated for about 17 hours at 37°C in an orbital shaker at 220 rpm. For nitrogen- starvation, mycelium was harvested by filtration, rinsed with starvation medium and divided in equal portions, which were transferred to 100ml supplemented minimal medium without any nitrogen source. After 3 hours of incubation at 37°C, mycelium was harvested again and frozen in liquid nitrogen.

The *Cladosporium fulvum pyr*$^-$ strain (race 4-4.2, Marmeisse et al., 1993) was grown on potato dextrose agar (PDA) or in liquid B5 (Duchefa) medium in shake cultures at 23°C. Ten days old PDA cultures of *C. fulvum* were used to prepare spore suspensions ($5x10^5$ conidiospores/ml) for inoculation of plants and for liquid cultures. Modified B5 medium without nitrogen (B5-N) was prepared as described previously (Van den Ackerveken et al., 1992). For *C. fulvum* nitrogen starvation experiments, 25ml B5 medium was inoculated with 10^7 conidiospores and grown for 48 hours at 23°C and 100 rpm. Subsequently, mycelium was harvested and incubated for 24 hours in B5-N medium.

Construction of plasmids

For the construction of the plasmids used in this study, see Figure 1. Only relevant parts are depicted. pCF004 contains the *A. nidulans pyr*G gene in pBluescript (KS-).

Generation and analysis of *C. fulvum* and *A. nidulans* transformants

C. fulvum mycelium grown for 48 hours in liquid B5 medium was harvested by filtration and used for the isolation of protoplasts (Harling et al., 1988). Transformations (Oliver et al., 1987) were performed with 10^7 protoplasts and 5µg DNA. Transformation of *A. nidulans* was performed as previously described (Wernars et al., 1985). *C. fulvum pyr*$^+$ transformants were analysed by PCR on conidiospores, using primer P1, specific for the *uid*A gene (5'-GAATGCCCACAGGCCGTCGAG-3') and primer P2 specific for the *Avr*9 gene (5' CTCTTCTACTCTACTGGTTAC-3'). *A. nidulans arg*B$^+$ transformants were analysed by Southern blotting for the presence, location and copy number of the introduced construct.

Determination of β-glucuronidase activity

β-glucuronidase (GUS)-activity in *C. fulvum* growing *in planta* was assayed at ten days after inoculation on 14-days-old tomato plants as described previously (Van den Ackerveken et al., 1992).

GUS activity of *C. fulvum* mycelium growing *in vitro* was determined visually after adding 0.5mg/ml X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide, Sigma) in 50mM sodium phosphate (pH 7.0), 1mM potassium ferri/ferro cyanide, 0.05% Triton X-100 to mycelium and incubated overnight at 37°C.

For *A. nidulans*, mycelium was ground in liquid nitrogen using a Mikro-Dismembrator II (Braun). Protein was extracted with a buffer containing 50mM sodium phosphate (pH7.0), 10mM Na_2EDTA, 0.1% sarkosyl, 0.1% triton X-100, 25μg/ml phenylmethane sulfonyl fluoride (PMSF). After 2 centrifugation steps (10 min, 4000 rpm, 4°C; 6 min, 13000 rpm, 4°C), 50μl of the cleared protein extract was used for a fluorimetric assay using methyl umbelliferyl glucuronide (MUG) as a substrate (Jefferson et al., 1987). Specific activity was defined as produced MU in nM/min/mg protein at 37°C. Protein concentration was determined using the Bradford assay (Bradford, 1976).

Acknowledgements: The authors would like to thank Dr. P.J. Punt for providing the *A. nidulans* strains G324[PTRAN3-1A] and SAA9003 [PTRAN3-1A] and Dr. M.H.A.J. Joosten for critically reading the manuscript. The investigations were supported by the Dutch Earth and Life Science Foundation (ALW; Projectnumber: 805.45.006), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Chapter three

Promoter analysis of avirulence gene *Avr9* of the phytopathogenic fungus *Cladosporium fulvum*

S. S. Snoeijers, T. Goosen, A. Pérez-García, M. H. A. J. Joosten, H.W. J. Van den Broek and P. J. G. M. De Wit

An adapted version of this chapter has been submitted for publication

Summary

The promoter of avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* contains twelve sequences within a region of 0.6 kb that are reminiscent of the binding sequences of the GATA-type regulator involved in nitrogen utilisation of the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*. Mutational analysis of this 0.6 kb promoter region, fused to the GUS reporter gene, revealed that two domains, each containing two TAGATA-boxes in inverted orientation and overlapping by two base pairs, are important for induction of *Avr9* promoter activity in *A. nidulans*. Each overlapping TAGATA-box differentially affected *Avr9* promoter activity when shifted apart by nucleotide insertions. The other regions, which do not contain two overlapping TAGATA-boxes have no, or only a limited, contribution to the inducibility of promoter activity. The mutated *Avr9* promoter fragments which did not show any activity in *A. nidulans* were fused to the *Avr9* coding region and introduced into strains of *C. fulvum* lacking *Avr9*. *C. fulvum* transformants containing these constructs were avirulent on tomato genotypes carrying the matching *Cf-9* resistance gene, indicating that these transformants still produce the AVR9 elicitor. *In vitro* studies revealed that *Avr9* gene expression is induced when the transformants are grown in rich, liquid media. This result suggests that, although not determined by Southern blot analysis, due to multiple integrations of the construct in the genome, *Avr9* expression is no longer only induced by nitrogen-dependent factors.

Introduction

Specific elicitor AVR9, which is the product of avirulence gene *Avr9* of the imperfect pathogenic fungus *Cladosporium fulvum*, is produced to high levels during colonisation of leaflets of its host, tomato (*Lycopersicon esculentum*) (De Wit, 1995; Joosten and De Wit, 1999). Histological studies on expression of the *Avr9* gene *in planta*, using transgenic *C. fulvum* containing an *Avr9* promoter-*uid*A (coding for the reporter gene ß-glucuronidase, GUS) fusion, revealed that the promoter of this gene is induced immediately upon penetration of tomato leaves through stomata (Van den Ackerveken et al., 1994). Tomato genotypes containing the *Cf-9* resistance gene specifically recognise the AVR9 peptide and respond by mounting a hypersensitive response (HR) that eventually leads to resistance against the fungus (Joosten and De Wit, 1999).

Expression of the *Avr9* gene is induced under conditions of nitrogen starvation *in vitro*, as growth on synthetic liquid media containing low amounts of any nitrogen source induces *Avr9* expression (Van den Ackerveken et al., 1994). Knowledge on nitrogen metabolism of *C. fulvum* during growth *in vitro* and during colonisation of tomato leaves is limited. For the filamentous fungi *Aspergillus nidulans* and *Neurospora crassa*, the control of nitrogen-metabolic circuits has been extensively studied and was shown to be largely identical (Marzluf, 1997). Both fungi contain a major transcription factor, designated AREA in *A. nidulans* and NIT2 in *N. crassa*, that activates expression of many genes whose products are required for uptake and utilisation of nitrogen from various sources, when primary nitrogen sources such as ammonia or glutamine become limiting.

AREA and NIT2 belong to the GATA-family of DNA-binding proteins. They contain a DNA-binding domain, consisting of a single zinc finger and basic region, which binds to specific domains that are located in the promoter region of nitrogen-regulated genes. Each domain either contains the consensus sequence TAGATA, the core sequence GATA or their complements (Chiang and Marzluf, 1995; Punt et al., 1995). It was shown that almost any base substitution within the core GATA sequences abolished NIT2 binding (Chiang et al., 1994). The presence of regulatory proteins with a similar structure and function has also been reported for other filamentous fungi such as *A. niger* (MacCabe et al., 1998), *A. oryzae* (Christensen et al., 1998) and *Penicillium chrysogenum* (Haas et al., 1995), the lung tissue pathogen *A. fumigatus* (Hensel et al., 1995), the phytopathogens *Magnaporthe grisea* (Froeliger and Carpenter, 1996) and *Gibberella fujikuroi* (Tudzynski et al., 1999), and the insect pathogen *Metarhizium anisopliae* (Screen et al., 1998).

Individual genes controlled by AREA and NIT2 can be expressed at different levels and with different kinetics, which, among others, may be due to a different number, orientation or location of the (TA)GATA-boxes in the promoters of these genes (Chiang and Marzluf, 1995; Punt et al., 1995).

Within a region of 0.6 kb of the *Avr9* promoter, immediately upstream of the main transcription start site, twelve (TA)GATA-boxes are present. This 0.6 kb region is sufficient for regulated expression of *Avr9 in planta* and *in vitro* (Van den Ackerveken et al., 1994). As *Avr9* expression is also induced under nitrogen starvation, likely also in *C. fulvum*, an AREA/NIT2-like transcription factor is involved in regulation of *Avr9* expression.

In a previous study we have shown that in transgenic *A. nidulans* the *Avr9* promoter is also induced *in vitro* during nitrogen starvation, suggesting that the nitrogen control circuit is conserved in both *A. nidulans* and *C. fulvum*. Moreover, we demonstrated that *Avr9* promoter activity in *A. nidulans* is dependent on active AREA and on the presence of the 0.6 kb region containing the (TA)GATA-boxes (Snoeijers et al., 1999).

Here we present a detailed mutational analysis of the *Avr9* promoter in *A. nidulans*. The importance of the (TA)GATA-boxes was determined by introducing specific point mutations and small insertions. The two overlapping TAGATA-boxes most proximal to the transcriptional start site, appeared to be crucial for inducibility of *Avr9* promoter activity. The mutated *Avr9* promoter fragments which did not show any activity in *A. nidulans*, were fused to the *Avr9* coding region and introduced into different strains of *C. fulvum* lacking the *Avr9* gene. Unexpectedly, these transformants were avirulent on tomato plants only containing the functional *Cf-9* gene, suggesting that the AVR9 elicitor is still produced by the transformants. Northern blot analysis revealed that *Avr9* gene expression is induced when the transformants are grown in rich liquid media, in which *Avr9* is normally not induced in *C fulvum* wild-type strains (Van den Ackerveken et al., 1994). These results indicate that, probably due to multiple integrations of the constructs in the *C. fulvum* genome, in the various transformants *Avr9* expression is no longer only induced by nitrogen-dependent factors.

Results

The effect of mutations in one or more (TA)GATA-boxes on *Avr9* promoter activity in *A. nidulans*

A region of 0.6 kb of the *Avr9* promoter, immediately upstream of the start codon contains twelve (TA)GATA-boxes. Based on the constellation of these (TA)GATA-boxes, this region was divided into five putative binding domains (A to E, Figure 1A).

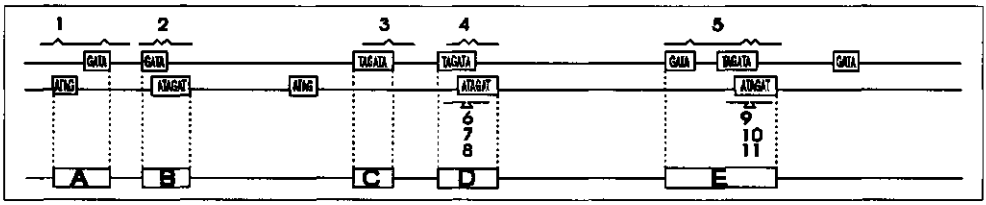


Figure 1A. Diagram of the 0.6 kb promoter region of *Avr9*, delineating the five putative binding domains (A to E) which were mutagenised. Primers used to introduce nucleotide substitutions (top) or insertions (below) are listed in Table 1.

Initially, to investigate whether these (TA)GATA-boxes play a role in the induction of the *Avr9* promoter, construct pGW1204 (Figure 1B), containing the 0.6 kb region of the *Avr9* promoter, was used to introduce mutations in eight of the twelve (TA)GATA-boxes (Figure 2A; construct pGW1281). This was done by replacing the distal A nucleotide, which is essential in functional GATA-boxes (Chiang et al., 1994) by a C nucleotide, using primers 2 to 5 at the same time in the Altered Sites *in vitro* mutagenesis system (Table 1; see Experimental procedures). The mutated *Avr9* promoter was subsequently fused to the coding region of the *uid*A gene, present in plasmid pGW1202 and the resulting construct, pGW1281 (Figure 1B), was transformed to the *A. nidulans arg*B^{minus} strain G324. *A. nidulans arg*B^{+} transformants that gave the expected product after performing a PCR with primers P1 and P2 specific for the *uid*A gene and the *Avr9* promoter, respectively (see Experimental procedures), were further analysed by Southern blotting to confirm the presence of a single copy integration, targeted at the *arg*B locus (data not shown).

Table 1. Primers used to introduce mutations in the various putative binding domains of the *Avr9* promoter. Nucleotides that are changed and insertions are underlined. The promoter domains (A to E) are classified as in Figure 1A. Mutated (TA)GATA sequences or their complements are depicted in bold.

primer	sequence	domain	mutation(s)
1	5'-GCAAACGC**GA**TC**G**GGTCTTGGA**T**C**G**GCGGGCA-3'	A	T → G; A → C
2	5'-GGCGGGCAAGA**GC**TCTATCGGCTG-3'	B	T → G; A → C
3	5'-AAACC**TAGAT**C**G**CTAGTTGAC-3'	C	A → C
4	5'-ATATTGGC**TAGA**G**C**TCTACCTAGAGC-3'	D	T → G; A → C
5	5'-AACTT**GAT**C**T**TAAC**TAGA**G**C**TCTACCTAGGCA-3'	E	A → C; T → G A → C
6	5'-CTTGATATTAAC**TAGATA**TATCTACCTAGGCAGTAGAT-3'	D	shift overlap apart
7	5'-CTTGATATTAAC**TAGATA**GGCTATCTACCTAGGCAGTAGAT-3'	D	shift overlap half a helical turn
8	5'-CTTGATATTAAC**TAGATA**GGCTGTCCTATCTACCTAGGCAGTA GAT-3'	D	shift overlap a full helical turn
9	5'-CTTCATATTGGC**TAGATA**TATCTACCTAGAGCAATACA-3'	E	shift overlap apart
10	5'-CTTCATATTGGC**TAGATA**GGCTATCTACCTAGAGCAATACA-3'	E	shift overlap half a helical turn
11	5'CTTCATATTGGC**TAGATA**GGCTGTCCTATCTACCTAGAGCAA TACA-3'	E	shift overlap a full helical turn

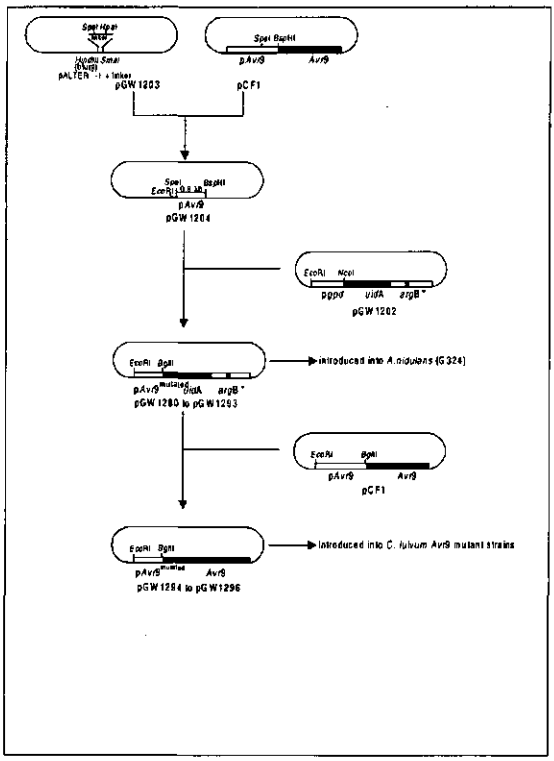


Figure 1B. Construction of plasmids. A suitable plasmid (pGW1203) was created by ligation of a linker containing a *Spe*I and a *Hpa*I site, into a *Hind*III- and *Sma*I-digested, Klenow-treated pALTER™-1 plasmid. Subsequently, a 0.8 kb *Spe*I-*Bsp*HI *Avr9* promoter fragment from pCF1 containing the various (TA)GATA-boxes (Van den Ackerveken et al., 1992) was Klenow-treated and cloned into plasmid pGW1203, creating plasmid pGW1204. Plasmid pGW1202 was constructed by inserting a *Xba*I fragment containing a mutated *A. nidulans arg*B allele (Snoeijers et al., 1999) into the Klenow-treated *Hind*III site of pCF20 (Van den Ackerveken et al., 1992). After mutagenesis (see Experimental procedures), *Avr9* promoter regions were excised with *Eco*RI and *Bsp*HI and inserted in *Eco*RI-*Nco*I-digested pGW1202 (resulting in pGW1280 to pGW1293; see Figures 2A and 2B). The *Eco*RI-*Bgl*II *Avr9* promoter fragments from the plasmids pGW1280, pGW1281 and pGW1287 were ligated into *Eco*RI-*Bgl*II digested pCF1, resulting in the plasmids pGW1294, pGW1295 and pGW1296, respectively.

From two independent *A. nidulans* transformants mycelium was grown, nitrogen-starved and the GUS activity of mycelial extracts was determined. When GUS activity of the non-mutated

*Avr*9 promoter (pGW1280) was set at 100%, the activity of the mutant construct (pGW1281) was completely abolished (Figure 2A). This result indicates that the (TA)GATA-boxes are important for the induction of *Avr*9 promoter activity.

To assess the relative importance of the individual (TA)GATA-boxes, mutations were introduced separately into each of the putative binding domains (Figure 1A, domains A to E). Single copy transformants of *A. nidulans* with a targeted integration of the various constructs at the *arg*B locus were selected as before and analysed for GUS activity after nitrogen starvation. Figure 2A shows that there is no significant difference in *Avr*9 promoter activity between the transformant containing the wild-type *Avr*9 promoter (pGW1280) and transformants carrying the constructs in which the (TA)GATA-boxes of either domains A, B or C are mutated (pGW1282, pGW1283 and pGW1284). However, mutation of either domain D (pGW1285) or domain E (pGW1286) leads to a decrease in GUS activity to 10-15% of the level of the wild-type *Avr*9 promoter. Mutating both domains D and E (pGW1287) completely abolished inducibility, indicating that the TAGATA-boxes in these domains are crucial for induction of *Avr*9 promoter activity.

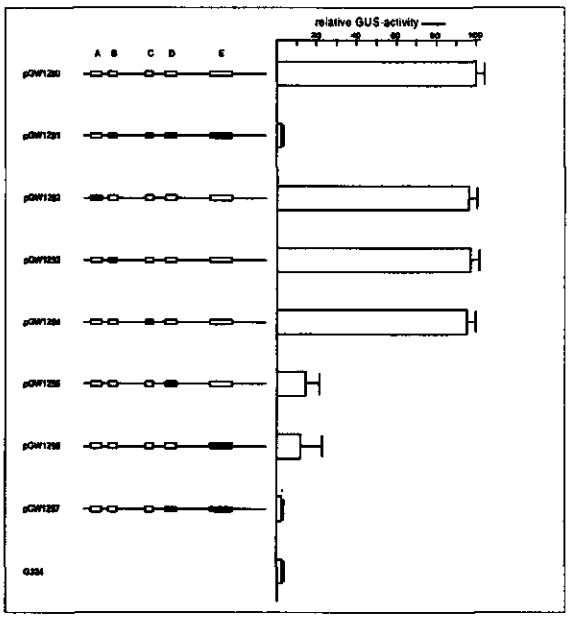


Figure 2A. Inducibility of the *Avr*9 promoter mutated in (TA)GATA-boxes due to nucleotide substitutions (for details, see Table 1). GUS activity of single copy, targeted, *A. nidulans* transformants was measured after nitrogen starvation. Each bar represents the average of three independent determinations. Activity is expressed relative to that of the wild-type *Avr*9 promoter (100%; pGW1280). G324 is the untransformed *A. nidulans* strain. The domains of the *Avr*9 promoter are depicted as open boxes. The mutated domains are shown as black boxes.

Partial overlap of the TAGATA-boxes of domain D and E is required for *Avr9* promoter activity

Both domains D and E contain two TAGATA-boxes in inverted orientation and overlapping by two nucleotides (Figure 1A). To investigate the relevance of this feature, insertions were introduced that increased the distance between the two TAGATA-boxes in both domains D and E. Activity of these mutant *Avr9* promoters in single copy, targeted, *A. nidulans* transformants was analysed as before.

Figure 2B shows that insertion of two and five nucleotides into the TAGATA-boxes, thereby shifting the overlap either just apart or half a helical turn, respectively, in domain D (pGW1288 and pGW1289, respectively) reduced *Avr9* promoter activity almost to background level. Insertion of ten nucleotides (shifting the overlap a full helical turn, pGW1290) restored *Avr9* promoter inducibility to wild-type levels. This indicates that the position of the TAGATA sequences in domain D is crucial for induction of *Avr9* promoter activity.

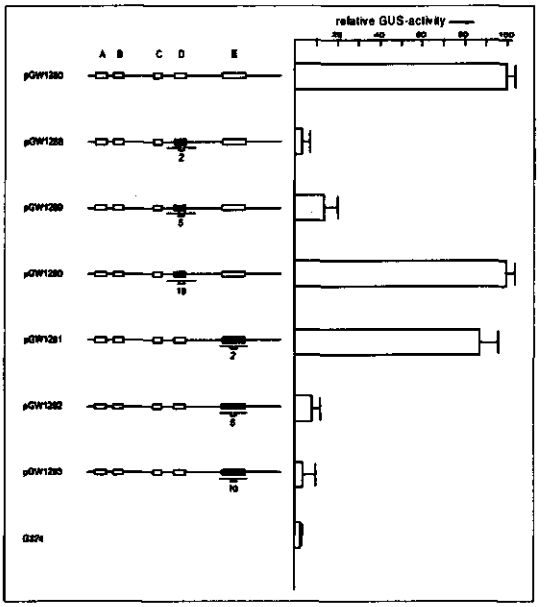


Figure 2B. Inducibility of the *Avr9* promoter mutated in (TA)GATA-boxes due to nucleotide insertions (for details see Table 1). GUS activity of single copy *A. nidulans* transformants was measured after nitrogen starvation. Each bar represents the average of three independent determinations. Activity is expressed relative to that of the wild-type *Avr9* promoter (100%; pGW1280). G324 is the untransformed *A. nidulans* strain. The domains of the *Avr9* promoter are depicted as open boxes. The shifted domains are shown as black boxes. The number of nucleotides inserted is depicted underneath the box.

In contrast, introduction of two nucleotides to shift the TAGATA-boxes of domain E (pGW1291) showed only a slight reduction of *Avr9* promoter activity. However, shifting the TAGATA-boxes of domain E five and ten nucleotides (pGW1292 and pGW1293) resulted in a strong reduction of expression, similar to that of the "loss-of-function" mutation (pGW1281,

Figure 2A). This suggests that for domain E the distance between the TAGATA sequences is important for *Avr9* promoter activity.

The effect of mutations in the (TA)GATA-boxes on expression of *Avr9* by *C. fulvum in planta*

The *Avr9* gene is highly expressed in the apoplast during colonisation by *C. fulvum* of leaves from susceptible tomato plants (Van den Ackerveken et al., 1994). To reveal whether the (TA)GATA-boxes are also important for induction of *Avr9* expression *in planta,* the *Avr9* promoter regions from constructs pGW1280 (non-mutated *Avr9* promoter), pGW1281 (binding domains B to E are mutated) and pGW1287 (binding domains D and E are mutated) were fused to the *Avr9*-coding region, resulting in constructs pGW1294, pGW1295 and pGW1296, respectively (Figure 1B). These three constructs were co-transformed with the pAN7-1 plasmid (containing the hygromycine B resistance cassette; Punt et al., 1987) to the natural *C. fulvum* strain race 2.5.9, lacking the *Avr9* gene, and the transformant of race 4 in which the *Avr9* gene has been deleted (Marmeisse et al., 1993).

For each of the three constructs, at least two independent hygromycin B-resistant transformants, giving the expected PCR product using primers Avr9II and Avr9III (see Experimental procedures), were used for inoculation of MM-Cf0 (containing no known resistance genes against *C. fulvum*) and MM-Cf9C (only containing the functional *Cf-9* gene; Parniske et al., 1997) tomato plants. Western blot analysis using antibodies raised against the extracellular protein ECP2, a marker for colonisation by *C. fulvum* (Laugé et al., 1997), indicated that all MM-Cf0 plants were equally colonised by the *C. fulvum* strains and transformants (data not shown). However, all *C. fulvum* transformants were avirulent on MM-Cf9C, indicating that still a sufficient amount of AVR9 is produced by transformants containing a mutated *Avr9* promoter region (data not shown).

Furthermore, Northern blot analysis showed that *Avr9* expression is induced in all transformants following nitrogen starvation *in vitro* (Figure 3A). However, when a selection of these transformants was grown in rich, liquid medium, *Avr9* expression was also apparent (Figure 3B).

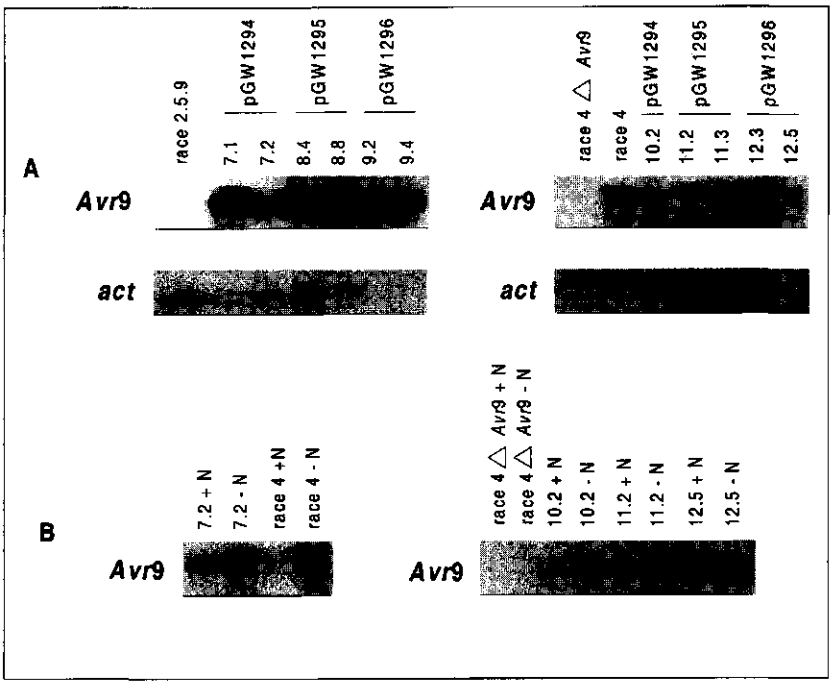


Figure 3. Analysis of *Avr9* expression of the *C. fulvum* strains and transformants *in vitro*. Ten µg of total RNA was separated on 1.5% denaturing formaldehyde-agarose, blotted onto Hybond N^+ membrane and hybridised with *Avr9* cDNA (Van Kan et al., 1991) and the *C. fulvum* actine (*act*) gene. The *act* gene is constitutively expressed in *C. fulvum* and is used as RNA loading-control. **(A)** Total RNA from nitrogen-starved mycelium from *C. fulvum* wild-type strain (race 4), the *Avr9*-lacking strains (race 2.5.9 and the race 4 *Avr9* deletion mutant) and the *C. fulvum* transformants 7.1, 7.2 (pGW1294 in the race 2.5.9 background), 8.4, 8.8 (pGW1295 in the race 2.5.9 background), 9.2, 9.4 (pGW1296 in the race 2.5.9 background), 10.2 (pGW1294 in *Avr9* deletion mutant), 11.2, 11.3 (pGW1295 in *Avr9* deletion mutant) and 12.3, 12.5 (pGW1296 in *Avr9* deletion mutant) was isolated and hybridised with labelled *Avr9* cDNA or the labelled *C. fulvum act* gene. **(B)** Total RNA from nitrogen-starved mycelium (lanes marked -N) or mycelium grown in rich, liquid medium (lanes marked +N) from a selection of the *C. fulvum* transformants presented in Figure 3A was isolated and hybridised with labelled *Avr9* cDNA.

Discussion

Sequence analysis revealed that the *Avr9* promoter contains in total twelve (TA)GATA-boxes which are known as binding sites for the transcription factors AREA and NIT2 in the filamentous fungi *A. nidulans* and *N. crassa*, respectively (Van den Ackerveken et al., 1994). These regulators belong to the GATA-family of transcription factors, which have also been found in other pathogenic fungi such as *Magnaporthe grisea* (Froeliger and Carpenter, 1996)

and *Metarhizium anisopliae* (Screen et al., 1998). AREA and NIT2 positively regulate the expression of various unlinked genes which specify nitrogen-catabolic enzymes (Davis and Hynes, 1987; Fu and Marzluf, 1990), including nitrate and nitrite reductases.

Expression of *nit-3*, coding for nitrate reductase in *N. crassa*, is completely dependent on the presence of NIT2. The *nit-3* promoter has two NIT2-binding domains, containing five (TA)GATA-boxes, located 1.2 kb upstream and a single NIT2-binding domain, containing three (TA)GATA-boxes, present 0.3 kb upstream of the transcription start site of the *nit-3* gene. Mutation analysis of the various (TA)GATA-boxes showed that all sequences are important for induction of *nit-3* activity (Chiang and Marzluf, 1995). In *A. nidulans*, expression of the *nia*D and *nii*A genes, coding for nitrate and nitrite reductase, respectively, which are divergently transcribed from an intergenic region of 1.3 kb, is regulated by AREA (Johnstone et al., 1990). Of the ten (TA)GATA-boxes present in this intergenic region, only three (TA)GATA-boxes, located 0.7 kb upstream of *nia*D and 0.6 kb upstream of *nii*A, are crucial for both *nia*D and *nii*A activity (Punt et al., 1995).

By mutational analysis we have demonstrated that regulation of *Avr9* expression is mediated by the (TA)GATA-boxes in its promoter. Mutation of all TAGATA-boxes completely abolished *Avr9* promoter activity in *A. nidulans* (Figure 2A). Subsequent detailed promoter analysis revealed that the proximal putative binding domains D and E are essential for induction of *Avr9* promoter activity in *A. nidulans* (Figure 2A). Mutations introduced into the more distal domains A, B and C did not influence promoter activity, when compared with the wild-type *Avr9* promoter (Figure 2A). This result suggests that the (TA)GATA-boxes present in these domains are not required for induction of *Avr9* promoter activity. However, one could envisage a role of these boxes under different environmental conditions, for example when the AREA protein is limiting.

As already mentioned, the two (TA)GATA-boxes present in domains D and E are essential for *Avr9* promoter activity in *A. nidulans*. Both domains contain two overlapping TAGATA sequences. Within both domains these sequences are in opposite orientation, while two nucleotides are overlapping (Figure 1A). By insertion of additional nucleotides, the TAGATA sequences are shifted and at the same time, the position on the helix will turn about 36° for every inserted nucleotide. These insertions had different effects on the two putative binding domains D and E (Figure 2B). Insertion of two nucleotides in the TAGATA-box present in domain D abolished promoter activity, while insertion of two nucleotides in the TAGATA-box in domain E hardly affected *Avr9* promoter activity (Figure 2B). Insertion of five nucleotides, which results in positioning the TAGATA sequences on opposite sites on the

helix, gave strongly reduced activity of the *Avr9* promoter, both for domain D and E (Figure 2B). Probably this shift changes the constellation of the TAGATA-box so drastically that binding of the transcription factor is strongly inhibited. Insertion of ten nucleotides gives a complete turn of 360°, leaving only a different spacing between the two TAGATA sequences. For promoter domain D this resulted in wild-type levels of inducible *Avr9* promoter activity. Apparently, a functional TAGATA-box is reconstituted which suggests that for *Avr9* promoter activity, in domain D, the position of the TAGATA sequences within the box is more important than the distance between the sequences.

In contrast, for domain E no restoration of promoter activity occurred when ten nucleotides were inserted to shift the TAGATA sequences. Here the distance between the sequences seems to be more important than the position of the sequences on the helix. One possible explanation could be that insertion of ten nucleotides results in an altered chromatin structure which prevents the binding of the transcription factor. Such "masking" of binding sites by nucleosome proteins in the promoter region has previously been observed for *Saccharomyces cerevisiae* genes. In this system, nucleosomes participate in promoter repression by interfering with binding of transcription factors (Svaren and Hörz, 1995).

In previous experiments with *C. fulvum* it was found that for the expression of *Avr9 in vitro* under nitrogen-limiting conditions, the (TA)GATA-boxes in the *Avr9* promoter are important (Snoeijers et al., 1999). In this study we have exploited *A. nidulans* to analyse the role of these boxes in induction of *Avr9* promoter activity in more detail. The most interesting promoter mutants were subsequently tested in *C. fulvum* grown *in planta*.

C. fulvum transformants containing mutated *Avr9* promoter fragments which did not show any inducibility in *A. nidulans*, fused to the *Avr9* coding region, were avirulent on tomato genotypes containing the functional *Cf-9* gene. This result indicates that still a sufficient amount of AVR9 is produced in these transformants. However, it has to be emphasised that in these *C. fulvum* transformants copy numbers of the constructs were not determined and the various constructs were not targeted to a specific locus in the genome. As a consequence, copy number and the unknown chromosomal environment of the introduced constructs are able to influence *Avr9* expression. This seems to be the case, as *in vitro* studies showed that *Avr9* expression is also induced in *C. fulvum* transformants growing in rich liquid medium. This observation made clear that a gene-targeting system is a necessity to perform reliable expression studies in *C. fulvum*. The development of such a system for *C. fulvum* is described in chapter four.

Experimental procedures

Strains and growth conditions

The *Aspergillus nidulans argB*^{minus} strain G324, for targeting the constructs at the *arg*B locus, and the natural isolate of race 2.5.9 of *Cladosporium fulvum* lacking the *Avr*9 gene and an *Avr*9 deletion mutant of race 4 of *C. fulvum* (Marmeisse et al., 1993) were used as acceptor strains for transformations. Growth of *A. nidulans* and *C. fulvum* in liquid shake cultures and *A. nidulans* nitrogen starvation experiments were performed as described previously (Snoeijers et al., 1999).

For *C. fulvum* nitrogen starvation experiments, 100 ml of B5 medium containing 50 mM glutamine was inoculated with approximately $4x10^7$ conidiospores and the culture was incubated for 2 days in a rotary shaker at 23°C, 100 rpm. Subsequently, mycelium was harvested, rinsed with B5 medium and transferred to 25 ml liquid B5 medium without any nitrogen source. After 24 hours of incubation in a rotary shaker at 23°C, 100 rpm, mycelium was harvested again and freeze-dried.

Construction of plasmids

The Altered Sites *in vitro* mutagenesis system (Promega, Madison, Wis, USA), to obtain site-specific mutations in the *C. fulvum Avr*9 promoter region, was used according to the manufacturer's instructions. The construction of the plasmids for pGW1280 to pGW1293 and pGW1294 to pGW1296 is shown in Figure 1B.

Primers used to introduce base substitutions and insertions in the *Avr*9 promoter are listed in Table 1. The sequence of the two, five, and ten nucleotide insertions are designed to keep the TAGATA-boxes intact, and to shift the overlap either just apart (two nucleotides), half a helical turn (five nucleotides), or a full helical turn (ten nucleotides). All *Avr*9 promoter regions were sequenced to confirm whether the appropriate mutations and insertions were introduced.

Generation and analysis of *A. nidulans* and *C. fulvum* transformants

Transformation of the plasmids pGW1280-1293 (Figure 1B) to the *A. nidulans argB*^{minus} strain, targeting the constructs at the *arg*B locus, was performed as previously described (Wernars et al., 1985). For transformation of *C. fulvum*, mycelium grown for 48 hours in liquid B5 (Duchefa) medium was harvested by filtration and used for the isolation of protoplasts (Harling et al., 1988). Co-transformations were performed with 10^7 protoplasts (Oliver et al., 1987) and 5 µg of either plasmid pGW1294, pGW1295 or pGW1296 (Figure 1B) in a 1:1 ratio with plasmid pAN7-1, containing the hygromycin B resistance gene (*hph*; Punt et al., 1987).

The initial screening of *A. nidulans argB*$^+$ transformants was performed by PCR on minipreps of genomic DNA (Thijs et al. 1995), using primer P1, specific for the *uidA* gene (5'-GAATGCCCACAGGCCGTCGAG-3') and primer P2, specific for the *Avr9* promoter (5'-CTCTTCTACTCTACTGGTTAC-3'). *A. nidulans* transformants which gave the expected PCR product were further analysed by Southern blotting for the presence, location and copy number of the introduced construct. Determination of GUS activity of the various *argB*$^+$ *A. nidulans* transformants was performed as described previously (Snoeijers et al., 1999).

Hygromycin B-resistant *C. fulvum* transformants were analysed by PCR, using the primers Avr9II, specific for the *Avr9* gene (5'-CATTGTAGCTTATGAAAGTCGC-3') and Avr9III, specific for the *Avr9* promoter (5'-GGGAGCTCCTTACACCTTGT-3'), on conidia.

Total RNA of the *C. fulvum* transformants was isolated according to the Clontech (Clontech Inc., Palo Alto, USA) protocol. Probes were labelled using the Random Primers Labeling System (Life Technologies Inc., Rockville, USA) including 20µCi α^{32}P-dATP (Amersham, Buckinghamshire, England).

Plants and inoculations

Ten-day-old PDA cultures of *C. fulvum* were used to prepare spore suspensions (approximately $5x10^5$ conidiospores/ml) for inoculation of plants. The tomato cultivar Moneymaker (MM-Cf0), susceptible to all known strains of *C. fulvum* and MM-Cf9C plants, only containing the functional *Cf-9* gene, which mediates recognition of the AVR9 peptide (Parniske et al., 1997) (transgenic seeds were kindly provided by Dr. M. Parniske, The Sainsbury Laboratory, John Innes Centre, Norwich, England) were used. Five-week-old plants were inoculated with aqueous conidial suspensions as described by De Wit (1977).

Isolation and analysis of apoplastic fluids

Apoplastic fluids (AFs) were isolated as described by De Wit and Spikman (1982). AFs were analysed on 15% (wt/vol) SDS polyacrylamide gels. Western blot analyses were carried out after electro transfer of the separated proteins to nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany). Polyclonal antibodies against ECP2 (Wubben et al., 1994), a marker for colonisation by *C. fulvum* (Laugé et al., 1997) were raised in rabbits (Veenendaal, The Netherlands).

Acknowledgements: S. S. Snoeijers was supported by the Dutch Earth and Life Science Foundation (ALW; Projectnumber: 805.45.006), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Chapter four

Development of a gene-targeting system for the tomato pathogen *Cladosporium fulvum*

S. S. Snoeijers, T. Goosen, B. Wennekes, R. Drissen, P. Schatz, C. Reis, M. H. A. J. Joosten and P. J. G. M. De Wit

Summary

Here we describe the development of a gene-targeting system for the tomato pathogen *Cladosporium fulvum*. For this purpose we isolated the *C. fulvum pyr*1 gene, which codes for the enzyme orotidine-5'-monophosphate decarboxylase (OMPdecase). OMPdecase is involved in the pyrimidine biosynthetic pathway and is considered to be a versatile selection marker for filamentous fungi. Isolation of the *C. fulvum pyr*1 gene was based on complementation of an *Aspergillus nidulans pyr*G-minus mutant strain with digested, genomic DNA of *C. fulvum*, co transformed with an autonomously-replicating plasmid ("instant gene bank" method). A transformation vector, containing the *C. fulvum pyr*1 gene with a defined mutation, was used to transform *C. fulvum pyr*1-mutant strains. Southern analysis of the obtained *pyr*1$^+$ transformants showed that site-directed integration of this vector at the *pyr*1 locus had occurred. A homologous *C. fulvum* transformation system, as described here, allows the targeting of constructs of interest to a certain site in the genome of the fungus.

Introduction

Selection markers which are available for transformation systems of filamentous fungi can be divided into dominant and auxotrophic markers. In general, dominant markers mediate

resistance against a toxic compound or allow utilisation of a substrate not used by the recipient strain. This type of markers has the advantage that they can be used in wild-type as well as mutant strains, as presence of a particular mutation in the recipient is not required. An example of a dominant selection marker is the *Aspergillus nidulans amd*S gene that codes for acetamidase, permitting growth on acetamide as sole carbon source (Kelly and Hynes, 1985; Beri and Turner, 1987).

Another type of dominant selection marker is based on mutant fungal genes, isolated from fungicide-resistant mutants, conferring resistance to fungicides, like oligomycin or benomyl (Ward et al., 1986; Bull et al., 1988; Ward et al., 1988). In case dominant markers are based on non-fungal genes that confer resistance to antimicrobial compounds, such as hygromycin B, bleomycin, neomycin or kanamycin, the coding sequence of these genes has to be fused to fungal promoter and terminator sequences to allow adequate expression to occur. Such hybrid-dominant selection markers have been used for the development of transformation systems for a wide variety of filamentous fungi (Goosen et al., 1992).

The use of auxotrophic selection markers is based on complementation of an auxotrophic mutation in the recipient strain with the corresponding wild-type gene. It has been demonstrated that not only the homologous gene (from the same species) but also the corresponding heterologous gene (from different species) can be used to complement the auxotrophy of the recipient. For example, the *arg*B gene of *A. nidulans*, which codes for ornithine carbamoyltransferease, can complement *arg*B mutants from several fungi (Buxton et al., 1985; Parson et al., 1987; Pentilla et al., 1987; Hahm and Bath, 1988). Similarly, it has been demonstrated that the nitrate reductase (*nia*D) gene of different filamentous fungi is functionally expressed in several heterologous systems (Unkles et al., 1989a and b; Daboussi et al., 1989; Campbell et al., 1989; Malardier et al., 1989; Whitehead et al., 1989; Johnstone et al., 1990; Sànchez-Fernàndez et al., 1991).

Once an auxotrophic mutant and the corresponding wild-type gene are available, it is possible to develop a gene-targeting system. The use of such a system provides the possibility of site-directed integration of a construct of interest through homologous recombination. In particular, for detailed studies on gene expression, site-specific integration is important to allow reliable conclusions to be drawn (Van Gorcom et al., 1985; Miller et al., 1987; Van Gorcom and Van den Hondel, 1988; Hamer and Timberlake, 1987; Punt et al., 1990).

Based on research on various filamentous fungi, the *pyr* gene, which codes for orotidine-5'-monophosphate decarboxylase (OMPdecase), is considered to be a versatile auxotrophic marker (Ballance et al., 1983; Cullen and Leong, 1986; Diez et al., 1987; Goosen

et al., 1987), as mutants lacking OMPdecase-activity are not able to grow in media without uridine or uracil. OMPdecase converts orotidine-5'-phosphate into uridine-5'-phosphate in the pyrimidine biosynthetic pathway.

In this study we describe a strategy to develop a gene-targeting system for the fungal tomato pathogen *Cladosporium fulvum* based on the *pyr*1 gene. In addition to *C. fulvum pyr*1-mutant strains, that have been described previously (Marmeisse et al., 1993), the corresponding wild-type *pyr*1 gene of *C. fulvum* still had to be isolated.

We used the *A. nidulans* "instant gene bank" strategy (Gems et al., 1994) to isolate this gene. This strategy is based on functional complementation of an *A. nidulans pyr*G-mutant strain, using digested genomic *C. fulvum* DNA in combination with an autonomously-replicating vector, designated pHELP1 (Gems and Clutterbuck, 1993). A targeting construct, containing the *C. fulvum pyr*1 gene with a defined mutation was used to transform *C. fulvum pyr*1-mutant strains. Southern analysis of the obtained *pyr*1$^+$ transformants showed that site-directed integration of this vector at the *pyr*1 locus had occurred.

Results

Isolation of the *C. fulvum pyr*1 gene

The first step of the "instant gene bank" strategy to isolate the *C. fulvum pyr*1 gene involved transformation of a *pyr*G-mutant strain of *A. nidulans* (WG499) with *Bam*HI-digested *C. fulvum* (race 5) chromosomal DNA, together with *Bam*HI-digested pHELP1 plasmid (see Experimental procedures).

This resulted in 8 independent *A. nidulans pyr*G$^+$ transformants, that were expected to have resulted from the uptake of the pHELP1 plasmid in which the *C. fulvum* wild-type *pyr*1 gene had been integrated by *in vivo* ligation. The *AMA1*-sequence on the pHELP1 plasmid allows autonomous replication. The second step involved reisolation of the recombinant pHELP1 plasmid from total DNA isolated from the *A. nidulans pyr*G$^+$ transformants.

Only from one *A. nidulans pyr*G$^+$ transformant, transformation of 1µg of total DNA, resulted in ca. 1000 ampicillin-resistant *Escherichia coli* colonies. To confirm whether the plasmid replicated autonomously in these *pyr*G$^+$ transformants, the *E. coli* transformants were pooled and plasmid DNA was isolated. Subsequently, the mixture of plasmids was retransformed to WG499, resulting in approximately 40 *A. nidulans pyr*G$^+$ transformants/µg plasmid DNA. Total DNA of one of these *A. nidulans pyr*G$^+$ transformants was isolated and

used for retransformation of *E. coli*. From the resulting ampicillin-resistant *E. coli* colonies, 3 different plasmids were isolated, which varied in size and showed different restriction patterns. One of these plasmids, pHELP1 with an insert of ± 7 kb (designated pGW1241), complemented WG499, indicating that the entire functional *C. fulvum pyr*1 gene is present on this insert.

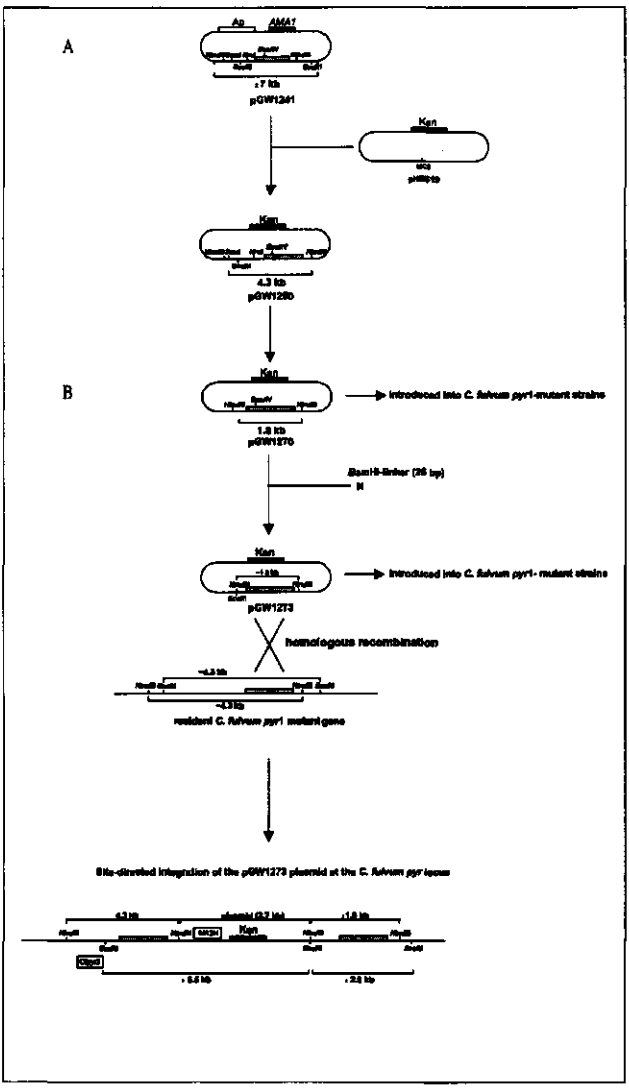


Figure 1. Subcloning of the ± 7 kb genomic *Bam*HI-fragment present in pGW1241 and strategy to obtain a plasmid for site-specific integration. **(A)** pGW1241 was digested with *Hind*III and the 4.3 kb fragment containing the functional *C. fulvum pyr*1 gene, was ligated into the pHSS19 vector, resulting in plasmid pGW1250. *C. fulvum pyr*1 sequence information was obtained from this plasmid. **(B)** Subsequently, pGW1250 was digested with *Sma*I and *Nru*I, resulting in pGW1270 after religation. To obtain a defined mutation in the *pyr*1 gene, pGW1270 was digested with *Eco*RV to introduce the *Bam*HI linker, resulting in plasmid pGW1273. *C. fulvum pyr*1$^+$ transformants will be obtained after integration at the *pyr* locus by a single crossover event (depicted as dotted lines), following transformation with pGW1273. A tandem duplication of the *pyr*1 sequence (one is defective because both mutations are present) will be separated by vector sequence. Expected fragment lengths after digestion of the *C. fulvum pyr*1$^+$ transformants with *Eco*RI and *Hind*III, and hybridisation with the *pyr*1 gene are also shown. **Ap**: Ampicillin resistance gene; **Kan**: Kanamycin resistance gene; The *C. fulvum pyr*1 gene is depicted as a grey box; the introduced mutation is shown as a black box.

To determine the location of the *pyr*1 gene on the insert, pGW1241 was digested with various restriction enzymes. The resulting mixture of restriction fragments were transformed to WG499 and complementation was obtained with *Hind*III-digested pGW1241, indicating that the entire functional *C. fulvum pyr*1 gene is present on one of these fragments. Subsequently, the pGW1241 *Hind*III-fragments were separately transformed to WG499, resulting in *pyr*G$^+$ transformants after transformation with a 4.3 kb *Hind*III-fragment. This fragment was cloned into vector pHSS19 (carrying a kanamycin-resistance gene; Nickoloff and Reynolds, 1991) resulting in plasmid pGW1250 (Figure 1A). From pGW1250 the *C. fulvum pyr*1 gene was sequenced (Figure 2).

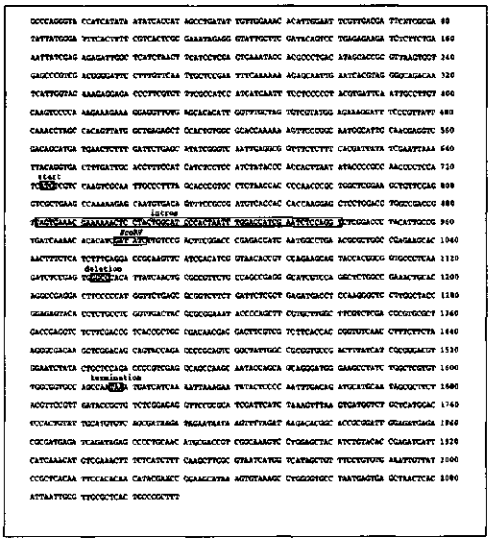


Figure 2. Nucleotide sequence of the *C. fulvum pyr*1 gene. Start codon, intron, the deletion present in the two *C. fulvum pyr*1-mutant strains, the *Eco*RV restriction site and the termination codon are indicated.

The sequenced region comprises 2110 bp, which includes 722 bp upstream of the open reading frame (ORF) and 491 bp sequence downstream of the ORF. Analysis, employing Dnastar and EditSeq software, revealed the presence of an ORF of 837 bp encoding OMPdecase. The coding region is interrupted by an intron of 60 bp, that is conserved, both in size and position, among most *pyr* genes of filamentous fungi (Figure 2). The mutation present in the *pyr*1-mutant strains of race 4 and race 5 of *C. fulvum* was found to be identical and appeared to be a deletion of 4 nucleotides, resulting in a frame-shift (Figure 2).

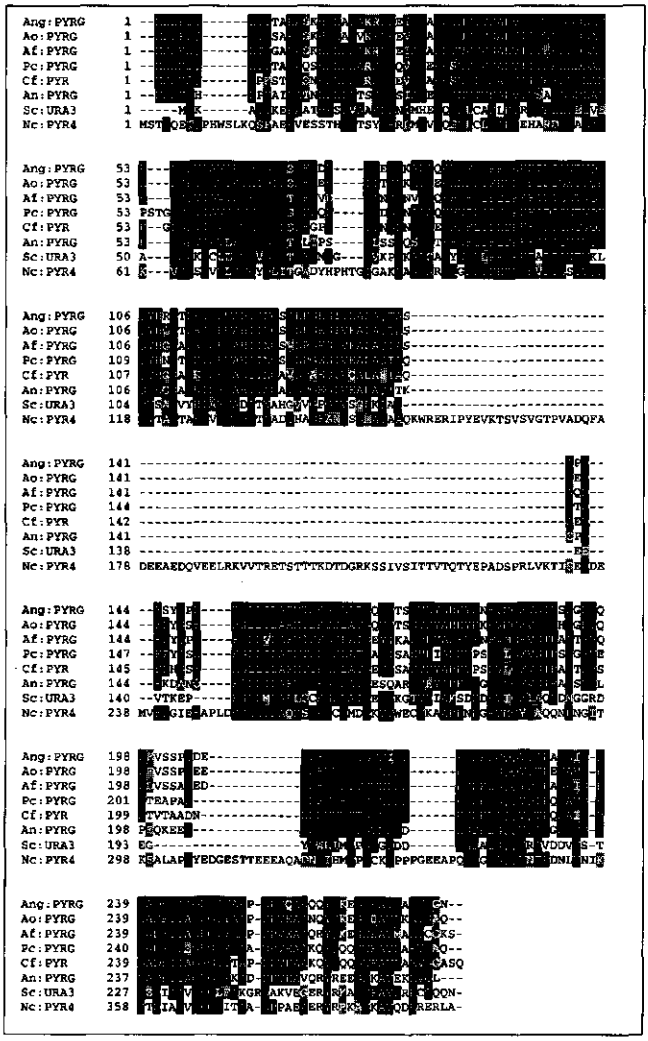


Figure 3. Alignment of the amino acid sequences of orotidine-5' monophosphate decarboxylase (OMPdecase) proteins from various fungi and the yeast *Saccharomyces cerevisae*. Ang: *Aspergillus niger* (Accession No S79674NID); Ao: *Aspergillus oryzae* (Accession No. AB017705NID); Af: *Aspergillus fumigatus* (Accession No. Y11303NID); Pc: *Penicillium chrysogenum* (Accession No. A15364NID); Cf: *Cladosporium fulvum*; An: *Aspergillus nidulans* (Accession No. M19132NID); Sc: *Saccharomyces cerevisae* (Accession No. NC_001137NID); Nc: *Neurospora crassa* (Accession No. X05993NID). Conserved amino acids are repressed as black boxes. Amino acid sequences were compared using ClustalW 1.8.

The OMPdecase protein encoded by the *pyr*1 gene of *C. fulvum* was compared with the OMPdecase proteins from *A. niger*, *A. oryzae*, *A. fumigatus*, *Penicillium chrysogenum*, *A.*

nidulans, *Neurospora crassa* and the yeast *Saccharomyces cerevisiae*. The *C. fulvum* protein reveals the highest homology with the *P. chrysogenum* protein (85% identical amino acids) and the lowest with the *N. crassa* protein (35% identical amino acids), which is due to the fact that the *N. crassa* protein contains a middle part of additional amino acids which is lacking in the other proteins (Figure 3).

Strategy to obtain site-specific integration at the *C. fulvum pyr* locus

Figure 1B shows the cloning strategy followed to obtain a plasmid that can be employed for site-specific integration of any gene of interest at the *C. fulvum pyr*1 locus. To reduce the size of the 4.3 kb *pyr*1-containing fragment present in pGW1250 into a 1.8 kb, the plasmid was digested with *Sma*I and *Nru*I and religated, resulting in plasmid pGW1270. Transformation of pGW1270 to the two *C. fulvum pyr*1-mutant strains, resulted in *pyr*1$^+$ transformants, indicating that the functional part of the *C. fulvum pyr*1 gene is present on the 1.8 kb fragment present in plasmid pGW1270 (Table 1).

To obtain a plasmid containing the *C. fulvum pyr*1 gene carrying a defined mutation in the coding region, pGW1270 was digested with *Eco*RV, followed by insertion of a *Bam*HI-linker, resulting in plasmid pGW1273 (Figure 1B). Upon transformation of *pyr*1-mutants of *C. fulvum* with pGW1273, complementation can only occur when single crossover takes place at the *pyr*1 locus. This will result in a tandem duplication of the *pyr*1 sequence, of which one is defective due to the presence of two mutations (Figure 1B). Table 1 shows the number of transformants that were obtained upon transfer of plasmid pGW1273 to the two *C. fulvum pyr*1-mutant strains.

Table 1. Number of *C. fulvum* transformants obtained after introduction of plasmids pGW1270 or pGW1273 into the two *C. fulvum pyr*1-mutant strains.

plasmids used for transformation	**number of *C. fulvum pyr*$^+$ transformants**	
	recipient race 4 *pyr*1 mutant	recipient race 5 *pyr*1 mutant
pGW1270	38	23
pGW1273	5	2

To determine whether pGW1273 had integrated at the resident *C. fulvum pyr*1 locus, PCR on transgenic conidiospores was performed, using primer (Cfpyr2; see Experimental

procedures), annealing to sequence upstream of the *pyr*1 gene present in the 4.3 kb *Hind*III fragment and primer (M13R; see Experimental procedures) which anneals to the pHSS19 vector sequence (Figure 1B). Three *C. fulvum pyr*1$^+$ transformants, designated 4I, 4II (derived from the race 4 *pyr*1 mutant) and 5I (derived from the race 5 *pyr*1 mutant) showed the expected PCR fragment of 2.1 kb, indicating that the construct had integrated at the resident *C. fulvum pyr*1 locus (data not shown).

Southern blot analysis of the *C. fulvum pyr*$^+$ transformants

In addition to PCR, Southern blot analysis was carried out to confirm that homologous recombination had occurred at the *pyr* locus. For this purpose, genomic DNA of the 3 *C. fulvum pyr*$^+$ transformants and the race 4 *pyr*1-mutant strain was isolated and digested with *Eco*RI or *Hind*III, respectively.

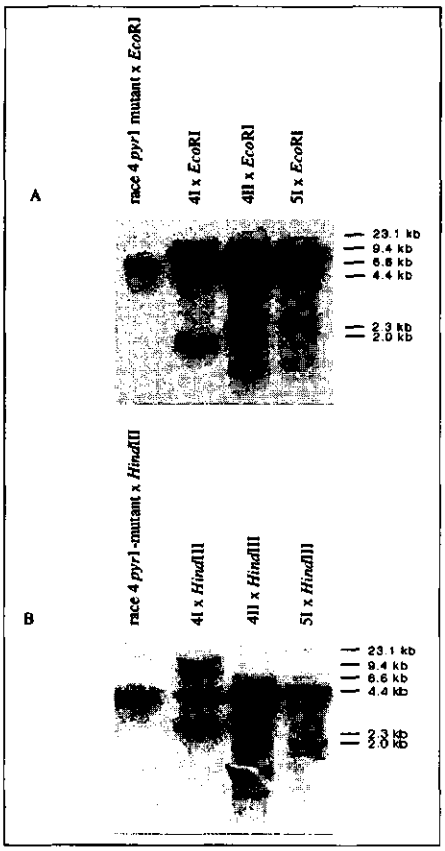


Figure 4. Southern blot analysis of the *C. fulvum pyr*1-mutant strain (race 4) and the *C. fulvum pyr*$^+$ transformants 4I, 4II and 5I. Genomic DNA was digested with (**A**) *Eco*RI or (**B**) *Hind*III, separated on 0.8% agarose gel, transferred to Hybond N$^+$ and probed with a ^{32}P-labelled *pyr*1 probe (1.8 kb *Hind*III fragment). Washes were performed at 65° C, in 0.2xSSC, 0.1% SDS. Transformants 4II and 5I show *Eco*RI fragments of approximately 6.5 and 2.9 kb and *Hind*III fragments of 4.3 and approximately 1.9 kb following hybridisation with the probe.

In case of site-directed integration has occurred at the *C. fulvum pyr*1 locus, probing with the 1.8 kb *Hind*III fragment from pGW1270, carrying the *pyr*1 gene, is expected to result in hybridising *Eco*RI fragments of approximately 6.5 and 2.9 kb and *Hind*III fragments of 4.3 and approximately 1.9 kb (Figure 1B).

Transformants 4II and 5I indeed show the expected fragments after hybridisation with the *pyr*1-probe, confirming occurrence of site-directed integration (Figures 4A and B), whereas transformant 4I shows several additional hybridising fragments, indicating that in addition to homologous recombination, ectopic integration of pGW1273 had occurred.

Discussion

The interaction between the biotrophic fungus *C. fulvum* and tomato is a well established model to study plant-pathogen interactions. Until now, several *C. fulvum* genes have been isolated which are highly expressed during growth of the fungus in the tomato plant (Joosten and De Wit, 1999). One of such genes is avirulence gene *Avr*9, of which expression is highly induced when *C. fulvum* colonises the intercellular spaces of tomato leaves (Van Kan et al., 1991). The *Avr*9 promoter contains several sequences which are known to act as binding sites for a major nitrogen regulatory protein in the filamentous fungi *A. nidulans* and *N. crassa* (designated AREA and NIT2, respectively) (Van den Ackerveken et al., 1994). For detailed studies on regulation of the *Avr*9 gene, it is essential that subtle differences in mutant *Avr*9 promoter activity can be detected. For these studies, it is a prerequisite that *C. fulvum* transformants carry the mutant *Avr*9 promoter constructs as a single copy and in an identical chromosomal environment.

Until now, *C. fulvum* is genetically poorly characterised. Here we describe the development of a homologous transformation system, designed for targeting constructs of interest to a one known site in the genome of the fungus. As *pyr*1 mutants of *C. fulvum* were already available (Marmeisse et al., 1993), we decided to develop such a targeting system based on this marker gene. An additional advantage of the *pyr* gene is that only very low frequencies of spontaneous reversion have been reported in literature, suggesting that the *pyr* gene can be considered as a strong selection marker (Goosen et al., 1987). Successful targeting based on homologous recombination at the *pyr* locus has been reported for *A. niger*, *A. nidulans* and *A. awamori* (Van Gorcom and Van den Hondel, 1988; Gouka et al., 1995).

For isolation of the *C. fulvum pyr*1 gene, "instant gene bank" strategy was used, which involves *in vivo* ligation of the genomic fragment containing the functional *C. fulvum pyr*1 gene into the autonomously-replicating pHELP1 plasmid (Gems and Clutterbuck, 1993). The advantage of this strategy is that complementation occurs by a plasmid-borne gene. Therefore, the entire gene, including essential regulatory regions, must be present on the complementing recombinant pHELP1 plasmid. This method was used in *A. nidulans* to isolate the *trp*C gene of *Penicillium canescens* (Gems et al., 1994) and the *pyr*G gene of the plant pathogen *Gaeumannomyces graminis* (Bowyer et al., 1994), supporting that this is an efficient method which obviates the need for conventional gene library construction.

On the other hand, the strategy still has several unpredictable steps. From only one, out of 8 *A. nidulans pyr*$^+$ transformants, a sufficient amount of pHELP1 plasmids could be recovered. No pHELP1 plasmids could be rescued in *E. coli* from the other *A. nidulans pyr*$^+$ transformants, which is probably the result of disruption of vector sequences present in the recombinant pHELP1 plasmid that are essential for propagation in bacteria.

In a targeting experiment, seven *C. fulvum pyr*$^+$ transformants were obtained upon introduction of the construct containing the functional *C. fulvum pyr*1 gene with a defined mutation into both race 4 and race 5 *C. fulvum pyr*1-mutant strains. Southern blot analysis revealed that two of these transformants had a single integration at the *C. fulvum pyr*1 locus, indicating that site-specific, single copy *pyr*1$^+$ transformants were obtained.

From previous observations it is known that homologous recombination, is not very efficient in *C. fulvum* (Marmeisse et al., 1993). However, the limited number of *pyr*1$^+$ transformants by using the targeting construct, pGW1273, was possibly due to the fact that the introduced mutation in the construct, described here, is very close to the resident mutation. The small distance between both mutations reduces the chance of homologous recombination, and as a consequence, the efficiency of the targeting system. To increase the chance of homologous recombination, the defined mutation in the *pyr*1 gene of the targeting construct should be more distal from the mutation in the resident gene.

An other possibility to obtain a larger distance between both mutations is transformation of *C. fulvum pyr*1 wild-type strains with a construct containing the *pyr*1 gene with a defined mutation which is located more to the 5' or 3' end of the *pyr*1 mutation which is present on the targeting construct. After transformation, *C. fulvum* transformants can be selected for 5-fluoro-orotic acid resistance, indicating that the *pyr*1 gene is not functional and that possibly replacement of the resident wild-type *C. fulvum pyr*1 gene, with the defined *pyr*1

mutant gene, has occurred. The latter principle is described for *A. awamori* (Gouka et al., 1995).

We can conclude from the research described here, that, although a greater distance between both *pyr*1 mutations would increase efficiency, gene-targeting, based on site-directed integration at the *pyr*1 locus, is possible for *C. fulvum*. The *pyr*-based targeting system used to study the regulation of several cloned genes of *C. fulvum* such as the cloned *Avr* and *Ecp* genes (Joosten and De Wit, 1999)

Experimental procedures

Strains and growth conditions

For complementation cloning, the *Aspergillus nidulans pyr*G-mutant strain WG499 (*yA*1, *pyr*G89, *acr*A1, *pyro*A4, *mei*A4) was used. For cultivation of mycelium, approximately $2x10^8$ conidia were transferred to 100ml supplemented minimal medium (1.5g/l KH_2PO_4 (pH 6.0), 0.5g/l $MgSO_4.7H_2O$, 0.5g/l KCl and 10g/l D-glucose, 0.1mg/l pyridoxine and 1g/l L-proline). Cultures were incubated for ca. 17 hours at 37°C in an orbital shaker at 220 rpm.

The *Cladosporium fulvum pyr*1-mutant strains (race 4 and race 5; Marmeisse et al., 1993), were grown on potato dextrose agar (PDA) or in liquid B5 (Duchefa) medium, supplemented with 2g/l uridine, in shake cultures at 23°C. Ten-day-old PDA cultures of *C. fulvum* were used to prepare conidial suspensions ($5x10^5$ conidia/ml) for liquid cultures.

Generation and analysis of *A. nidulans* and *C. fulvum* transformants

For co transformations of *A. nidulans*, aimed at complementation of the *pyr*G mutation, 100µg *Bam*HI-digested *C. fulvum* (race 5) chromosomal DNA in combination with 40µg of linear *Bam*HI-digested pHELP1 plasmid was used to transform 2.10^7 protoplasts (Wernars et al., 1985). The pHELP1 plasmid carries a 6.1 kb *A. nidulans* sequence (*AMA*1) responsible for autonomous replication and the *Escherichia coli* ampicillin resistance gene (Gems and Clutterbuck, 1993).

C. fulvum mycelium, grown for 48 hours in liquid B5 medium, was harvested by filtration and used for the isolation of protoplasts (Harling et al., 1988). Transformations (Oliver et al., 1987) were performed with 10^7 protoplasts and 10µg plasmid DNA. As a first screen, *C. fulvum pyr*1$^+$ transformants were analysed by PCR on conidia, using primers Cfpyr2 (5'-CGCGATATTATGGGATTTCA-3'; annealing to the 5'-end of the *pyr*1 gene, not present in plasmid

pGW1273, which is used for targeting the construct to the *C. fulvum pyr*1 locus, see Figure 1B) and M13R (5'-CAGGAAACAGCTATGAC-3'; annealing to vector (pHSS19) sequence.

DNA manipulations

Standard DNA manipulation techniques were as described by Sambrook et al. (1989). The *E. coli* strain DH5α F' was used to propagate the plasmids. Isolation of *A. nidulans* and *C. fulvum* DNA was performed as described by Yelton et al. (1984).

Following restriction enzyme digests, DNA fragments were separated on 0.8% agarose gels and transferred to Hybond N^+ membranes, using 0.4 M NaOH according to the manufacturer's instructions (Amersham). Membranes were probed with random-primed [α-^{32}P]dATP-labelled DNA fragments and washes were performed at 65° C, in 0.2xSSC, 0.1% SDS).

Phylogenetic analysis

Protein alignments were performed using ClustalW 1.8. The alignment file was analysed using the Boxshade 3.21 program (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html). Phylogenetic analysis was performed using the Clustal method with PAM250 residue weight table (MegAlign, DNAstar program).

Acknowledgements: The investigations were supported by the Dutch Earth and Life Science Foundation (ALW; Projectnumber: 805.45.006), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Chapter five

Expression of the avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* is regulated by the global nitrogen response factor NRF1

A. Pérez-García, S. S. Snoeijers, M. H. A. J. Joosten, T. Goosen and P. J. G. M. De Wit

An adapted version of this chapter has been submitted for publication

Summary

Here we describe the role of the *Cladosporium fulvum Nrf*1 (for nitrogen response factor 1) gene in regulation of the expression of avirulence gene *Avr*9 and virulence on tomato. The *Nrf*1 gene, which was isolated by a PCR-based strategy, is predicted to encode a protein of 919 amino acid residues. The protein contains a putative zinc finger DNA-binding domain that is 98% identical to the zinc finger of the major nitrogen regulatory proteins AREA and NIT2 of *Aspergillus nidulans* and *Neurospora crassa*, respectively. Functional equivalence of *Nrf*1 to *are*A was demonstrated by complementation of an *A. nidulans are*A loss-of-function mutant with *Nrf*1. *Nrf*1-deficient transformants of *C. fulvum* that were obtained by homologous recombination, were unable to utilise nitrate and nitrite as a nitrogen source. Expression analysis in liquid media revealed that in *Nrf*1-deficient strains, the *Avr*9 gene is not induced under nitrogen starvation conditions, which is in contrast to what is observed in wild-type *C. fulvum*. On susceptible tomato plants the *Nrf*1-deficient strains were as virulent as wild-type strains of *C. fulvum* although the expression of the *Avr*9 gene was strongly reduced. In addition, *Nrf*1-deficient strains were still avirulent on tomato plants containing the functional *Cf-9* resistance gene, indicating that *in planta* apparently still sufficient quantities of stable AVR9 elicitor are produced. Our results suggest that NRF1 is a major regulator of the *Avr*9 gene, but that *in planta* at least one additional positive regulator of *Avr*9 is active.

Introduction

Plant pathogenic bacteria and fungi generally have limited host ranges, and are often confined to members of a single plant species or genus. This appears to result from the products of avirulence (*Avr*) gene(s), present in the pathogen, restricting the host range rather than from positive factors which allow the pathogen to infect many hosts. *Avr* gene products are recognised by plants that carry the matching resistance (*R*) genes. When a pathogen, carrying an *Avr* gene, attacks a plant with the matching *R* gene, a hypersensitive response (HR) is triggered which results in localised host-cell death, preventing onset of the disease and spread of the pathogen. In absence of either one or both members of the *Avr/R* gene pair, the plant fails to recognise the pathogen, HR is not triggered and the host will become diseased (Vivian and Gibbon, 1997; Laugé and De Wit, 1998).

The expression of *Avr* genes seems to be subject to environmental stimuli and nutritional signals. Expression of *Avr* genes from *Pseudomonas syringae* and *Erwinia amylovora* has shown to be induced *in vitro* by culturing bacteria on minimal media (Shen and Keen 1993; Lorang and Keen 1995; Gaudriault et al., 1997; Bogdanove et al., 1998). Both expression of *P. syringae Avr* and *hrp* (hypersensitive response and pathogenicity) genes occurs under similar environmental conditions. In this plant-pathogenic bacterium, *Avr* and *hrp* genes are under control of a multi-component regulatory system, consisting of the *hrp* genes *hrpS*, *hrpR* and *hrpL*. The products of these genes control expression of *hrp*-responsive genes under conditions similar to those encountered by bacteria *in planta*, such as low pH and low nutrient concentration (Salmeron and Staskawicz, 1993; Xiao and Hutcheson, 1994; Xiao et al., 1994).

However, very little is known of regulation of fungal *Avr* genes. An exception is *Avr9* of the fungal pathogen *Cladosporium fulvum*, which encodes a 28 amino acid peptide (Van Kan et al., 1991; Van den Ackerveken et al. 1992). Virulent races transformed with the *Avr9* gene become avirulent on tomato genotypes carrying the *R* gene *Cf-9*. The *Avr9* gene is highly expressed in *C. fulvum* while growing *in planta*, whereas expression of *Avr9* during growth in rich liquid media *in vitro* is very low (Van Kan et al., 1991). Several observations suggest that *Avr9* expression is nitrogen-controlled. First, nitrogen limitation *in vitro* induces *Avr9* expression. Secondly, in the promoter of *Avr9* several copies of the sequence (TA)GATA are present, which in *Aspergillus nidulans* and *Neurospora crassa* are known to represent binding sites for the major nitrogen regulatory proteins AREA and NIT2, respectively (Van den Ackerveken et al., 1994). In previous experiments we have shown that

the *A. nidulans* AREA protein induces *Avr9* promoter activity in *A. nidulans* (Snoeijers et al., 1999).

These nitrogen regulatory proteins are members of the GATA family of transcription factors and contain a remarkably similar DNA-binding domain, which consists of a single Cys2/Cys2-type zinc finger motif with a central loop of 17 amino acids and an adjacent basic region. In both *A. nidulans* and *N. crassa*, these GATA-type transcription factors activate expression of many genes whose products are required for the uptake and utilisation of nitrogen from various sources (e.g., nitrate, nitrite, purines, amides, most amino acids, and proteins), when primary nitrogen sources, such as ammonia or glutamine, are not available or during conditions of nitrogen starvation (Marzluf, 1997). The presence of regulatory proteins with a structure and function similar to AREA and NIT2 has been reported for other filamentous fungi, such as many *Aspergillus* and *Penicillium* species (Fu and Marzluf, 1990; Kudla et al., 1990; Haas et al., 1995; Ellis, 1996; Christensen et al., 1998; Hensel et al., 1998; MacCabe et al., 1998; Gente et al., 1999). Furthermore, the plant pathogens *Magnaporthe grisea* (Froeliger and Carpenter, 1996) and *Gibberella fujikuroi* (Tudzynski et al., 1999) and the insect pathogen *Metarhizium anisopliae* (Screen et al., 1998) have been shown to contain AREA/NIT2-like proteins.

Some of these genes have also a role in expression of secondary metabolites. The NRE protein of *P. chrysogenum* binds to GATA sequences in the intergenic promoter regions of the penicillin biosynthetic gene cluster (Haas and Marzluf, 1995), whereas the *G. fujikuroi* AREA protein is involved in regulation of gibberellin synthesis (Tudzynski et al., 1999).

Here we describe the isolation of the gene that encodes a GATA factor of *C. fulvum*, designated *Nrf*1 (for nitrogen responsive factor 1), and report on its role in regulation of *Avr*9 expression *in vitro* and *in planta*. We found that *Avr*9 expression in *Nrf*1-deficient transformants was abolished *in vitro*, following nitrogen starvation, and was strongly repressed on plants normally susceptible to the parent *C. fulvum* strains. However, *Nrf*1-deficient transformants did not show significant reduction in virulence, nor did they loose avirulence on *Cf-9* containing plants.

Taken these data together, we conclude that the NRF1 nitrogen factor is a major regulator of *Avr*9 expression but that upon growth *in planta* at least one additional positive regulator of *Avr*9 is active.

Results

Isolation of the *C. fulvum areA/nit-2* homologous gene

To isolate the *C. fulvum areA/nit-2*-homologous gene, a PCR-based strategy was followed. Initially, PCR with degenerated oligonucleotide primers based on the zinc finger domain of the *areA* and *nit-2* gene of *A. nidulans* and *N. crassa*, respectively, (see Experimental procedures; Haas et al., 1995) were performed. Amplification by PCR resulted in a product of 141 bp. The DNA sequence of this PCR-product revealed high homology with the region that encodes the DNA-binding zinc finger domain of other known AREA/NIT2-like proteins. Subsequently, PCR with specific zinc finger primers in combination with primers annealing to the vector sequences (see Experimental procedures) was carried out to isolate sequences flanking the zinc finger domain. For this approach, a cDNA library prepared from *C. fulvum* mycelium grown under nitrogen starvation conditions was used as template. PCR on this library resulted in products of 1.2 kb and 300 bp, respectively. Both PCR-products were sequenced and again showed high homology to other fungal *areA/nit-2* like-genes.

A genomic 1.2 kb PCR-product, which was obtained by using two additional specific primers (see Experimental procedures), was used as homologous probe to screen a genomic library of *C. fulvum*. Following two purification steps, six plaques were identified as putative candidates containing at least a part of the *areA/nit-2* homologue of *C. fulvum*. All six clones showed a similar restriction pattern (data not shown), whereas Southern blot analysis showed the same hybridisation pattern for all clones, using the genomic 1.2 kb PCR-product as probe, indicating that similar genomic DNA fragments were present in these clones (data not shown).

Functional analysis of the *C. fulvum areA/nit-2* homologue

To determine whether the *C. fulvum* genomic clones contain a functional *areA/nit-2* homologue, clones 1 to 6 were transformed to the *A. nidulans areA*-loss-of-function mutant *areA18* (Arst et al., 1989). As for *A. nidulans* a functional AREA protein is required for nitrate assimilation (Caddick et al. 1994), selection was performed by determination whether growth of the transformants occurred on minimal medium, with nitrate as sole nitrogen source. A positive control, the wild-type *A. nidulans areA* gene, which is present on the

pAR4-322-1 plasmid (Davis and Hynes, unpublished) was transformed. All *C. fulvum* genomic clones resulted in *A. nidulans* transformants which were able to grow on this nitrate-selective medium.

Twenty independent *A. nidulans* transformants containing either genomic clones 1 or 3 of *C. fulvum* were chosen for analysis of additional growth properties on minimal medium supplemented with various nitrogen sources (Table 1). In all cases, the growth properties of the transformants resembled those of the *A. nidulans areA* wild-type strain and the (positive control) pAR4-322 transformants. Therefore, we concluded to have isolated the *C. fulvum areA/nit-2* homologue, which we designate *Nrf1* (for nitrogen response factor 1).

Table 1. Growth properties of strains of *Aspergillus nidulans* and transformants of this fungus on minimal medium, supplemented with various nitrogen sources. Growth properties of the *A. nidulans* wild-type (A4) and *areA*-loss-of-function mutant *areA18* are shown, in addition to the growth properties of transformants of the *areA18* mutant.

supplemented nitrogen source (5mM)	***A. nidulans* strains**		***A. nidulans areA18* strain transformed with:** [a]		
	A4	***areA18***	pAR4	clone 1	clone 3
ammonia	+++[b,c]	+++	+++	+++	+++
nitrate	+++	-	+++	+++	+++
nitrite	+++	-	+++	++	++
glutamate	+++	-	+++	+++	+++
histidine	+/-	-	+/-	+/-	+/-
asparagine	+++	-	+++	+++	+++
adenine	+++	-	+++	++	++
hypoxanthine	+/+	-	+	+	+
uric acid	+	-	+	+	+
glutamine	+++	+++	+++	+++	+++

[a] Plasmid pAR4-322-1 contains the *A. nidulans areA* wild-type gene;
C. fulvum clones 1 and 3 contain the putative *areA/nit-2* homologue of *Cladosporium fulvum* (see text for details) were transformed to *A. nidulans areA18*.

[b] +++: strong growth; ++: good growth; +: growth; +/-: weak growth; -: no growth

[c] For the growth tests 20 representative *A. nidulans* transformants were chosen.

Sequence analysis of the *C. fulvum Nrf1* gene

Genomic clone 1 of *C. fulvum* was found to contain an insert of 5206 bp, which includes 1171 bp upstream of the predicted ATG, 2754 bp of coding sequence and 1217 bp of downstream non-coding sequence. In addition, DNA sequence analysis showed that clone 3 contains a promoter region of approximately 500 bp, indicating that, at least in *A. nidulans*, this fragment

contains sufficient *Nrf*1 promoter elements to allow *Nrf*1 expression and utilisation of secondary nitrogen sources.

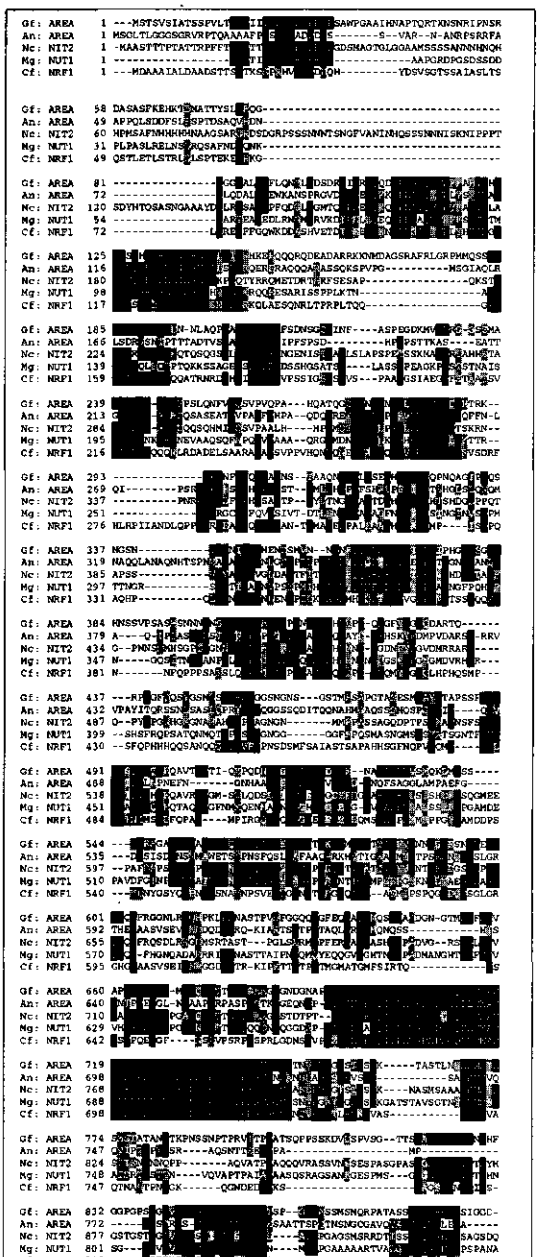


Figure 1. Comparison of the amino acid sequence of *Cladosporium fulvum* NRF1 with AREA/NIT2-like proteins from various other fungi. Gf: *Gibberella fujikuroi* (AREA; Accession Y11006NID); An: *Aspergillus nidulans* (AREA; Accession X52491NID); Nc: *Neurospora crassa* (NIT2; Accession M33956NID); Mg: *Magnaporthe grisea* (NUT1; Accession U60290NID); Cf: *Cladosporium fulvum*. Conserved amino acids are indicated in black boxes. Homologous amino acids are indicated in grey boxes. Amino acid sequences were compared using ClustalW 1.8.

A dash (-) represents a gap position.

The *Nrf*1 gene is predicted to encode a 918 amino acid protein. The coding region is interrupted by one intron of 64 bp that occurs at approximately the same position as the intron that is conserved among all fungal *areA*/*nit*-2-like genes. Furthermore, the intron shows 5' and 3'-splicing sites which are typical for filamentous fungi. Comparison of the *C. fulvum* NRF1 with the major nitrogen regulatory proteins of *Gibberella fujikuroi*, *A. nidulans*, *N. crassa* and *Magnaporthe grisea* reveals significant homology at the amino acid level (Figure 1).

Within the zinc finger domain 48 out of 50 amino acids are identical among all AREA/NIT2-like proteins analysed so far. Ten C-terminal amino acids are also highly conserved. In *N. crassa* this C-terminal region has been shown to interact with the NMR protein (for nitrogen metabolic repression), which is involved in nitrogen metabolite repression (Marzluf, 1997).

Disruption of the *C. fulvum Nrf*1 gene

To inactivate the *Nrf*1 gene by gene replacement following homologous recombination, a *Nrf*1-disruption construct, designated pNRF1KO, was obtained by introducing the hygromycin B resistance expression cassette (*hph*, Punt et al., 1987) into the domain coding for the *Nrf*1 zinc finger (Figure 2A).

Approximately 900 hygromycin B-resistant transformants were generated following transformation of the *C. fulvum* wild-type strains race 4 and race 5 with pNRF1KO. As for nitrate assimilation a functional NRF1 protein is expected to be required, all hygromycin B-resistant transformants were tested for growth on *C. fulvum* minimal medium with sodium nitrate as sole nitrogen source. Thirty-seven hygromycin B-resistant transformants were not able to grow on this nitrate-selective medium. Subsequently, these transformants were analysed by PCR, using *Nrf*1-(p9 and p10; see Experimental procedures) and *hph* (p12; see Experimental procedures)-specific primers, on their genomic DNA.

Three out of the thirty-seven transformants gave a PCR-product, indicative of a disrupted *Nrf*1 gene. The three transformants, two originating from race 4 (4.94 and 4.125) and one from race 5 (5.224), were analysed by Southern blotting for presence, location and copy number of the introduced construct. Transformant 4.125 contained one additional integration of pNRF1KO in the genome and was not used for further analyses (data not shown). Both 4.94 and 5.224 are single copy transformants with a unique integration of the

2.8 kb *hph* cassette in the *Nrf*1 locus (Figure 2B). *Nrf*1-disruptants 4.94 and 5.224 were used for further study.

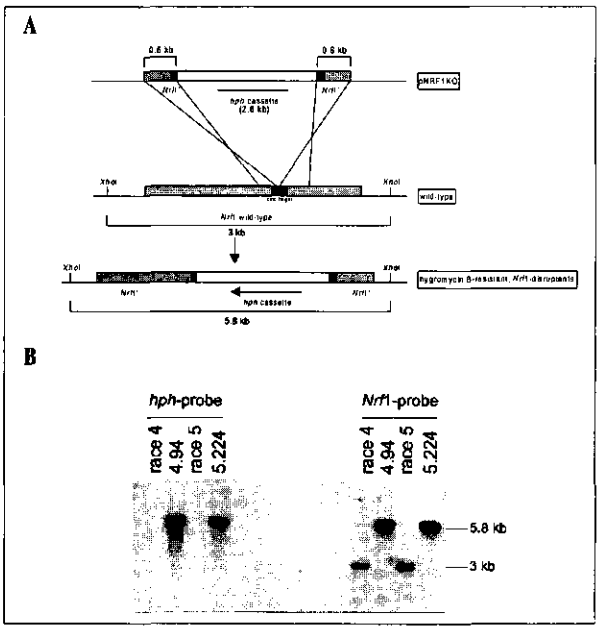


Figure 2. Disruption by replacement of the *Nrf*1 gene in *C. fulvum*. **(A)** The 2.8 kb blunted *Hin*dIII/*Sst*I fragment of pAN7-1 (Punt et al., 1987), containing the hygromycin B resistance gene (*hph* cassette; depicted as a white box), was ligated into the blunted *Not*I site of the sequence coding for the zinc finger domain (depicted as black box) of the genomic 1.2 kb *Nrf*1 PCR-product (depicted as grey box) which was cloned in the TA cloning vector pCR 2.1, resulting in pNRF1KO. Single copy integration of pNRF1KO at the *Nrf*1 locus will result in a 5.8 kb *Xho*I fragment hybridising with the *hph* cassette or the *Nrf*1 gene. **(B)** Southern blot analysis of *Xho*I-digested genomic DNA isolated from *C. fulvum* wild-type strains (race 4 and race 5) and the *Nrf*1-disruptants 4.94 and 5.224, using the 2.8 kb *hph* cassette or the genomic 1.2 kb *Nrf*1 PCR fragment as probe, showed that in 4.94 and 5.224 the 2.8 kb *hph* cassette had replaced the *Nrf*1 gene.

To characterise the *C. fulvum Nrf*1-disruptants, growth properties of disruptants 4.94 and 5.224 on minimal medium supplemented with various nitrogen sources were compared to those of the wild-type strains, race 4 and race 5 (Table 2).

Table 2. Growth properties of *C. fulvum* wild-type strains, race 4 and race 5, and the corresponding *Nrf*1-disruptants 4.94 and 5.224, respectively.

supplemented nitrogen source (5mM)	*C. fulvum* wild-type strains		corresponding *Nrf*1-disruptants	
	race 4	race 5	4.94	5.224
ammonia	+++[a)]	+++	+	+
nitrate	+++	+++	-	-
nitrite	+++	+++	-	-
glutamate	+++	+++	+	+
histidine	++	++	+/-	+/-
asparagine	+++	+++	+	+
adenine	+/-	+/-	-	-
hypoxanthine	+/-	+/-	-	-
uric acid	++	++	+/-	+/-
glutamine	+++	+++	+++	+++

[a)]+++: strong growth; ++: good growth; +: growth; +/-: weak growth; -: no growth

As shown in Table 2, *C. fulvum* race 4 and race 5 displayed strong growth on most of the various nitrogen sources. In contrast, the *Nrf*1-disruptants were unable to utilise nitrate and nitrite, but still utilised glutamine and to a lesser extent other nitrogen compounds, such as ammonia, glutamate and asparagine.

Avr9 expression of the *Nrf*1-disruptants of *C. fulvum* is abolished under nitrogen starvation *in vitro*

Avr9 expression can be induced under conditions of nitrogen limitation *in vitro*. In the promoter of the *Avr9* gene several copies of putative binding sites for AREA/NIT2 factors are present (Van den Ackerveken et al., 1994), suggesting that the NRF1 factor can bind to these sites, and induce *Avr9* expression. Previous experiments have shown that in *A. nidulans*, the activity of the *Avr9* promoter is induced under nitrogen starvation conditions and that the expression of the *Avr9* gene is fully dependent on presence of AREA (Snoeijers et al., 1999). Northern blot analysis was performed in order to determine whether the *Avr9* gene is induced in the *Nrf*1-disruptants 4.94 and 5.224 under nitrogen starvation conditions, *in vitro*.

Conidiospores from the wild-type strains race 4 and race 5 and the corresponding *Nrf*1-disruptants were grown in B5 medium, supplemented with 50 mM glutamine. After 2 days, mycelium was harvested, divided into equal portions and transferred to B5 medium supplemented either with 50 mM glutamine (*are*A-repressing nitrogen source in *A. nidulans*) or 50 mM nitrate (*are*A-inducing nitrogen source in *A. nidulans*), or without any nitrogen

source (*are*A-inducing condition in *A. nidulans*). After 24 hours of incubation, total RNA was isolated and *Avr*9 expression was followed by Northern blot analysis. As shown in Figure 3, in the wild-type strains, *Avr*9 is not only induced under nitrogen starvation but also, albeit to a much lower extent, in medium supplemented with nitrate as sole nitrogen source. In both *Nrf*1-disruptants, no *Avr*9 was observed under any of the conditions applied.

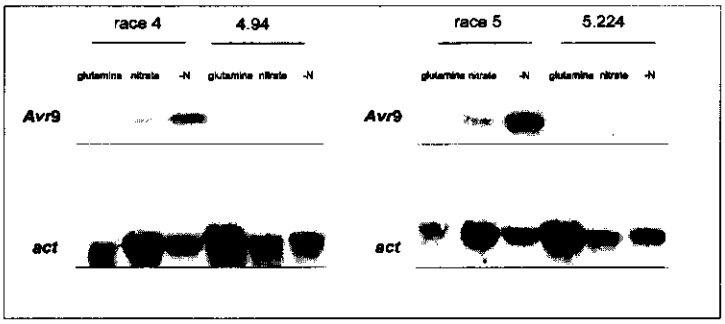


Figure 3. Analysis of *Avr*9 expression *in vitro* of the *C. fulvum* wild-type strains and the corresponding *Nrf*1-disruptants. *Avr*9 expression in *C. fulvum* grown *in vitro* in liquid B5 medium containing 50 mM glutamine (lanes marked glutamine), 50 mM nitrate (lanes marked nitrate) or no nitrogen source (lanes marked -N). Total RNA (10 µg) was separated on 1.5% denaturing formaldehyde-agarose, blotted onto Hybond N^+ membrane and hybridised with *Avr*9 cDNA (Van Kan et al., 1991) and the *C. fulvum* actine (*act*) gene. The *act* gene is constitutively expressed in *C. fulvum* and is used as RNA loading-control.

C. fulvum wild-type strains and *Nrf*1-disruptants show no difference in virulence on susceptible tomato plants

Using a standard inoculation assay of susceptible MM-Cf0 tomato plants (see Experimental procedures), sporulation and disease symptoms caused by the single copy *Nrf*1-disruptants 4.94 and 5.224 were comparable to those caused by wild-type strains. Two weeks after inoculation conidiophores emerged from the stomata at the lower side of leaves and started to produce conidiospores. Western blot analysis of apoplastic fluid (AF), isolated from infected leaves 20 days after inoculation, probed with antibodies raised against ECP2 (Laugé et al., 1997), showed similar concentrations of ECP2 in MM-Cf0 plants irrespective whether they were inoculated with wild-type strains, or with *Nrf*1-disruptants (data not shown).

In addition, Southern blotting performed with DNA isolated from MM-Cf0 leaves infected by wild-type strains and the *Nrf*1-disruptants digested with *Xho*I. and probed with the

genomic 1.2 kb *Nrf*1 PCR-product, gave no signals indicating the true nature of both disruptants (data not shown).

Avr9 expression in *C. fulvum Nrf*1-disruptants is reduced *in planta*

When susceptible tomato plants were inoculated with *Avr9*-containing *C. fulvum* strains, the *Avr9* gene is highly expressed during growth *in planta* (Van Kan et al., 1991; Van den Ackerveken et al., 1994). To determine whether the *Nrf*1-disruptants still produce AVR9 *in planta*, AFs were isolated from MM-Cf0 tomato leaves, 20 days after inoculation with the wild-type strains race 4 and race 5 and the corresponding *Nrf*1-disruptants 4.94 and 5.224, all containing the *Avr9* gene.

Although Western blot analysis (see above) showed that colonisation of MM-Cf0 plants by both wild-type strains and corresponding *Nrf*1-disruptants was comparable, injections of the various AFs into leaves of MM-Cf9 plants revealed that the production of AVR9, determined by necrosis-inducing activity (NIA) of AFs from the *Nrf*1-disruptants was lower when compared with that of the wild-type strains (Figure 4).

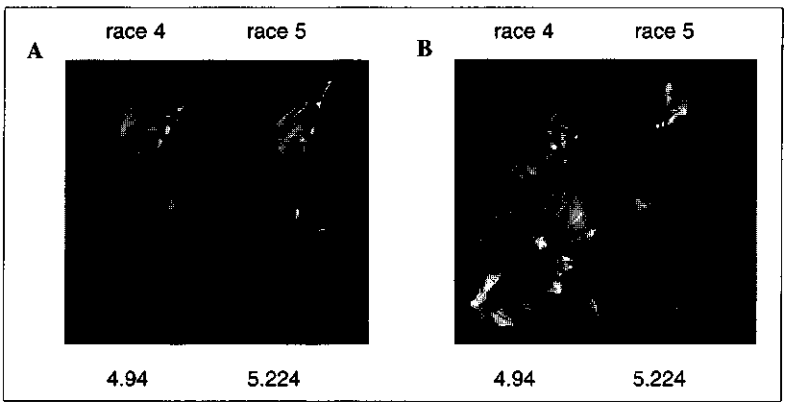


Figure 4. Hypersensitive response (HR) induced in leaves of MM-Cf9 plants upon injection with undiluted and four times diluted apoplastic fluids (AFs) isolated from leaves of MM-Cf0 plants, inoculated with *C. fulvum* wild-type strains race 4 and race 5 and the corresponding *Nrf*1-disruptants 4.94 and 5.224. (**A**) necrosis developed after injection of undiluted AFs isolated from MM-Cf0 plants inoculated with the *C. fulvum* parent strains and the *Nrf*1-disruptants. (**B**) necrosis developed after injection of four times diluted AFs from the same *C. fulvum* strains/disruptants as shown in A. AFs were isolated 20 days post inoculation. Photographs were taken six days after injection.

However, both 4.94 and 5.224 were still avirulent on MM-Cf9C plants (transgenic for the functional *Cf-9* gene), indicating that still sufficient AVR9 peptide is produced in the *Nrf*1-disruptants to be recognised by these plants (data not shown).

In addition, Northern blot analysis of total RNA isolated from leaves infected by the wild-type strains and corresponding *Nrf*1-disruptants was performed to investigate whether the lower concentrations of AVR9 produced by the *Nrf*1-disruptants *in planta*, are due to reduced *Avr9* expression. Figure 5 shows that *Avr9* mRNA is abundantly present at 20 days post inoculation, when tomato leaves were infected by *C. fulvum* wild-type strains. Although significantly reduced when compared to the wild-type strains, some *Avr9* expression could still be detected in the *Nrf*1-disruptants.

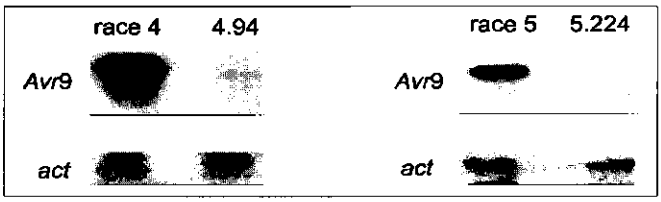


Figure 5. *Avr9* expression *in planta* of the *C. fulvum* wild-type strains and the corresponding *Nrf*1-disruptants. Total RNA from MM-Cf0 plants infected by the *C. fulvum* wild-type strains race 4 and race 5 and the corresponding *Nrf*1-disruptants 4.94 and 5.224, respectively, was isolated 20 days post inoculation. Total RNA (10 μg) was loaded on a 1.5% denaturing formaldehyde-agarose gel, blotted onto Hybond N^+ membrane and hybridised with *Avr9* cDNA (Van den Ackerveken et al., 1992) and the *C. fulvum* actine (*act*) gene. The *C. fulvum act* gene is constitutively expressed in *C. fulvum* and is used as a marker for fungal colonisation. Note the strongly reduced expression of *Avr9* in the *Nrf*1-disruptants 4.94 and 5.224 as compared to the parent strains race 4 and 5, respectively.

Discussion

A better understanding of the function and regulation of avirulence (*Avr*) genes of pathogens is essential, as the trigger of active plant resistance cannot be their primary function. Our results suggest that regulation of *Avr* genes is subject to environmental stimuli and nutritional signals. *Avr9* of *C. fulvum* and *inf*1 of *Phytophthora infestans* are induced *in vitro* under nitrogen- and carbon-limiting conditions, respectively (Van den Ackerveken et al., 1994; Kamoun et al., 1997).

During nitrogen metabolite repression, preferential utilisation of favoured nitrogen sources occurs due to repression of enzymes and permeases required for utilisation of nitrogen sources other than ammonia or glutamine (Wiame et al., 1985; Marzluf, 1997). Nitrogen metabolite repression not only controls genes encoding enzymes of primary metabolism, but also regulates genes involved in secondary metabolism, like penicillin and gibberellin biosynthesis (Haas and Marzluf, 1995; Tudzynski et al., 1999). In the present study, the putative nitrogen regulation of the *Avr*9 gene of *C. fulvum* has been examined in detail. We have cloned the *are*A/*nit*-2 homologue of *C. fulvum*, designated *Nrf*1, and we have studied *Avr*9 expression in *C. fulvum Nrf*1-disruptants.

Several lines of evidence suggest that *Nrf*1 is indeed the major nitrogen regulatory gene from *C. fulvum*. First, NRF1 shares strong homology with reported fungal, positively-acting nitrogen regulatory proteins from *G. fujikuroi* (AREA), *A. nidulans* (AREA), *N. crassa* (NIT2) and *M. grisea* (NUT1) (Fu and Marzluf, 1990; Kudla et al., 1990; Froeliger and Carpenter, 1996; Tudzynski et al., 1999). Secondly, *Nrf*1 is able to complement an *are*A-loss-of-function mutant of *A. nidulans*, in restoring wild-type growth of transformants on a variety of nitrogen sources. These results suggest that NRF1 recognises similar specific (TA)GATA elements in the promoter of *A. nidulans* AREA-regulated genes and that NRF1 and AREA respond to similar external nutritional signals of nitrogen metabolite repression. Thirdly, *C. fulvum Nrf*1-disruptants are unable to utilise a wide range of nitrogen sources. Therefore, we conclude that the *Nrf*1 gene of *C. fulvum* encodes a protein with a similar function as the positively-acting global nitrogen regulatory proteins reported for other fungi.

Although the *C. fulvum Nfr*1-disruptants were unable to grow on a variety of nitrogen sources, they were still able to utilise, in addition to glutamine, secondary nitrogen sources such as glutamate and asparagine. As both in *A. nidulans* and *A. fumigatus* the AREA protein is not required for ammonia utilisation (Kudla et al., 1990; Hensel et al., 1998), it was expected that the *Nrf*1-disruptants would reveal wild-type growth on ammonia. Interestingly, in the plant-pathogenic fungus *Colletotrichum gloeosporioides*, the gene coding for glutamine synthetase (GS), designated *CgGS*, has been shown to be induced during pathogenesis on its host *Stylosanthes guianensis*, but also *in vitro* under nitrogen-limiting conditions (Stephenson et al., 1997). In general, the GS enzyme is required for ammonia assimilation in a broad variety of organisms. An explanation for poor growth of the *Nrf*1-disruptants on ammonia could be that in *C. fulvum* the gene encoding GS is under control of NRF1.

*are*A/*nit*-2-like genes play a major role in regulating expression of genes involved in utilisation of various nitrogen sources and in regulation of genes involved in secondary

metabolism. As *C. fulvum* is a biotrophic pathogen of tomato, we hypothesised that disruption of *Nrf*1 in this fungus would have a major effect on both regulation of the *Avr*9 gene, and disease development. However, using a standard inoculation assay, disease symptoms caused by the *Nrf*1-disruptants were similar to those caused by *C. fulvum* wild-type strains. This indicates that disruption of *Nrf*1 does, at least under greenhouse conditions, not affect virulence. A similar observation was reported for the rice blast pathogen *M. grisea*. Disruption of the *M. grisea areA/nit-2* homologous gene, *nut*1, did not result in reduced virulence (Froeliger and Carpenter, 1996). However, the mechanisms employed by *C. fulvum* and *M. grisea* to infect plants are different. In a compatible interaction (plant susceptible, fungus virulent), *M. grisea* grows intracellularly (Valent and Chumley, 1991), whereas *C. fulvum* grows extracellularly in the intercellular space (De Wit, 1977). Full colonisation of susceptible tomato plants by *C. fulvum Nrf*1-disruptants, suggests that primary nitrogen sources, such as glutamine, are available in the apoplast to allow fungal growth. Possibly, *C. fulvum* possesses other nitrogen control systems that function independently of *Nrf*1, as has been suggested for *M. grisea* where other regulatory genes involved in both nitrogen metabolism and virulence are present (Lau and Hamer, 1996).

*Avr*9 expression in *Nrf*1-disruptants of *C. fulvum* was completely abolished following nitrogen starvation *in vitro*, suggesting that induction of *Avr*9 expression *in vitro* is fully under control of the NRF1 protein. In addition, using 50 mM nitrate as sole nitrogen source, a weak induction of *Avr*9 could be detected in the wild-type *C. fulvum* strains, which is in contrast to previous results observed by Van den Ackerveken et al. (1994).

However, upon inoculation of *Nrf*1-disruptants on susceptible tomato plants, AVR9 still accumulated in the apoplast, albeit at very low levels, as compared to the wild-type strains. In addition, Northern blot analysis confirmed that expression of *Avr*9 was indeed significantly reduced in leaves colonised by the *Nrf*1-disruptants. Taken together, these results indicate that, although the NRF1 protein is a major factor, at least one additional factor is involved in induction of *Avr*9 expression *in planta*.

The counterpart of *areA/nit*-2-like genes, is the *nmr* gene (for nitrogen metabolic repression). This gene was first isolated from *N. crassa* (Young et al., 1990) and recently from *A. nidulans* (Adrianopoulos et al., 1998) and *G. fujikuroi* (Tudzynski, personal comm.). In *N. crassa* and *A. nidulans nmr*-mutants, show a derepressed phenotype in the presence of ammonia or glutamine, which normally completely represses nitrogen-induced activity (Tomsett et al., 1981; Adrianopoulos et al., 1998). The NMR protein is supposed to act by binding to the AREA/NIT2-like protein, thereby preventing DNA-binding when sufficient

concentrations of primary nitrogen sources, such as glutamine, are present (Marzluf, 1997). A gene with significant homology to the *nmr* gene was recently isolated from *C. fulvum* in our group (A. Pérez-García et al., unpublished). We hypothesise that disruption of this gene in *C. fulvum* may lead to derepression of avirulence genes which are under control of NRF1.

In addition to *are*A/*nit*-2 and *nmr* genes, other genes are involved in regulation of nitrogen metabolism. The *tam*A gene of *A. nidulans*, for example, encodes a protein required for full expression of genes under *are*A control (Kinghorn and Pateman, 1975; Davis et al., 1996). Recently, it has been shown that TAMA fused to a DNA-binding domain is able to activate expression of nitrogen regulated genes. It has been proposed that the TAMA fusions are recruiting AREA to the relevant promoters (Small et al., 1999).

The positively-acting pathway specific proteins NIRA in *A. nidulans* and NIT4 in *N. crassa* are required for expression of nitrate assimilation genes (Burger et al., 1991a, b; Yuan et al., 1991). For *C. fulvum* one could speculate that *Avr*9 expression is not only under control of NRF1 but also under control of an additional positively-acting pathway specific regulator. More data on *C. fulvum* nitrogen metabolism are needed to fully understand the mechanism of regulation of *Avr*9 expression.

Experimental procedures

Fungal strains and culture conditions

The following *Aspergillus nidulans* strains were used: A4 (wild-type) and the *are*A loss-of-function mutant *are*A18 (yA2, *paba*A1, *ino*B2, *are*A18; Arst., 1989). *A. nidulans* strains were grown routinely on solid complete medium, supplemented with the appropriate requirements as described by Pontecorvo et al. (1953). For mycelial growth, approximately $2x10^8$ conidiospores were transferred to 100ml supplemented liquid minimal medium containing 1.5g/l KH_2PO_4 (pH 6.0), 0.5g/l $MgSO_4.7H_2O$, 0.5g/l KCl and 10g/l D-glucose, 0.1mg/l pyridoxine and 1g/l L-proline (Pontecorvo et al., 1953). Cultures were incubated for about 17 hours at 37°C in an orbital shaker at 220 rpm.

Strains of *C. fulvum* (race 4 and race 5) were grown on potato dextrose agar (PDA) or in liquid B5 medium (Duchefa Biochemie BV, Haarlem, The Netherlands) in shake cultures at 23°C (100 rpm). Ten-day-old PDA plates of *C. fulvum* were used to prepare spore suspensions for plant inoculations and for liquid cultures. *C. fulvum Nrf*1-deficient transformants were grown on PDA and B5 medium supplemented with 10 mM glutamine.

For *C. fulvum* nitrogen starvation experiments, 100 ml of B5 medium (supplemented with 50 mM glutamine) was inoculated with approximately $4x10^7$ conidiospores and the culture was incubated

for 2 days at 23°C (100 rpm). Subsequently, mycelium was harvested, rinsed with B5 medium, and divided into equal portions, which were transferred to 25 ml liquid B5 medium supplemented with 50 mM nitrate, 50 mM glutamine or without any nitrogen source. After 24 hours of incubation at 23°C and shaking at 100 rpm, mycelium was harvested again, freeze-dried and stored at -80°C.

PCR-strategy to isolate the *C. fulvum areA/nit-2* homologous gene

The *areA/nit*-2-like gene is conserved in various fungi, suggesting that a similar gene is also present in *C. fulvum*. Initially, degenerated oligonucleotide primers, based on the region coding for the zinc finger domain of the *are*A and the *nit*-2 gene of *A. nidulans* and *N. crassa*, respectively, p1 (5'-TGTACNAAYTGYTTYACNCA-3') and p2 (5'-TTCTTPATNACPTCNGTYTT-3'), as described by Haas et al. (1995) were used. PCR resulted in a 141 bp fragment which was subsequently ligated into the TA cloning vector pCR 2.1 (Invitrogen, Carlsbad, USA) and sequenced. The DNA sequence of this fragment revealed indeed high homology with the domain that encodes for the conserved DNA-binding zinc finger domain of other known AREA/NIT2-like proteins.

Based on the DNA sequence of the 141 bp fragment, specific primers were designed to isolate the flanking sequences. The specific zinc finger primers p3 (5'-TGTACTAATTGTTTTACGCA-3') and p4 (5'-TTCTTGATGACGTCGGTCTT-3') were used, in combination with primers annealing to vector sequence p5 (5'-TACCGGGCCCCCCCTCGATT-3') and p6 (5'-TCTAGAACTAGTGGATCCCC-3'), respectively. PCR, using these specific primers, on a cDNA library, in Uni-ZAP XR (Stratagene, La Jolla, USA) prepared from *C. fulvum* mycelium grown under nitrogen starvation conditions was performed. Fragments of 1.2 kb (using primers p3 and p5) and 300 bp (using primers p4 and p6) were amplified and ligated into the TA cloning vector pCR 2.1. Subsequently, these fragments were sequenced and based on the DNA sequences, the primers p7 (5'-TGAATACTCCGCAATGGACG-3') and p8 (5'-CAGGGGAACTCGTGGGTACG-3') were designed to amplify a genomic 1.2 kb fragment, which was used as homologous probe to screen a λBlueStar (Novagen, Madison, USA) *C. fulvum* genomic library (R. Oliver, unpublished).

Generation and analysis of *A. nidulans* and *C. fulvum* transformants

Transformation of *A. nidulans* was performed as previously described by Wernars et al. (1985). For *A. nidulans* complementation experiments, transformed protoplasts were plated on selective minimal medium, containing sodium nitrate (10 mM) as sole nitrogen source. *A. nidulans* transformants were analysed for their ability to grow on minimal medium supplemented with various nitrogen sources. The gene which was able to complement was designated *Nrf*1 (for nitrogen response factor 1)

In order to obtain a *Nrf*1-disruption construct, the 2.8 kb *Hin*dIII-*Sst*I fragment containing the hygromycin B resistance gene (*hph* cassette), originating from plasmid pAN7-1 (Punt et al., 1987), was blunted and inserted into the, blunted, *Not*I site of the sequence coding for the zinc finger domain, resulting in pNRF1KO. In pNRF1KO, the *hph* cassette is flanked on both sides by approximately 0.6 kb *Nrf*1 sequence (Figure 2A). Subsequently, pNRF1KO was transformed to the *C. fulvum* wild-type strains race 4 and race 5.

For *C. fulvum* transformation, mycelium grown for 3 days in liquid B5 medium was harvested by filtration and used for the isolation of protoplasts according to the method of Harling et al. (1988). Transformations were performed as previously described by Oliver et al (1987).

Initially, hygromycin B-resistant *C. fulvum* transformants were selected for the inability to grow on *C. fulvum* minimal medium containing 0.5 g/l KCl, 0.5 g/l Mg-glycerophosphate, 1 mg/l $FeSO_4$, 0.35 g/l K_2SO_4, 15 g/l sucrose, 15 g/l agar (pH 6.0) with sodium nitrate (0.51 g/l) as sole nitrogen source. Genomic DNA of *C. fulvum* hygromycin B-resistant transformants which did not grow on this nitrate-selective medium was analysed by PCR using *Nrf*1-specific primers p9 (5'-GGCTCACCACAGTGGTTTCAA-3') and p10 (5'-GCACAGAGTTGCCACTTCCG-3'), and the *hph* specific primer p11 (5'-CATACACCGGGCAAAGCAGG-3'), to determine whether the insertion of the *hph* gene had occurred at the *Nrf*1 locus. Finally, DNA isolated from the putative *C. fulvum Nrf*1-disruptants was analysed by Southern blotting for the presence, location and copy number of the introduced construct.

Isolation and analysis of nucleic acids

Total DNA of *C. fulvum* and tomato leaves infected with the fungus was isolated according to Laugé et al (1997). Total RNA was isolated according to the Clontech (Clontech Inc., Palo Alto, USA) protocol. Southern and Northern blotting procedures were performed essentially according to Sambrook et al. (1989). Probes were labelled using the Random Primers Labeling System (Life Technologies Inc., Rockville, USA) including 20μCi α^{32}P-dATP (Amersham, Buckinghamshire, England).

Plants and inoculations

Ten-days-old PDA cultures of *C. fulvum* were used to prepare spore suspensions (approximately $5x10^5$ conidiospores/ml) for inoculation of plants. For this purpose, the tomato cultivar Moneymaker (MM-Cf0), susceptible to all known strains of *C. fulvum* and MM-Cf9C plants, only containing the functional *Cf-9* gene (*Hcr9-9C*; Parniske et al., 1997), mediating recognition of the AVR9 peptide (transgenic seeds were kindly provided by Dr. M. Parniske, The Sainsbury Laboratory, John Innes

Centre, Norwich, England), were used. Five-week-old plants were inoculated with conidial suspensions as described by De Wit (1977).

Isolation and analysis of apoplastic fluids

For detection of AVR9 in *C. fulvum*-infected tomato leaves, apoplastic fluids (AFs) were isolated as described by De Wit and Spikman (1982) and injected into leaflets of five-week-old MM-Cf9 plants, containing the complete *Cf-9* locus (Parniske et al., 1997). AFs were analysed on 15% (wt/vol) SDS polyacrylamide gels. Western blot analyses were carried out after electro transfer of the separated proteins to nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany). Polyclonal antibodies against ECP2, a marker for colonisation by *C. fulvum* (Wubben et al., 1994; Laugé et al., 1997) were raised in rabbits. GARAP antibodies were obtained from Bio-Rad (Veenendaal, The Netherlands).

Acknowledgements: We are indebted to Richard P. Oliver (Carlsberg Laboratory) for providing *Cladosporium fulvum* cDNA and genomic DNA libraries. We also thank Mark X. Caddick (University of Liverpool) for providing us with the *Aspergillus nidulans are*A18 strain. A. Pérez-García was supported by the EC-TMR Program ERBFMBICT 961539. S. S. Snoeijers was supported by a grant from the Dutch Earth and Life Science Foundation (ALW; Projectnumber: 805.45.006), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Chapter six

Avirulence gene *Avr9* of the tomato pathogen *Cladosporium fulvum* is repressed *in planta* by elevated levels of nitrate

S. S. Snoeijers, A. Pérez-García, M. H. A. J. Joosten and P. J. G. M. De Wit

An adapted version of this chapter has been submitted for publication

Summary

Strains of the fungal tomato pathogen *Cladosporium fulvum* that carry avirulence gene *Avr9* are avirulent on tomato genotypes carrying matching resistance gene *Cf-9*. Expression of the *Avr9* gene is strongly induced upon nitrogen starvation *in vitro* and during colonisation of the intercellular spaces of tomato leaves. In order to determine whether the amount of nitrogen available affects expression of *Avr9* by the fungus *in planta*, tomato plants containing high levels of nitrate were inoculated with transgenic strains of *C. fulvum* containing the *Avr9* promoter fused to the GUS reporter gene. We used either plants with a defective nitrate reductase gene or plants that had been supplied with additional nitrate. Nitrate levels in leaves of such plants were 3-8 times higher than in wild-type plants. Plants containing both the *Cf-9* gene and the defective nitrate reductase gene or plants containing *Cf-9* that were supplied with additional nitrate, were more susceptible to strains of *C. fulvum* containing the *Avr9* gene, than wild-type Cf9 plants. This indicates that the *Avr9* gene is repressed in plants with elevated levels of nitrate and strongly suggests that *in planta*, nitrogen-limiting conditions induce expression of the *Avr9* gene. Although the promoter of the *Avr9* gene contains several (TA)GATA sequences that are present in promoters of various genes involved in nitrogen metabolism, we have no indications yet that the *Avr9* gene itself has a role in nitrogen metabolism.

Introduction

The interaction between tomato and the biotrophic, imperfect fungal pathogen *Cladosporium fulvum* is well characterised. Runner hyphae of the fungus penetrate tomato leaves through stomata, and colonise the apoplastic space of the mesophyll. The strictly extracellular growth of *C. fulvum* allows the isolation and identification of fungal components secreted in the apoplast of infected plants (Laugé and De Wit, 1998).

Tomato plants that carry one, or more, resistance (*Cf*) gene(s) against the fungus perceive secreted protein(s) (so-called elicitors), that are encoded by matching avirulence (*Avr*) genes of *C. fulvum*. After recognition, the plant mounts a hypersensitive response (HR) that eventually leads to resistance against the fungus (Joosten and De Wit, 1999).

Cf-9/*Avr9* is a well-studied gene pair. MM-Cf9, a near isogenic line derived from the tomato cultivar Moneymaker (MM-Cf0) which does not contain any known *Cf* gene against *C. fulvum*, contains an introgression segment originating from *Lycopersicon pimpinellifolium* which carries five *Cf* gene homologues. These homologous genes are referred to as *Hcr9-9*s (for homologues of *C. fulvum* resistance gene *Cf-9*, present at the *Cf-9* locus; Parniske et al., 1997). Homologue *Hcr9-9C* is the functional *Cf-9* gene, which mediates specific HR-associated recognition of the AVR9 peptide (Jones et al., 1994). Previous experiments have shown that MM-Cf9 plants show partial resistance to strains of *C. fulvum* lacking *Avr9*, indicating that one, or more, of the other *Hcr9-9*s are also functional (Parniske et al., 1997; Laugé et al., 1998) in recognising strains of *C. fulvum* carrying *Avr* genes other then *Avr9*.

The *Avr9* gene encodes a peptide of 63 amino acids which is processed to a stable 28 amino acid peptide that accumulates *in planta*. The expression of the *Avr9* gene is highly induced when *C. fulvum* colonises the intercellular spaces of tomato leaves (Van Kan et al., 1991). Van den Ackerveken et al. (1994) observed that *Avr9* expression can also be induced under nitrogen-limiting conditions *in vitro*. They speculated that the apoplast might represent a nitrogen-limiting environment, inducing *Avr9* expression. The observation that the *Aspergillus nidulans* major nitrogen regulatory protein, designated AREA, is able to induce *Avr9* promoter activity (Snoeijers et al., 1999) supports this hypothesis. So far, the intrinsic function of AVR9 for the fungus is not known. The structure of AVR9 is very similar to that of the potato carboxypeptidase inhibitor (Vervoort et al., 1997). However, protease inhibition assays showed that AVR9 has no inhibitory activity (Van den Hooven, unpublished data).

Marmeisse et al. (1993) found that strains of *C. fulvum* in which the *Avr9* gene had been deleted, did not show reduced virulence when compared to *Avr9*-containing strains. This result indicates that the *Avr9* gene has, at least under greenhouse conditions, only a limited role in virulence. Nevertheless, plant breeders consider the *Cf-9* gene as a stable resistance gene as fungal strains virulent on *Cf-9*-containing plants most probably also lack competitive ability, as no serious epidemics of strains lacking the *Avr9* gene have been reported in the past. Furthermore, loss of only the *Avr9* gene is not sufficient to overcome the additional functional *Hcr9-9*s (Parniske et al., 1997; Laugé et al., 1998) present on the *Cf-9* cluster.

In this report we have studied the effect of varying nitrate levels on the expression of *Avr9 in planta* during infection by *C. fulvum*. Nitrate reductase-deficient tomato plants, which lack *Cf* genes and accumulate elevated levels of nitrate in the apoplast (Schoenmakers et al., 1991) and MM-Cf9 plants crossed to such plants, have been used to investigate whether *Avr9* expression is suppressed during colonisation of these plants by *C. fulvum*. In addition, MM-Cf0 plants transformed with *Hcr9-9C* were supplied with potassium nitrate (KNO_3), to artificially increase nitrate levels in the apoplast. Plants containing both the *Cf-9* resistance gene and elevated levels of nitrate in the apoplast, were inoculated with an *Avr9*-containing transformant of *C. fulvum*, constitutively expressing the *uid*A (GUS) reporter gene (encoding ß-glucuronidase), allowing monitoring of fungal colonisation (Oliver et al., 1993). We observed increased growth of *C. fulvum* in plants with elevated levels of nitrate when compared to control plants with normal nitrate levels, which is most probably due to repression of *Avr9* expression.

Results

The *Avr9* promoter of *C. fulvum* is suppressed upon infection of plants with elevated levels of nitrate

To determine whether elevated levels of nitrate in the apoplast repress *Avr9* induction, five-week-old nitrate reductase-deficient tomato plants (A29 plants), which lack *Cf* genes and accumulate elevated levels of nitrate in the apoplast, and MM-Cf0 plants were inoculated with conidia of the P*Avr9-uid*A transformant of *C. fulvum* (Van den Ackerveken et al., 1994). In this transformant, GUS activity is correlated with *Avr9* promoter activity.

Figure 1.

Figure 2.

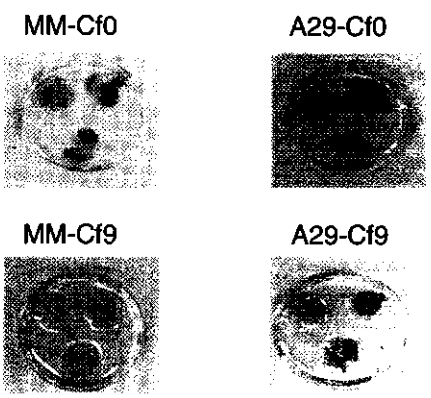


Figure 3.

Figure 4.

Figure 5.

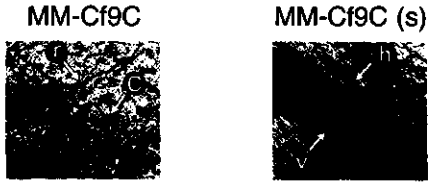


Figure 1. *Avr9* promoter activity of the P*Avr9-uid*A transformant, inoculated onto plants with low (MM-Cf0) and elevated levels of nitrate in the apoplastic fluid (A29). Leaf disks were taken 20 days after inoculation, stained for GUS activity and analysed by light microscopy. GUS activity (blue staining) is a reflection of *Avr9* promoter activity. **(h)** intercellular hyphea; **(v)** vascular tissue; **(c)** conidiophores emerging through the stomata; **(s)** stoma

Figure 2. Colonisation of leaves of tomato genotypes MM-Cf0, A29-Cf0, MM-Cf9, and A29-Cf9 by the *C. fulvum* P*gpd-uid*A transformant (carrying *Avr9*). Leaf disks were taken 20 days after inoculation and stained for GUS activity. The blue patches on the leaf disks of the infected plants represent mycelium of *C. fulvum*.

Figure 3. Colonisation of the mesophyll of MM-Cf9 and A29-Cf9 tomato plants by the *C. fulvum* P*gpd-uid*A transformant (carrying *Avr9*). Leaf disks were taken 20 days after inoculation, stained for GUS activity and analysed by light microscopy. **(c)** inoculated conidiospores; **(r)** runner hyphae; **(v)** vascular tissue; **(h)** intercellular hyphae

Figure 4. Colonisation of leaves of tomato genotypes MM-Cf9C and MM-Cf9C (s) by the *C. fulvum* P*gpd-uid*A transformant (carrying *Avr9*). Leaf disks were taken 20 days after inoculation and stained for GUS activity. The blue patches on the leaf disks of the infected plants represent *C. fulvum* mycelium.

Figure 5. Colonisation of leaves of the mesophyll of MM-Cf9C and MM-Cf9C (s) tomato plants by the *C. fulvum* P*gpd-uid*A transformant (carrying *Avr9*). Leaf disks were taken twenty days after inoculation, stained for GUS activity and observed by light microscopy. **(c)** inoculated conidiospores; **(r)** runner hyphae; **(h)** intercellular hyphae; **(v)** vascular tissue

Two weeks after inoculation, symptoms started to develop in both plants. Conidiophores emerged through the stomata at the lower side of leaves and sporulation on both A29 and MM-Cf0 plants was similar (data not shown). Twenty days after inoculation, the concentration of nitrate in apoplastic fluid (AF) of A29 plants was 38 mM, compared to 7 mM for MM-Cf0 plants. At this time point, leaf disks of MM-Cf0 plants showed abundant blue-stained mycelium in the intercellular spaces, indicating that the *Avr9* promoter is highly induced (Figure 1). However, in the apoplastic space of A29 plants no blue-stained mycelium was detected while conidiophores were blue (Figure 1), suggesting that in the apoplast of A29 plants *Avr9* promoter activity is suppressed.

Suppression of *Avr9* expression in *Cf-9*-containing plants with elevated levels of nitrate, allows increased fungal growth

If *Avr9* expression is suppressed as a result of high nitrate levels, an *Avr9*-containing strain of *C. fulvum* is expected to show increased colonisation of Cf9 plants with elevated levels of nitrate in the apoplast as compared to control plants. To test this hypothesis, MM-Cf9 plants were crossed to A29 plants and as a control, a cross between MM-Cf0 and A29 plants was performed. As expected, about 25% of the F2 plants, resulting from a selfing of the F1 of each cross, showed the recessive nitrate reductase-deficient phenotype. Plants with this phenotype and also containing the *Cf-9* resistance gene (as determined by injection with AF containing AVR9), were designated A29-Cf9, whereas nitrate reductase-deficient plants present in the F2 of the cross between MM-Cf0 and A29 were designated A29-Cf0.

Five-week-old MM-Cf0, A29-Cf0, MM-Cf9 and A29-Cf9 plants were inoculated with the *C. fulvum* (race 4) P*gpd-uid*A transformant (carrying the *Avr9* gene), which is virulent on MM-Cf4 and avirulent on MM-Cf5 plants, allowing determination of fungal growth by GUS-staining. The nitrate concentration in AF of these plants was determined at the day of inoculation (t_0) whereas at twenty days post inoculation (t_{20}), the nitrate concentration was determined of AF isolated from the A29-Cf0 and A29-Cf9 plants. In addition, the nitrate concentration of AF isolated from uninoculated A29-Cf0 plants incubated under the same conditions as the inoculated plants, was determined. Table 1 shows that at t_0 nitrate concentrations in AF were 5-8 times higher in plants with the A29 phenotype (A29-Cf0 and A29-Cf9 plants) than in MM-Cf0 and MM-Cf9 plants. At t_{20}, nitrate concentrations in AF of the various A29 plants, either inoculated with *C. fulvum* or not, were similar.

Table 1. Nitrate concentration of apoplastic fluid (AF) and fungal colonisation after inoculation of various tomato genotypes with the *C. fulvum* P*gpd-uid*A transformant. Nitrate concentration of AF was determined at day zero (t_0) and twenty days (t_{20}) after inoculation. Nitrate concentrations presented are the average of three independent determinations. Fungal growth was determined twenty days (t_{20}) after inoculation by GUS-staining.

tomato genotypes	nitrate concentration of AF (mM)		fungal colonisation at t_{20}
	t_0	t_{20}	
MM-Cf0	6±3	n.d.[a)]	+++[b)]
A29-Cf0	30±5	30±3	+++
MM-Cf9	5±2	n.d.	-
A29-Cf9	41±7	30±4	+
A29-Cf0, n.i.[c)]	35±3	21±8	n.a.[d)]

[a)]n.d.: not determined, [b)]+++: the apoplast was fully colonised by *C. fulvum*; +: partial colonisation of the apoplast by *C. fulvum*; -: no mycelium detected; [c)]n.i.: not inoculated; [d)]n.a.: not applicable

MM-Cf0 and A29-Cf0 plants were found to be fully susceptible to the P*gpd-uid*A transformant; at t_{20}, sporulating patches of mycelium were observed on the lower side of the leaves (data not shown). No difference in infection could be detected between the two genotypes, indicating that the elevated level of nitrate in the apoplast has no influence on the susceptibility of these plants. Furthermore, ELISA on AF isolated at t_{20}, employing antibodies raised against ECP2, a marker for colonisation by *C. fulvum* (Laugé et al., 1997), indicated that similar concentrations of ECP2 were present in the MM-Cf0 and A29-Cf0 plants (data not shown). However, no sporulating patches of mycelium could be observed on the leaves of the inoculated MM-Cf9 and A29-Cf9 plants.

Figure 2 shows the presence of mycelium of *C. fulvum* in leaf disks of MM-Cf0, A29-Cf0, MM-Cf9, and A29-Cf9 plants, inoculated with the P*gpd-uid*A transformant, after staining for GUS activity at t_{20}. Leaf disks of infected MM-Cf0 and A29-Cf0 plants show

plants. Microscopic observation revealed that fungal colonisation of the MM-Cf9C (s) plants mainly occurred in the vicinity of the vascular tissue. In leaf disks of MM-Cf9C plants no fungal growth inside the plant was observed (Figure 5).

After GUS-staining, only very small blue spots were observed on leaf disks of MM-Cf5 and MM-Cf5 (s) plants, indicating that nitrate-supply had no effect on the outcome of an incompatible interaction not dependent on *Avr*9 expression (Table 2).

Discussion

Little is known about basic metabolic control circuits in phytopathogenic fungi and nutritional conditions in plants affecting disease development. Induction of fungal genes, both *in vitro* under conditions of nitrogen limitation and *in planta*, has been reported before (Pieterse et al., 1994; Lau and Hamer, 1996; Talbot et al., 1997; Stephenson et al., 1997; Segers et al., 1999). Van den Ackerveken et al. (1994) hypothesised that *in planta* expression of the *C. fulvum* *Avr*9 gene might depend on nitrogen concentrations in the apoplastic space of tomato leaves. The observation that the *Avr*9 promoter contains various copies of the sequence (TA)GATA, which bind major fungal nitrogen regulators (designated AREA in *Aspergillus nidulans*), would support this hypothesis.

Previously, we have shown that in an *A. nidulans are*A wild-type strain transformed with an *Avr*9 promoter-*uid*A (GUS) fusion, this promoter is induced following nitrogen starvation, whereas this induction does not occur in a strain of *A. nidulans* lacking the *are*A gene (Snoeijers et al., 1999). This suggests that an AREA-like transcription factor is likely involved in the induction of *Avr*9 expression when nitrogen levels are low.

Hardly any induction of the *Avr*9 promoter was observed following inoculation with a *C. fulvum* transformant, carrying the *Avr*9 promoter-*uid*A fusion, of susceptible nitrate reductase-deficient plants (A29 plants; Schoenmakers et al., 1991), that showed 5-6 times more nitrate in the AF than MM-Cf0 plants. This result suggests that elevated levels of nitrate in the apoplast indeed cause *Avr*9 suppression *in planta*. Interestingly, conidiophores emerging from stomata of these plants showed strong GUS activity. This migh suggest that (i) nitrogen outside the plant is soon limiting and (ii) that *Avr*9 expression is strongly induced during the production of conidiophores which is probably independent of a nitrogen factor. A similar phenomenon was observed before, when *C. fulvum* transformants carrying the *uid*A gene fused to promoter sequences of the *Ecp*1 or *Ecp*2 gene, coding for extracellular proteins 1 and 2, respectively, were inoculated on susceptible tomato plants (Wubben et al., 1994).

Both the promoter of *Ecp*1 and *Ecp*2 were strongly induced in conidiophores emerging from stomata. *Ecp* genes of *C. fulvum* are normally highly expressed during pathogenesis and it has been shown that at least some of them are virulence factors (Laugé et al., 1997).

Significantly more growth of *C. fulvum* (carrying the *Avr*9 gene) was observed in the apoplast of inoculated A29-Cf9 plants, containing the *Cf-9* locus in a nitrate reductase-deficient background, than in inoculated MM-Cf9 plants. Microscopic analysis showed highly branched and slightly thickened hyphae around the vascular tissue in the apoplast of A29-Cf9 plants, which are characteristic for a compatible interaction. However, a fully compatible interaction was not expected, as additional resistance gene(s) have been reported to be present at the *Cf-9* locus (Parniske et al., 1997; Laugé et al., 1998).

To circumvent the influence of these additional resistance gene(s), experiments were carried out with transgenic MM-Cf0 tomato plants only containing the *Hcr9-9C* gene (the functional *Cf-9* gene), designated MM-Cf9C. Inoculation of MM-Cf9C plants, containing elevated levels of nitrate in the apoplast, with *C. fulvum* (carrying the *Avr*9 gene), however, did not result in more colonisation compared to A29-Cf9 plants.

Based on these results we conclude that *Avr*9 expression is not fully suppressed in plants containing elevated levels of nitrate in the apoplast. Nevertheless, partial growth of *C. fulvum* in the apoplast of the leaves of *Cf-9* genotypes indicates that during the first phase of pathogenesis the fungus is not recognised. Possibly, a minimal concentration of AVR9 elicitor is necessary for recognition and induction of a defence response. The partial growth of *C. fulvum* suggests a delayed defence response of the plant in a later phase of the infection. As a result of the increased fungal biomass, a sufficient amount of AVR9 is produced for recognition. If this is true, the continuous stress to host cells during the first phase of the infection would possibly lead to high levels of pathogenesis related (PR) protein accumulation as observed for partial resistance by Laugé et al. (1998).

Recently the *C. fulvum are*A homologue, designated *Nrf*1 for nitrogen response factor 1, has been cloned (Pérez-García et al., unpublished). However, *Nrf*1-disruptants were still avirulent on MM-Cf9C plants, indicating that AVR9 is still produced in these disruptants. This implies that *Avr*9 expression *in planta* is not fully dependent on the global nitrogen regulatory AREA-like protein. Further research is required to determine whether other (host) factor(s) are able to induce *Avr*9 expression *in planta*, causing low, but constitutive expression of *Avr*9.

Experimental procedures

Plant genotypes

Several genotypes, derived from the tomato (*Lycopersicon esculentum* Mill.) cultivar Moneymaker (MM-Cf0) were used in this study. MM-Cf9 is a near-isogenic line (NIL) of MM-Cf0, containing an introgression segment of *L. pimpinellifolium*, PI126933, carrying the actual *Cf-9* resistance gene (*Hcr9-9C*), in addition to four *Cf-9*-homologous genes (*Hcr9-9A*, *-9B*, *-9D* and *-9E*). MM-Cf5 is a NIL derived from MM-Cf0, containing an introgression segment of *L. esculentum* var. *cerasiforme*, PI187002, carrying the *Cf-5* gene (Stevens and Rick, 1988).

A29 is a nitrate reductase-deficient mutant obtained by mutagenizing seeds of MM-Cf0 with ethylmethanesulphonate (EMS) (Schoenmakers et al., 1991; seeds were kindly provided by M. Koornneef). The mutation is monogenic and recessive and nitrate reductase deficiency probably results from the inability of the enzyme to bind the molybdenum cofactor (MoCo) necessary for nitrate reductase activity. A29-Cf9 plants are the result of a cross between A29 and MM-Cf9 plants; they are nitrate reductase-deficient and contain the five *Hcr9-9*s. Selection for the A29 phenotype was performed visually (A29 plants grow slower and have a chlorotic appearance) and by determination of the nitrate concentration present in apoplastic fluid (AF) isolated from the leaves (De Wit and Spikman, 1982). The presence of the *Cf-9* resistance gene in the A29 background was confirmed by injection of AF containing AVR9 and subsequent scoring for a hypersensitive response (HR) visible as necrosis two days after injection. Seeds from transgenic MM-Cf0 plants, containing the functional *Cf-9* gene (*Hcr9-9C*; designated MM-Cf9C plants), were kindly provided by Dr. M. Parniske (The Sainsbury Laboratory, John Innes Centre, Norwich, England).

In experiments where plants were supplied with nitrate, daily approximately 100ml of 100mM KNO_3 were added to the soil from ten days before inoculation, till the end of the inoculation experiment.

Fungal strains and plant inoculations

Two *C. fulvum* transformants were used. One (transformant P*Avr9-uid*A) was obtained by transforming *C. fulvum* race 5 (containing the *Avr*9 gene) with the pCF24 plasmid containing 800 bp of the *Avr*9 promoter fragment fused to the *uid*A (GUS) reporter gene (coding for ß-glucuronidase; Van den Ackerveken et al., 1994). The other transformant (P*gpd-uid*A) was obtained by introducing the pNOM12 plasmid, containing the constitutively-expressed *A. nidulans gpd* promoter fused to the *uid*A gene, into *C. fulvum* race 4 (containing both the *Avr*5 and *Avr*9 gene; Van den Ackerveken et al., 1994). Culture conditions for *C. fulvum*, as well as the plant inoculation procedure were as described

by De Wit (1977). The development of symptoms was followed in time and representative photographs were taken twenty days after inoculation.

Visualisation of fungal growth *in planta*

Growth of the *C. fulvum* transformants *in planta* was visualised by GUS-staining. Leaf disks were infiltrated *in vacuo* with a solution of 0.2 mg ml^{-1} X-Gluc (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide, Duchefa) in 50 mM sodium phosphate buffer (pH 7.0), containing 1 mM potassium ferri/ferrocyanide and 0.05% (v/v) Triton X-100 (Jefferson, 1987). After infiltration leaf disks were incubated at 37°C in the dark and subsequently cleared by successive washes in 70% ethanol and finally analysed by light microscopy, using a Nikon UFX-II microscope.

Isolation and analysis of apoplastic fluids

Apoplastic fluids (AFs) were isolated at the day of inoculation and twenty days later. From each plant, approximately 10 leaflets were randomly chosen and AF was obtained by *in vacuo* water infiltration, followed by centrifugation at 2,000 x g according to De Wit and Spikman (1982). For quantification of the extracellular *C. fulvum* protein ECP2, ELISA was performed in Nunc-Immuno Plate Maxisorp plates (Nunc, Roskilde, Denmark), using polyclonal antibodies raised against ECP2 in rabbits (Laugé et al., 1997). Optical density was determined at 405 nm, employing an EL312 microplate reader (Bio-Tek, Winooski, VT).

Nitrate concentrations were determined following the method described by Cataldo et al. (1975), with minor modifications. To 200 µl of a two-fold dilution of AF in distilled water, 800 µl of 5% (w/v) salicylic acid in 96% (w/v) sulphuric acid was added in a 25 ml glass tube. The mixture was incubated for 20 minutes at room temperature (RT), and subsequently 19 ml of 2M NaOH was slowly added. After incubation for 30 minutes at RT, absorbance was measured against the blank (1 ml of distilled water) at 410 nm. Nitrate concentration was calculated using a standard curve obtained with a concentration series of KNO_3.

Acknowledgements: S. S. Snoeijers was supported by a grant from the Dutch Earth and Life Science Foundation (ALW; Projectnumber: 805.45.006), which is subsidised by the Netherlands Organisation for Scientific Research (NWO).

Chapter seven

General discussion

Introduction

Plants are the largest and most important nutritional source for prokaryotic and eukaryotic organisms. Like other organisms, plants are continually exposed to the threat of diseases caused by viruses, bacteria, fungi, nematodes and insects. One possibility to reduce diseases caused by pathogens and pests is through the use of selective agrochemicals. A big disadvantage of using such compounds is their toxicity towards and persistence in the environment.

An alternative to reduce and control plant diseases is to exploit natural resistance of plants. Plants carry resistance genes that can be introgressed into crop plants by classical breeding or introduced using by tools. To obtain the full benefit of molecular resistance breeding, a detailed understanding of the molecular communication between plants and their pathogens is required.

The interaction between the fungal pathogen *Cladosporium fulvum* (syn *Fulvia fulva*), the causal agent of leaf mould disease, and its only host tomato is used as a model system to study avirulence and resistance in *C. fulvum* and tomato, respectively. The fungus only colonises the extracellular spaces of tomato leaves, allowing the identification of all proteins that are involved in a compatible plant-pathogen interaction, by isolation of apoplastic fluid (AF) of *C. fulvum*-infected leaves. One compound that causes avirulence of *C. fulvum* is the peptide AVR9, which is a specific elicitor encoded by avirulence gene *Avr9*. The peptide is secreted into the intercellular space and is specifically recognised by tomato plants carrying the matching resistance gene *Cf-9*.

To increase our understanding of the molecular basis of the *Avr9* function and regulation, we set out to investigate in detail the expression of this gene both *in vitro* and *in planta*. Prior to the study described in this thesis, it had already been observed that *Avr9* is

strongly expressed during growth of the fungus *in planta* and under nitrogen-limiting conditions *in vitro*.

Avirulence genes and their role in pathogenicity

Although there was no understanding of the molecular basis of host resistance and pathogen avirulence, at that time, the occurrence of genotype/cultivar-specific resistance of plants to fungal pathogens were already described a century ago. In 1905, Biffen proved that resistance of wheat to yellow rust was genetically determined and followed Mendel's laws of inheritance (Biffen, 1905). However, the first major breakthrough came in the nineteen-forties when Flor provided the genetic basis for the outcome of an interaction between host and pathogen (Flor, 1946), which later became known as the gene-for-gene hypothesis. He demonstrated that both resistance in flax to the fungal pathogen *Melampsora lini*, the causal agent of flax rust, and avirulence of the fungus, inherited as dominant monogenic factors. Since this theory was proposed, many scientists have struggled to find a biochemical and molecular basis for this theory. Recombinant DNA-techniques made it possible to prove this hypothesis at the molecular level. The evidence came in the nineteen-eighties, with the cloning of the first avirulence (*Avr*) gene from the bacterium *Pseudomonas syringae* pv. *glycinea*, a pathogen of soybean, by Staskawicz et al. (1984). Although many gene-for-gene systems have been described in genetic terms, very few biochemical mechanisms have been developed so far.

If the host plant carries matching resistance (*R*) genes, the products of *Avr* genes (which are termed elicitors) are recognised by the host, which will subsequently mount a defence reaction which is known as the hypersensitive response (HR). HR involves genetically programmed, local death of a few plant cells at the site of invasion. This restricts further growth of the pathogen in the plant and thereby inhibits any further infection. Other defence responses include the production of reactive oxygen species, release of hydrolytic enzymes, the production of phytoalexins, cell wall modification, and accumulation of pathogenesis-related (PR) proteins. Although the general principle is understood, there are still many questions remaining concerning elicitor perception and subsequent induction of defence responses. In addition, little is known about the intrinsic function of avirulence genes for the pathogen and factors controlling these genes.

The puzzling question is: why would pathogens expressing dominant genes which allow them to be recognised and repressed by their hosts? Pathogens are highly adapted micro-organisms, with survival strategies that usually require multiplication on, or within,

another living organism. The apparent need of the pathogen to possess *Avr* genes, even when its product is recognised by individual resistant host plants, suggests an important intrinsic function for these genes during pathogenesis.

One current theory is that avirulence gene products are required for growth of the pathogen in the host. According to this hypothesis, avirulence genes might encode a "survival protein". Such a protein could possibly provide the pathogen with nutrients while growing in the host. This theory is supported by the observation that expression of various bacterial and fungal (a)virulence/pathogenicity genes is highly induced during nutrient- (nitrogen-) starvation *in vitro*, suggesting that these conditions might mimic the environment in the host (reviewed in **Chapter one**). The abundant production of "survival proteins" by the pathogen, will trigger the surveillance system of the host and as a result of selection pressure on the host, HR-based resistance will develop by recognising one or more of these particular proteins. According to this theory, a virulence/pathogenicity gene, encoding such a protein could become an *Avr* gene as soon as its product is recognised by the host (Leach and White, 1996; Joosten and De Wit, 1999; Laugé et al., 2000). When this hypothesis is true, individual HR-based resistance against these "survival proteins" would be expected to be present in natural host populations.

Only from a few phytopathogenic bacterial and fungal avirulence genes their importance in pathogenesis has been established. One such a gene is the *avr*Bs2 gene from the phytopathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*), causing leaf spot on pepper. Studies by Kearny and Staskawicz (1990) and Swords et al. (1996) have shown that *avr*Bs2 mutant strains induce disease symptoms on susceptible pepper plants that are significantly reduced compared to *Xcv* strains that contain the wild-type *avr*Bs2 gene. The structure of *avr*Bs2 shows homology to agrocinopine synthase genes of *Agrobacterium tumefaciens*, and probably provides the bacterium with a carbon and nitrogen source during colonisation of its host plant.

One fungal *Avr* gene with a function in pathogenicity is the *nip*1 gene of the barley pathogen *Rhynchosporium secalis*. This gene encodes a specific elicitor (NIP1) that induces accumulation of pathogenesis-related (PR) proteins in barley cultivars carrying the matching resistance gene *Rrs*1. *R. secalis nip*1 mutant strains are less pathogenic on barley plants than wild-type strains that do produce the NIP1 protein (Rohe et al., 1995; Knogge, 1996).

For *Avr* gene *Avr-Pita* (formerly called *Avr2-YAMO*) of the rice blast fungus *Magnaporthe grisea*, a possible role in pathogenesis has been claimed. Mutational analysis and sequencing of virulent and avirulent *Avr-Pita* alleles indicated that the conservation of a

putative protease motif in the encoded protein is essential for avirulence (Bryan et al., 1999). The deduced amino acid sequence of the AVR-Pita protein has 29% identity to NpII, a neutral zinc metalloprotease from *Aspergillus oryzae* (Tatsumi et al., 1991). However, biochemical evidence for metalloprotease activity is lacking.

Pathogenicity and virulence of *C. fulvum*

As mentioned earlier, *C. fulvum* only colonises the extracellular spaces of tomato leaves, allowing the identification of fungal proteins that are secreted into the apoplast, by isolation of apoplastic fluid (AF) of *C. fulvum*-infected leaves (De Wit and Spikman, 1982).

Two extracellular proteins ECP1 (for extracellular protein 1) and ECP2 (Van den Ackerveken et al., 1993) are abundantly secreted by the fungus during infection of tomato. Both proteins, produced by all *C. fulvum* strains that have been analysed so far, seem to play an essential role during pathogenesis. *Ecp*1- and *Ecp*2-deficient strains were still able to cause disease, however, they were blocked in a later stage of the disease development (Laugé et al., 1997).

Three more *C. fulvum* extracellular proteins have been isolated so far (ECP3, ECP4 and ECP5; Laugé et al., 2000). All ECPs seem to be unique with no homology to other proteins.

Avirulence of *C. fulvum* and resistance of tomato

From *C. fulvum*, the race-specific avirulence genes *Avr*2, *Avr*4 and *Avr*9 (Takken et al., unpublished; Joosten et al., 1994; Van Kan et al., 1991; Van den Ackerveken et al., 1992) have been cloned. Natural *C. fulvum* mutant strains or transformants not producing the AVRs did not show reduced virulence under greenhouse conditions when compared to wild-type strains, implicating that these peptides are not required for full virulence (Marmeisse et al., 1993).

Many *R* genes, conferring resistance against different types of pathogens, have been cloned from various plant species (Hammond-Kosack and Jones, 1997; De Wit, 1997). The homology between *R* genes in many unrelated plant species suggests the existence of a general surveillance system in plants, providing protection against putative pathogens. From tomato, four *R* genes, *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9*, conferring resistance to *C. fulvum* strains carrying the *Avr*2, *Avr*4, *Avr*5 and *Avr*9 genes, respectively, have been cloned (Jones et al.,

1994; Dixon et al., 1996; Thomas et al., 1997). All four *Cf* gene products share extensive structure and sequence similarities. The structure of the loci suggests that sequence duplication, gene recombination, gene conversion and diversifying selection has occurred in natural populations of *Lycopersicon* species. The various events, result in new recognitional specificities of fungal factors (Parniske et al., 1997). This is confirmed by the observation that, using a functional screen for wild tomato species, a resistance gene has been detected for every *C. fulvum* ECP (Laugé et al., 1999; Laugé et al., 2000), indicating that virulence factors can become avirulence factors once mounting an HR in a particular genotype.

Regulation of the expression of the *C. fulvum Avr9* gene

The mechanisms by which the expression of most bacterial and fungal avirulence genes is regulated, are still largely unknown. To obtain a better insight in the intrinsic role of avirulence genes in the pathogenic process, it is necessary to understand the regulation and regulators of these genes. We set out to study the regulation of the *Avr9* gene (Van Kan et al., 1991; Van den Ackerveken et al., 1992).

Van den Ackerveken et al. (1994) observed that specific TAGATA sequences, which have been described in the filamentous fungi *A. nidulans* and *N. crassa* as binding sites for a positively acting major global nitrogen regulator (named AREA and NIT2, respectively), are present in the *Avr9* promoter. In addition, Van den Ackerveken et al. (1994) found that *Avr9* expression is specifically induced *in vitro* under conditions of nitrogen starvation. Combining both observations made it tempting to speculate that (i) the *Avr9* gene is regulated by a nitrogen factor that is similar to AREA and NIT2, implying that the TAGATA sequences are important for *Avr9* regulation and that (ii) *in planta* nitrogen limitation also induces *Avr9* expression.

As there are *are*A mutants (lacking the AREA protein) and gene-targeting systems available for *A. nidulans*, initially this fungus was used as a model system to test whether the *A. nidulans* AREA protein is able to regulate *Avr9* expression. Experiments with single copy transgenic *A. nidulans*, containing the *Avr9* promoter fused to the GUS-reporter gene, integrated at the *arg*B locus, revealed that *Avr9* promoter activity is indeed induced in *A. nidulans*, following nitrogen starvation. This result suggests that nitrogen regulation in both *A. nidulans* and *C. fulvum* is based on the same mechanism. The observation that, after nitrogen starvation, no *Avr9* promoter activity was induced in *A. nidulans are*A null mutants,

indicated that the *Avr*9 promoter is fully induced by the *A. nidulans* AREA protein (described in **Chapter two**).

Disruption of the *C. fulvum areA/nit*-2-like gene, designated *Nrf*1, had a drastic effect on *Avr*9 expression *in planta* and completely abolished *Avr*9 expression *in vitro*, indicating that NRF1 is a major positive regulatory factor of the *Avr*9 gene. However, the observation that there is still, although reduced, *Avr*9 expression in *Nrf*1-disruptants when colonising susceptible tomato plants, suggests that there is an additional (host)factor, which is able to induce *Avr*9 expression *in planta* (described in **Chapter five**). The low *Avr*9 expression of the *Nrf*1-disruptants, however, still caused avirulence of the disruptants on *Cf-9* plants, suggesting that the AVR9 peptide is very stable and accumulates to sufficient levels in plants, even when influenced by strains that express *Avr*9 at very low levels.

Interestingly, the virulence of the *Nrf*1-disruptants was not reduced compared to wild-type *C. fulvum*, when inoculated onto susceptible tomato plants, implying that factors which are required for virulence are not under control of this global nitrogen regulator. However, preliminary experiments have shown that expression of *Ecp*5, which also contains AREA/NIT2-binding sites in the promoter, is regulated by the NRF1 factor *in planta* (unpublished data).

M. grisea and *C. fulvum* differ from each other in their mode of infection. *M. grisea* forms appressoria, penetrating into the cells encountering a different environment in comparison with *C. fulvum* that is strictly confined to the apoplastic space. Infection studies revealed that pathogenicity was not affected in *M. grisea nut*1-disruptants, where *nut*1 is the *areA/nit*-2-like gene of *M. grisea* (Froeliger and Carpenter, 1996). However, infection was highly reduced in *M. grisea npr*1- and *npr*2-mutants. Both *npr*1 and *npr*2, which are unlinked genes, are thought to encode a major nitrogen regulator, suggesting that pathogenesis and nitrogen metabolic pathways have overlapping regulation circuits (Lau and Hamer, 1996). Unfortunately, DNA sequence information on *npr*1 and *npr*2 is still lacking. It would be interesting to know whether *npr*1 and *npr*2 homologous genes are present in *C. fulvum*, and whether disruption of those genes in *C. fulvum* causes simultaneous loss or down regulation of multiple (a)virulence/pathogenicity genes, including *Avr*9.

Deletion studies on the putative binding domains present in the *Avr*9 promoter, revealed that these are important for *Avr*9 promoter activity in *A. nidulans* and *C. fulvum* (described in **Chapter two**). Introduction of specific point mutations in the TAGATA sequences showed that two TAGATA-boxes, most proximal to the start codon, both containing two invertedly orientated TAGATA sequences, are crucial for the inducibility of

*Avr*9 promoter activity in *A. nidulans*. (described in **Chapter three**). The mutated *Avr*9 promoter sequences, which did not give any activity in *A. nidulans*, were fused to the coding sequence of *Avr*9 and transformed to *C. fulvum* strains which lack the *Avr*9 gene. However, *C. fulvum* transformants carrying *Avr*9 promoter mutant constructs are still recognised by tomato plants only carrying the functional *Cf-9* gene, indicating that there is still sufficient AVR9 produced by these transformants (described in **Chapter three**). However, the *Avr*9 gene was also induced when the transformants were grown in rich, liquid media, a condition which normally suppresses *Avr*9 gene expression. It could be that, although we lack Southern data, multiple integrations have caused the loss of nitrogen-dependent *Avr*9 regulation both *in vitro* and *in planta*.

To determine subtle differences in the activity of different *Avr*9 promoter sequences, without strong effects of the chromosomal environment on expression, a gene-targeting system was developed to target the integration of mutated *Avr*9 promoters of interest to a specific location in the genome of *C. fulvum*. As there are *C. fulvum pyr*1 mutants available (Marmeisse et al., 1993), we isolated the *C. fulvum pyr*1 gene, which is coding for the enzyme orotidine-5'-monophosphate decarboxylase (OMPdecase). OMPdecase is involved in the pyrimidine biosynthetic pathway and is considered to be a versatile selection marker for filamentous fungi (Ballance et al., 1983; Cullen and Leong, 1986; Diez et al., 1987; Goosen et al., 1987). In **Chapter four** we have demonstrated that targeting, based on site-directed integration at the *C. fulvum pyr*1 locus, is possible. For future research, this system can be exploited to study expression of wild-type and mutant genes of *C. fulvum*.

For *A. nidulans* and *N. crassa* the accepted model for nitrogen-dependent gene regulation is that a negative regulator, designated NMR, complexes with AREA or NIT2, respectively, in the presence of sufficient primary nitrogen sources. NMR interacts both with residues in the DNA-binding domain and in the carboxyl terminus of AREA and NIT2. In this way NMR prevents AREA/NIT2 binding to DNA (Xiao et al., 1995; Platt et al., 1996). Recently the putative *nmr* gene from *C. fulvum* has been isolated; amino acid sequence analysis of the encoded protein showed a high homology with both the NMR proteins from *A. nidulans* and *N. crassa* (A. Pérez-García et al., unpublished). Additional experiments can now be performed to prove whether NMR is also a negative regulator in *C. fulvum* and if so, whether it is able to influence virulence.

Speculations about plant-pathogen systems and future research

The *C. fulvum* AVR and ECP proteins described so far, are divergent in composition, although, they do share certain characteristics, such as relatively low molecular weight, an extracellular location and an even number of cysteine residues.

Interestingly, the AVRs and ECPs show structural homologies to the exogenous proteins of the S receptor complex, which are involved in recognition and rejection of self pollen in *Brassica* stigmas (Schopfer et al., 1999). It is hypothesised that, in the case of self-pollination, these proteins act as a stable, exogenous signal that, upon perception by a matching S receptor kinase present in the S receptor complex, initiates a signalling pathway in the stigma eventually leading to pollen rejection. This phenomenon has similarities to the incompatibility resulting from perception of pathogen-produced elicitors in the *C. fulvum*-tomato interaction.

Expression of all *in planta*-induced genes coding for *C. fulvum* AVRs and ECPs can also be induced *in vitro* under conditions of nutrient depletion (Laugé et al., 2000; Takken et al., unpublished), suggesting that an EST-library constructed from mycelium grown under these conditions might contain various *in planta* expressed genes of *C. fulvum* that have not been identified up till now (Coleman et al., 1997). One can speculate that some of these proteins are involved in providing the fungus with the appropriate nutrients. Hypothetically, these "nutrient survival proteins" are produced abundantly and are stable, and as a consequence, recognitional specificities have evolved towards these proteins.

*Avr*9 expression is specifically induced *in vitro* under nitrogen starvation, suggesting that the AVR9 peptide might have a role in providing the fungus with sufficient nitrogen. This is supported by both the observation that *Avr*9 expression is under control of the *C. fulvum* major nitrogen regulator NRF1 (described in **Chapter five**) and that *Avr*9 expression is repressed when increased levels of nitrate are present in the apoplast, where the fungus resides (described in **Chapter six**).

Nevertheless, both the AVR9 peptide (Marmeisse et al., 1993) and the NRF1 nitrogen regulatory protein (described in **Chapter five**) are not required for full virulence. However, it has to be emphasised that infection studies were performed under greenhouse conditions, which are considered optimal for the host. For reliable virulence and fitness comparisons between wild-type and mutant strains of *C. fulvum*, both should be tested in wild tomato species growing in their natural environment. Alternatively, AVR9 (and possibly ECP5) could be a secondary component derived from a global nitrogen utilisation circuit,

simultaneously controlled by NRF1. If the AVR9 peptide would not be beneficial for the fungus it is still hard to understand why such high expression levels are obtained during colonisation of susceptible tomato plants (described in **Chapter five**). How could *Avr*9 survive strong selection pressure when the AVR9 peptide does not have a substantial function during growth of *C. fulvum in planta*? Possibly, in natural *Avr*9 mutant strains an additional redundant "survival protein", with an AVR9-like function, can complement the *Avr*9 gene.

For bacterial pathogens of mammals it is known that a single DNA region can convert the micro-organism into a pathogen. For example, the determinants responsible for invasion and intercellular spreading of *Shigella flexneri* are encoded within a large virulence plasmid, called a pathogenicity island (Parsot, 1994). Transfer of this plasmid to a laboratory strain of *E. coli* renders it invasive (Sansonetti et al., 1982). Similarly, a 35 kb region termed the locus of enterocyte effacement (LEE) in enteropathogenic *E. coli*, mediates the production of attachment and effacing lesions of intestinal epithelial cells (McDaniel et al., 1995). This phenotype can be reproduced in a laboratory strain of *E. coli* upon introduction of a plasmid carrying LEE (McDaniel and Kaper, 1997), suggesting that a nonpathogenic *E. coli*, which is a normal member of the human intestinal flora, harbors many of the genes necessary for interaction with human cells. Thus, they can easily become pathogens upon acquisition of a particular virulence gene cluster. In further support of this notion, many of the genes implicated in *Salmonella* virulence are also present in nonpathogenic strains of *E. coli*. These genes encode enzymes responsible for the biosynthesis of nutrients that are scarce within host tissues, transcriptional and post-transcriptional regulatory factors, proteins necessary for the repair of damaged DNA, and products necessary for defence against host microbicidal mechanisms. The presence of these genes in nonpathogenic species suggests that they promote survival within nutritionally deprived and/or potentially lethal environments that these micro organisms encounter inside and outside mammalian hosts.

Similarly, in nonpathogenic fungi genes could be present, which are homologous to avirulence genes of phytopathogenic fungi, and play a role as "survival genes" under nutrient-limiting conditions in phytopathogenic fungi. Based on this hypothesis, *Ecp* or *Avr* homologues could exist in, for example, *A. nidulans* and *N. crassa*. In this respect, the completion of the genome sequences of *A. nidulans* and *N. crassa* might provide a new impuls in the study of avirulence gene(s).

Both the isolation of a gene that is encoding a "survival protein" that is conserved among a broad range of (phytopathogenic) fungi and its matching resistance gene, would be a challenge for future research in molecular phytopathology. The matching resistance gene

could facilitate development of durable resistance against a broad range of phytopathogenic fungi, as these can not escape recognition without losing pathogenic abilities.

Literature cited

Agrios, GN (1997) Plant Pathology. Academic Press, San Diego (USA)

Ahman J, Ek B, Rask L and Tunlid A (1996) Sequence analysis and regulation of a gene encoding a cuticle-degrading serine protease from the nematophagous fungus *Arthrobotrys oligospora*. Microbiology 142:1605-1616

Albright LM, Huala E and Ausubel FM (1989) Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu Rev Genetic 23:311-336

Anderson M, Pollitt CE, Roberts IS and Eastgate JA (1998) Identification and characterization of the *Erwinia amylovora rpoS* gene: RpoS is not involved in induction of fireblight disease symptoms. J Bacteriol 24:6789-6792

Andrianopoulos A, Kourambas S, Sharp JA, Davis MA and Hynes MJ (1998) Characterization of the *Aspergillus nidulans nmrA* gene involved in nitrogen metabolite repression. J Bacteriol 180:1973-1977

Arst HN. Jr, Tollervey D and Caddick MXA (1989) Translocation associated, loss-of-function mutation in the nitrogen metabolite repression regulatory gene of *Aspergillus nidulans* can revert intracistronically. Mol Gen Genet 215:364-367

Balestra GM and Varvaro L (1997) Influence of nitrogen fertilization on the colonization of olive phylloplane by *Pseudomonas syringae* subsp.*savastanoi*. In: *Pseudomonas syringae* Pathovars and Related Pathogens. (Rudolph K, Burr TJ, Mansfield JW, Stead D, Vivian A and von Kietzell J, eds). pp 88-92, Kluwer Academic Publishers, Dordrecht (The Netherlands)

Ballance DJ, Buxton FP and Turner G (1983) Transformation of *Aspergillus nidulans* by the orotidine-5'-phosphate decarboxylase gene of *Neurospora crassa*. Biochem biophys res Commun 112:284-289

Bender CL, Alarcon-Chaidez F and Gross DC (1999) *Pseudomonas syringae* phytotoxins: mode of action, regulation, and biosynthesis by peptide and polyketide synthetases. Microbiol Mol Biol Rev 63:266-292

Beri RK and Turner G (1987) Transformation of *Penicillium chrysogenum* using the *Aspergillus nidulans amdS* gene as a dominant selective marker. Curr Genet 11:359-365

Biffen RH (1905) Mendel's laws of inheritance and wheat breeding. J Agric Sci 1:4-48

Bogdanove AJ, Bauer DW and Beer SV (1998) *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional *AvrE* homolog, through the Hrp (type III secretion) pathway. J Bacteriol 180:2244-2247

Bonas U (1994) *Hrp* genes of phytopathogenic bacteria. Curr Top Microbiol Immunol 192:79-98

Bonas U and Van den Ackerveken G (1997) Recognition of bacterial avirulence proteins occurs inside the plant cell: a general phenomenon in resistance to bacterial diseases? Plant J 12:1-7

Bowyer P, Osbourn AE and Daniels MJ (1994) An "instant gene bank" method for heterologous gene cloning: complementation of two *Aspergillus nidulans* mutants with *Gaeumannomyces graminis* DNA. Mol Gen Genet 242:448-454

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Bryan GT, Farall L, Kang S, Morgan-Hoffman SA and Valent B (1999) The *Magnaporthe grisea* avirulence gene *Avr-Pita* may encode a secreted metalloprotease. Unpublished data in Biology of Plant-Microbe interactions (Vol. 2), Molecular characterization of resistance gene/avirulence gene interactions in the rice blast system (De Wit PJGM, Bisseling T, Stiekema WJ, eds). pp 35-39

Bull JH, Smith DJ and Turner G (1988) Transformation of *Penicillium chrysogenum* with a dominant selectable marker. Curr Genet 13:377-382

Burger G, Strauss J, Scazzocchio C and Lang B (1991a) *nirA*, the pathway-specific regulatory gene of nitrate assimilation in *Aspergillus nidulans*, encodes a putative GAL4-type zinc finger protein and contains introns in highly conserved regions. Mol Cell Biol 11:5746-5755

Burger G, Tilburn J and Scazzocchio C (1991b) Molecular cloning and functional characterisation of the pathway-specific regulatory gene *nir*A, which controls nitrate assimilation in *Aspergillus nidulans*. Mol Cell Biol 11:795-802

Buxton FP, Gwynne DI and Davies RW (1985) Transformation of *Aspergillus niger* using the *arg*B gene of *Aspergillus nidulans*. Gene 37:207-214

Caddick MX, Peters P and Platt A (1994) Nitrogen regulation in fungi. Antonie van Leeuwenhoek 65:169-177

Campbell EI, Unkless SE, Macro JA, Van den Hondel C, Contreras R and Kinghorn JR (1989) Improved transformation efficiency of *Aspergillus niger* using the homologous *nia*D gene for nitrate reductase. Curr Genet 16:53-56

Cataldo DA, Haroon M, Schrader LE and Youngs VL (1975) Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid. Commun Soil Sci Plant Anal 6:71-80

Chérif M and Boubaker A (1997) Incidence of cultural practices and biological control agents on Botrytis bunch rot of grapes. 10th Congress of the Mediterranean Phytopathological Union. Montpellier (France). pp 681-687

Chiang T, Rai R, Cooper TG, Marzluf GA (1994) DNA binding site specificity of the *Neurospora* global nitrogen protein NIT2: Analysis with mutated binding sites. Mol Gen Genet 245:512-516

Chiang T, Marzluf GA (1995) Binding affinity and functional significance of NIT2 and NIT4 binding sites in the promoter of the highly regulated *nit-3* gene, which encodes nitrate reductase in *Neurospora crassa*. J of Bacteriology 177:6093-6099

Christensen T, Hynes MJ and Davis MA (1998) Role of the regulatory gene *are*A of *Aspergillus oryzae* in nitrogen metabolism. Appl Environ Microbiol 64:3232-3237

Coleman M, Henricot B, Arnau J and Oliver RP (1997) Starvation-induced genes of the tomato pathogen *Cladosporium fulvum* are also induced during growth *in planta*. Mol Plant-Microbe Interact 9:1106-1109

Cove DJ (1979) Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol Rev 54:291-327

Crawford NM and Arst HN (1993) The molecular genetics of nitrate assimilation in fungi and plants. Annu Rev Genet 27:115-146

Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7: 859-868

Cullen D, Leong S (1986) Recent advances in the molecular genetics of industrial filamentous fungi. Trends Biotechnol 4:285-288

Daboussi MJ, Djeballi A, Gerlinger C, Bouvier I, Cassan M, Lebrun MH, Parisot D and Brygoo Y (1989) transformation of seven species of filamentous fungi using the nitrate reductase gene of *Aspergillus nidulans*. Curr Genet 15:453-456

Daly JM (1949) The influence of nitrogen source on the development of stem rust of wheat. Phytopathology 39:386-394

Dangl JL (1994) The enigmatic avirulence genes of phytopathogenic bacteria. In: Dangl (ed.) Bacterial Pathogenesis of Plants and Animals. pp 99-114, Springer-Verlag, Berlin (Germany)

Davis MA and Hynes MJ (1987) Complementation of *are*A-regulatory gene mutations of *Aspergillus nidulans* by the heterologous regulatory gene *nit-2* of *Neurospora crassa*. Proc Natl Acad Sci USA 84:3753-3757

Davis MA, Small AJ, Kourambas S and Hynes M J (1996) The *tam*A gene of *Aspergillus nidulans* contains a putative zinc finger cluster motif which is not required for gene function. J Bacteriol. 178:3406-3409

De Wit PJGM (1977) A light and scanning electron-microscopic study of infection of tomato plants by virulent and avirulent races of *Cladosporium fulvum*. Neth J Plant Pathol 83:109-122

De Wit PJGM and Spikman G (1982) Evidence for occurence of race- and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions between *Cladosporium fulvum* and tomato. Physiol Plant Pathol 21:1-11

De Wit PJGM, Buurlage MB and Hammond KE (1986) The occurence of host, pathogen and interaction-specific proteins in the apoplast of *Cladosporium fulvum* (syn. *Fulvia fulva*) infected tomato leaves. Physiol Mol Plant Pathol 29:159-172

De Wit PJGM (1995) Fungal avirulence genes and plant resistance genes: unraveling the molecular basis of gene-for-gene interactions. Adv Bot Res 21:147-185

De Wit PJGM (1997) Pathogen avirulence and plant resistance: a key role for recognition. Trends Plants Sci 2:452-458

Diez B, Alvarez E, Cantoral JM, Barredo JL and Martin JF (1987) Selection and characterization of *pyr*G mutants of *Penicillium chrysogenum* lacking orotidine-5'-phosphate decarboxylase and complementation by the *pyr*4 gene of *Neurospora crassa*. Curr Genet 12:277-282

Dixon RA and Harrison MJ (1990) Activation, structure and organization of genes involved in microbial defense in plants. Adv Genet 28:166-234

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrison K and Jones JDG (1996) The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84:451-459

Durbin RD and Uchytil TF (1984) The role of intercellular fluid and bacterial isolate on the *in vivo* production of tabtoxin and tabtoxinine-β-lactam. Physiol Plant Pathol 24:25-31

Ellis CM (1996) Regulation of polyketide gene expression: the isolation and function of nitrogen regulatory factor NRFA from *Penicillium urticae*. Biological Sciences, University of Calgary (Canada)

Flor HH (1946) Genetics of pathogenicity in *Melampsora lini*. J Agric Res 73:3335-3357

Froeliger E and Carpenter B (1996) NUT1, a major nitrogen regulatory gene in *Magnaporthe grisea*, is dispensable for pathogenicity. Mol Gen Genet 25:647-656

Fu YH and Marzluf GA (1990) *nit*-2, the major positive-acting nitrogen regulatory gene of *Neurospora crassa*, encodes a sequence-specific DNA-binding protein. Proc Natl Acad Sci USA 87:5331-5335

Gallegly ME Jr and Walker JC (1949) Plant nutrition in relation to disease development. Am J Bot 36: 613-623

Gaudriault S, Malandrin L, Paulin JP and Barny MA (1997) DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of *Pseudomonas syringae*, is secreted via the Hrp secretion pathway in a DspB-dependent way. Mol Microbiol 26:1057-1069

Gems DH and Clutterbuck AJ (1993) Co-transformation with autonomously-replicating helper plasmids facilitates gene cloning from an *Aspergillus nidulans* gene library. Curr Genet 24:520-524

Gems D, Aleksenko A, Belenky L, Robertson S, Ramsden M, Vinetski Y and Clutterbuck AJ (1994) An 'instant gene bank' method for gene cloning by mutant complementation. Mol Gen Genet 242:467-471

Gente S, Poussereau N and Fevre M (1999) Isolation and expression of a nitrogen regulatory gene, *nmc*, of *Penicillium roqueforti*. FEMS Microbiol Lett 175:291-297

Goosen T, Bloemheuvel G, Gysler C, De Bie DA, Van den Broek HWJ and Swart K (1987) Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate decarboxylase gene. Curr Genet 11:499-503

Goosen T, Bos CJ and Van den Broek HWJ (1992) Transformation and gene manipulation in filamentous fungi: an overview. In handbook of Applied Mycology, vol 4, fungal biotechnology (eds Arora DK, Elander RP, Mukerji KG). pp 151-195, New York (USA)

Gouka RJ, Hessing JGM, Stam H, Musters W and Van den Hondel CAMJJ (1995) A novel strategy for the isolation of a site-specific integration system for *Aspergillus awamori*. Curr Genet 27:536-540

Groisman EA and Ochman H (1994) How *Salmonella* became a pathogen. Trends Microbiol 2:289-294

Haas H, Bauer B, Redl B, Stoffler G and Marzluf GA (1995) Molecular cloning and analysis of *nre*, the major nitrogen regulatory gene of *Penicillium chrysogenum*. Curr Genet 27:150-158

Haas, HB and Marzluf GA (1995) NRE, the major nitrogen regulatory protein of *Penicillium chrysogenum*, binds specifically to elements in the intergenic promoter regions of nitrate assimilation and penicillin biosynthetic gene clusters. Curr Genet. 28:177-183

Hahm YT and Bath CA (1988) Genetic transformation of an *arg*B mutant of *Aspergillus oryzae*. Appl Environ Microbiol 54:1610-1611

Hamer JE and Timberlake WE (1987) Functional organization of the *Aspergillus nidulans trp*C promoter. Mol Cell Biol 7:2352-2359

Hammond-Kosack KE and Jones JDG (1996) Resistance gene-dependent plant defense responses. Plant Cell 8: 1773-1791

Hammond-Kosack KE and Jones JDG (1997) Plant disease resistance genes. Annu Rev Plant Physiol Plant Mol Biol 48:573-607

Harling R, Kenyon L, Lewis BG, Oliver RP, Turner JG and Coddington A (1988) Conditions for efficient isolation and regeneration of protoplasts from *Fulvia fulva*. J Phytopathol 122:143-146

Heiskanen P, Taira S and Rhen M (1994) Role of *rpo*S in the regulation of *Salmonella* plasmid virulence (*spv*) genes. FEMS Microbiol Lett 123:125-130

Hensel M, Tang CM, Arst HN and Holden DW (1995) Regulation of fungal extracellular proteases and their role in mammalian pathogenesis. Can J Bot 73 (Suppl. 1): S1065-1070

Hensel M, Arst HN Jr, Aufauvre-Brown A and Holden DW (1998) The role of the *Aspergillus fumigatus are*A gene in invasive pulmonary aspergillosis. Mol Gen Genet 258:553-557

Horns T and Bonas U (1996) The *rpo*N gene of *Xanthomonas campestris* pv. *vesicatoria* is not required for pathogenicity. Mol Plant-Microbe Interact 9:856-859

Huber DM and Watson RD (1974) Nitrogen form and plant disease. Annu Rev Phytopathol 12:139-165

Huynh TV, Dahlbeck D and Staskawicz BJ (1989) Bacterial blight of soybean: regulation of a pathogen gene determining host cultivar specificity. Science 245:1374-1377

Innes RW, Bent AF, Kunkel BN, Bisgrove SR and Staskawicz BJ (1993) Molecular analysis of avirulence gene *avr*Rpt2 and identification of a putative regulatory sequence common to all known *Pseudomonas syringae* avirulence genes. J Bacteriol 175:4859-4869

Iriarte M, Stainier I and Cornelis GR (1995) The *rpo*S gene from *Yersinia enterocolitica* and its influence on expression of virulence factors. Infect Immun 63:1840-1847

Isaacs NW (1995) Cystine knots. Curr Opin Struct Biol 5:391-395

Jefferson RA, Kavanagh TA and Bevan MW (1987) GUS fusions: ß-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901-3907

Johnstone LL, McCabe PC, Greaves P, Gurr SJ, Cole GE, Brow MAD, Unkles SE, Clutterbuck AJ, Kinghorn JR and Innes MA (1990) Isolation and characterisation of the *crn*A-*nii*A-*nia*D gene cluster for nitrate assimilation in *Aspergillus nidulans*. Gene 90:181-192

Jones DA, Thomas CM, Hammond-Kosack, KE, Balint-Kurti PJ and Jones JDG (1994) Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science 266:789-793

Joosten MHAJ, Cozijnsen AJ and De Wit PJGM (1994) Host resistance to a fungal tomato pathogen lost by a single base-pair change in an avirulence gene. Nature 367:384-386

Joosten MHAJ, Honée G, Van Kan, JAL and De Wit PJGM (1997) In: The gene-for-gene concept in plant-pathogen interactions: tomato-*Cladosporium fulvum*. The Mycota V Part B Plant Relationships (Caroll/Tudzynski , eds). pp 3-16

Joosten MHAJ and De Wit PJGM (1999) The tomato-*Cladosporium fulvum* interaction: a versatile experimental system to study plant-pathogen interactions. Annu Rev Phytopathol 37:335-367

Kamoun S, Van West P, De Jong AJ, De Groot KE, Vleeshouwers VG and Govers F (1997) A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection in potato. Mol Plant-Microbe Interact 10:13-20

Kearny B and Staskawicz BJ (1990) Widespread distribution and fitness contribution of *Xanthomonas campestris* avirulence gene, *avr*Bs2. Nature 346:385-386

Keen NT, Tamaki S, Kobayashi D, Gerhold D, Stayton M, Shen H, Gold S, Lorang J, Thordal-Christensen H, Dahlbeck D and Staskawicz B (1990) Bacteria expressing avirulence gene *avr*D produce a specific elicitor of the soybean hypersensitive reaction. Mol Plant-Microbe Interact 3:112-121

Kelly JM and Hynes MJ (1985) Transformation of *Aspergillus niger* by the *amd*S gene of *Aspergillus nidulans*. EMBO J 4:475-479

Kershaw MJ and Talbot NJ (1998) Hydrophobins and repellents: proteins with fundamental roles in fungal morphogenesis. Fungal Genet Biol 23:18-33

Kinghorn JR and Pateman JA (1975) Studies of partially repressed mutants at the *tam*A and *are*A loci in *Aspergillus nidulans*. Mol Gen Genet 140:137-147

Knogge W (1996) Fungal infections of plants. Plant Cell 8:1711-1722.

Knoop V, Staskawicz B and Bonas U (1991) The expression of the avirulence gene *avrBs*3 from *Xhanthomonas campestris* pv. *vesicatoria* is not under control of *hrp* genes and is independent of plant factors. J Bacteriol 173:7142-7150

Kudla B, Caddick MX, Langdon T, Martinez-Rossi NM, Bennett CF, Sibley S, Davies RW and Arst HN (1990) The regulatory gene *are*A mediating nitrogen metabolite repression in *Aspergillus nidulans*. Mutations affecting specificity of gene activation alter a loop residue of a putative zinc-finger. EMBO J 9:1355-1364

Lam, HM, Coschigano K, Oliveira IC, Melo-Oliveira R and Coruzzi G (1996) The molecular-genetics of nitrogen assimilation into amino acids in higher plants. Annu Rev Plant Physiol Plant Mol Biol 47:569-593

Langdon T, Sheerins A, Ravagnani A, Gielkens M, Caddick MX and Arst HN (1995) Mutational analysis reveals dispensability of the N-terminal region of the *Aspergillus* transcription factor mediating nitrogen metabolite repression. Mol Microbiol 17:877-888

Lapwood, DH and Dyson PW (1966) An effect of nitrogen on the formation of potato tubers and the incidence of common scab (*Streptomyces scabies*). Plant Pathol 15:9-14

Last FT (1953) Some effects of temperature and nitrogen supply on wheat powdery mildew. Ann Appl Biol 40:312-322

Lau GW and Hamer JE (1996) Regulatory genes controlling *mpg*1 expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. Plant Cell 8:771-781

Laugé R, Joosten MHAJ, Van den Ackerveken GFJM, Van den Broek HWJ and De Wit PJGM (1997) The *in planta*-produced extracellular proteins ECP1 and ECP2 of *Cladosporium fulvum* are virulence factors. Mol Plant-Microbe Interact 10:725-734

Laugé R and De Wit PJGM (1998) Fungal avirulence genes: structure and possible functions. Fungal Genet Biol. 24:285-297

Laugé R, Dmitriev AP, Joosten MHAJ and De Wit PJGM (1998) Additional resistance gene(s) against *Cladosporium fulvum* present on the *Cf-9* introgression segment are associated with strong PR protein accumulation. Mol Plant-Microbe Interact 11:301-308

Laugé R, Joosten MHAJ, Haanstra JPW, Goodwin PH, Lindhout P and De Wit PJGM (1999) Succesful search for a resistance gene in tomato targeted against a virulence factor of a fungal pathogen. Proc Natl Acad Sci USA 95:9014-9018

Laugé R, Goodwin PH, Joosten MHAJ and De Wit PJGM (2000) Specific HR-associated recognition of secreted proteins from *Cladosporium fulvum* occurs in both host and non-host plants. Plant J (Accepted for publication)

Lea PJ (1992) Ammonia Assimilation in Higher Plants. In: Nitrogen Metabolism of Plants (Mengel K and Pilbeam DJ eds). pp 153-186, Oxord University Press, Oxford (UK)

Leach JE and White FF (1996) Bacterial avirulence genes. Annu Rev Phytopathol 34:153-179

Lindgren PB (1997) The role of *hrp* genes during plant-bacterial interactions. Annu Rev Phytopathol 35:129-152

Long SR and Staskawicz BJ (1993) Prokaryotic plant parasites. Cell 73:921-935

Lorang JM and Keen NT (1995) Characterization of *avr*E from *Pseudomonas syringae* pv. *tomato*: a *hrp*-linked avirulence locus consisting of at least two transcriptional units. Mol Plant-Microbe Interact 8:49-57

Lucas JA (1998) Plant Pathology and Plant Pathogens. Blackwell Science, Oxford (UK)

Magasanik B (1996) Regulation of gene expression in *Escherichia coli*. In: Regulation of Nitrogen Utilization (Lin ECC, and Lynch AS, eds). pp 1344-1356, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York (USA)

Malardier L, Daboussi MJ, Julien J, Roussel F, Scazzocchio C and Brygoo Y (1989) Cloning of the nitrate reductase gene (*nia*D) of *Aspergillus nidulans* and its use for transformation of *Fusarium oxysporum*. Gene 78:147-156

Marmeisse R, Van den Ackerveken GFJM, Goosen T, De Wit PJGM and Van den Broek HWJ (1993) Disruption of the avirulence gene *Avr9* in two races of the tomato pathogen *Cladosporium fulvum* causes virulence on tomato genotypes with the complementary resistance gene *Cf-9*. Mol Plant-Microbe Interact 6:412-417

Marzluf GA, Kanaan M, Fu YH (1992) Molecular analysis of DNA-binding, trans-acting regulatory proteins of *Neurospora crassa*. In: Molecular Biology of Filamentous Fungi (Stahl U and Tudzynski P, eds). pp 153-166, VCH, Weinheim (Germany)

Marzluf GA (1997) Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol Rev 61:17-32

McCabe AP, Vanhanen S, Gelpke MDS, Van de Vondervoort PJI, Arst HN and Visser J (1998) Identification, cloning and sequence of the *Aspergillus niger are*A wide domain regulatory gene controlling nitrogen utilisation. Biochem Biophys Acta 1396:163-168

McDaniel TK, Jarvis KG, Donnenberg MS and Kaper JB (1995) A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. Proc Natl Acad Sci USA 92:1664-1668

McDaniel TK and Kaper JB (1997) A cloned pathogenicity island from enteropathogenic *Escherichia coli* confers the attaching and effacing phenotype on *E. coli* K-12. Mol Microbiol 23:399-407

McElhaney R, Alvarez AM and Kado CI (1998) Nitrogen limits *Xanthomonas campestris* pv. *campestris* invasion of the host xylem. Physiol Mol Plant Pathol 52:15-24

McGuire RG, Jones JB, Stanley CD and Csizinsky AA (1991) Epiphyticpopulations of *Xanthomonas campestris* pv. *vesicatoria* and bacterial spot of tomato as influenced by nitrogen and potassium fertilization. Phytopathol 81:656-660

McNew GL and Spencer EL (1939) Effect of nitrogen supply of sweet corn on the wilt bacterium. Phytopathol 29:1051-1067

Mengaud J, Dramsi S, Gouin E, Vazquez-Boland JA, Milon G and Cossart P (1991) Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol Microbiol 5:2273-2283

Merrick MJ and Edwards RA (1995) Nitrogen control in bacteria. Microbiol Rev 59:604-622

Midland SL, Keen NT, Sims JJ, Midland MM, Stayton MM, Burton V, Smith MJ, Mazzola EP, Graham KJ and Clardy J (1993) The structures of syringolides 1 and 2, novel C-glycosidic elicitors from *Pseudomonas syringae* pv. *tomato*. J Org Chem. 58:2940-2945

Miller BL, Miller KY, Roberti KA and Timberlake WE (1987) Position dependent and -independent mechanisms regulate cell-specific expression of the *Spo*C1 gene cluster of *Aspergillus nidulans*. Mol Cell Biol 7:427-434

Mitchell RE (1976) Isolation and structure of chlorosis inducing toxin of *Pseudomonas phaseolicola*. Phytochemistry 15:1941-1947

Mitchell RE and Bielski RL (1977) Involvement of phaseolotoxin in halo blight of beans: transport and conversion to functional toxin. Plant Physiol 60:723-729

Moore RF, Niemezura WP, Kwok OCH and Patil SS (1984) Inhibitors of ornithine carbamoyltransferase from *P. syringae* pv. *phaseolicola*. Revised structure of phaseolotoxin. Tetrahedron Lett 25:3931-3934

Mylona P, Pawlowski K and Bisseling T (1995) Symbiotic nitrogen fixation. Plant Cell 7:869-885

Nickerson CA and Curtiss R 3rd (1997) Role of sigma factor RpoS in initial stages of *Salmonella typhimurium* infection. Infect Immun 65:1814-1823

Nickoloff JA and Reynolds RJ (1991) Subcloning with new ampicillin- and kanmycin-resistant analogs of pUC19. Biotechniques 10:469-472

Oliver RP, Roberts IN, Harling R, Kenyon L, Punt PJ, Dingemanse MA and Van den Hondel CAMJJ (1987) Transformation of *Fulvia fulva*, a fungal pathogen of tomato, to hygromycin B resistance. Curr Genet 12:231-233

Oliver RP (1992) A model system for the study of plant-fungal interactions: tomato leaf mold caused by *Cladosporium fulvum*. In: Molecular Signals in Plant-Microbe Communications (Verma, DPS, ed). p 97, CRC Press, Boca Raton (USA)

Oliver RP, Farman ML, Jones JDG and Hammond-Kosack KE (1993) Use of fungal transformants expressing ß-glucuronidase activity to detect infection and measure hyphal biomass in infected plant tissues. Mol Plant-Microbe Interact 6:521-525

O'Neal CR, Gabriel WM, Turk AK, Libby SJ, Fang FC and Spector MP (1994) RpoS is necessary for both the positive and negative regulation of starvation survival genes during phosphate, carbon, and nitrogen starvation in *Salmonella typhimurium*. J Bacteriol 176:4610-4616

Pallaghy PK, Nielsen KJ, Craik DJ and Norton RS (1994) A common structure motif incorporating a cystine knot and a triple-stranded beta sheet in toxic and inhibitory polypeptides. Protein Sci 3:1833-1839

Parniske M, Hammond-Kosack KE, Golstein C, Thomas CW, Jones DA, Harrison K, Wulff BBH and Jones JDG (1997) Novel disease resistance specificities result from sequence exchange between tandemly repeated genes at the *Cf-4/9* locus of tomato. Cell 91:821-832

Parson KA, Chumley FG, Valent B (1987) Genetic transformation of the fungal pathogen responsible for rice blast disease. Proc Natl Acad Sci USA 84:4161-4165

Parsot C (1994) *Shigella flexneri*: genetics of entry and intercellular dissemination in epithelial cells. Curr Top Microbiol Immunol. 192:217-241

Pentilla M, Nevalainen H, Ratto M, Salminen E and Knowles J (1987) A versatile transformation system for the cellulolytic filamentous fungus *Trichoderma reesei*. Gene 61:155-164

Pérez-García A, Cánovas FM, Gallardo F, Hirel B and De Vicente A (1995) Differential expression of glutamine synthetase isoforms in tomato detached leaflets infected with *Pseudomonas syringae* pv. *tomato*. Mol Plant-Microbe Interact 8:96-103

Pérez-García A, Pereira S, Pissarra J, García Gutiérrez A, Cazorla FM, Salema R, De Vicente A and Cánovas FM (1998) Cytosolic localization in tomato mesophyll cells of a novel glutamine synthtase induced in response to bacterial infection or phosphinothricin treatment. Planta 206:426-434

Pieterse CM, Derksen AM, Folders J and Govers F (1994) Expression of the putative pathogenicity genes *ipi*B and *ipi*O of *Phytophthora infestans in planta* and *in vitro*. Mol Gen Genet 244:269-277

Platt A, Langdon T, Arst HN Jr, Kirk D, Tollervey D, Mates Sanchez JM and Caddick MX (1996) Nitrogen metabolite signalling involves the C-terminus and the GATA domain of the *Aspergillus* transcription factor AREA and the 3' untranslated region of its mRNA. EMBO J 15:2791-2801

Pontecorvo G, Roper JA, Hemmonds, LM, McDonald K.D and Bufton AWJ (1953) The genetics of *A. nidulans*. Adv Genet 5:141-239.

Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH and Van den Hondel CAMJJ (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117-124

Punt PJ, Dingemanse MA, Kuyvenhoven A, Soede RD, Pouwels PH and Van den Hondel CA (1990) Functional elements in the promoter region of the *Aspergillus nidulans gpd*A gene encoding glyceraldehyde-3-phosphate dehydrogenase. Gene 93:101-109

Punt PJ, Greaves PA, Kuyvenhoven A, Van Deutekom JC, Kinghorn JR, Pouwels PH and Van den Hondel CAMJJ (1991) A twin-reporter vector simultaneous analysis of expression signals of divergently transcribed, contiguous genes in filamentous fungi. Gene 104:119-122

Punt PJ, Strauss J, Smit R, Kinghorn JR, Van den Hondel CAMJJ and Scazzocchio C (1995) The intergenic region between divergently transcribed *nii*A and *nia*D genes of *Aspergillus nidulans* contains multiple NirA binding sites which act bidirectionally. Mol Cell Biol 15:5688-5699

Rhame LG, Mindrinos MN and Panopoulos NJ (1992) Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolica*. J Bacteriol 174:3499-3507

Roberts IN, Oliver RP, Punt PJ and Van den Hondel CAMJJ (1989) Expression of the *Escherichia coli* ß-glucuronidase gene in industrial and phytopathogenic filamentous fungi. Curr Genet 15:177-180

Rohe M, Gierlich A, Hermann A, Hahn M, Schmidt B, Rosahl S and Knogge W (1995) The race-specific elicitor, NIP1, from the barley pathogen, *Rhynchosporium secalis*, determines avirulence on host plants of the *Rrs*1 resistance genotype. EMBO J 14:4168-4177

Rossier O, Wengelnik K, Hahn K and Bonas U (1999) The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens. Proc Natl Acad Sci USA 16:9368-9373

Salmeron JM and Staskawicz B J (1993) Molecular characterization and *hrp* dependence of the avirulence gene *avrPto* from *Pseudomonas syringae* pv. tomato. Mol Gen Genet 239:6-16

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (USA)

Sànchez-Fernàndez R, Unkles SE, Campbell EI, Macro JA, Cerdà-Olmedo E and Kinghorn JR (1991) Transformation of the filamentous fungus *Gibberella fujikuroi* using the *Aspergillus niger niaD* gene encoding nitrate reductase. Mol Gen Genet 225:231-233

Sansonetti PJ, Kopecko DJ and Formal SB (1982) Involvement of a plasmid in the invasive ability of *Shigella flexneri*. Infect Immun 35:852-860

Scazzocchio C (2000) The fungal GATA factors. Curr Opion in Microbiol 3:126-131

Scholtens-Toma IMJ and De Wit PJGM (1988) Purification and primary structure of a necrosis-inducing peptide from the apoplastic fluid of tomato infected with *Cladosporium fulvum* (syn. *Fulvia fulva*). Physiol Mol Plant Pathol 33:59-67

Schoenmakers HC, Koornneef M, Alefs SJ, Gerrits WF, Van der Kop D, Cherel I and Caboche, M (1991) Isolation and characterization of nitrate reductase-deficient mutants in tomato (*Lycopersicon esculentum* Mill.). Mol Gen Genet 227:458-464

Schopfer, CR, Nasrallah ME and Nasrallah JB (1999) The male determinant of self-incompatibility in *Brassica*. Science 286:1697-1700

Screen S, Bailey A, Charnley K, Cooper R and Clarkson J (1998) Isolation of a nitrogen response regulator gene (*nrr*1) from *Metarhizium anisopliae*. Gene 221:17-24

Segers GC, Hamada W, Oliver RP and Spanu PD (1999) Isolation and characterisation of five different hydrophobin-encoding cDNAs from the fungal tomato pathogen *Cladosporium fulvum*. Mol Gen Genet 261:644-652

Shen H and Keen NT (1993) Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. J Bacteriol 175:5916-5924

Shen H, Gold SE, Tamaki SJ and Keen NT (1992) Construction of a Tn7-lux system for gene expression studies in gram-negative bacteria. Gene 122:27-34

Showalter AM (1993) Structure and function of plant cell wall proteins. Plant Cell 5:9-23

Small AJ, Hynes MJ and Davis MA (1999) The TamA protein fused to a DNA-binding domain can recruit AREA, the major nitrogen regulatory protein, to activate gene expression in *Aspergillus nidulans*. Genetics 153:95-105

Smithson SL, Paterson IC, Bailey AM, Screen SE, Hunt BA, Cobb BD, Cooper RM, Charnley AK and Clarkson JM (1995) Cloning and characterisation of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. Gene 166:161-165

Snoeijers SS, Vossen P, Goosen T, Van den Broek HWJ and De Wit PJGM (1999) Transcription of the avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum* is regulated by a GATA-type transcription factor in *Aspergillus nidulans*. Mol Gen Genet 261:653-659

Spanu P and Whiteford J (2000) Fungal raincoats as dispersal aids. Abstract book (p 140), 5th European Conference on Fungal Genetics, Arcachon (France)

Spector MP (1998) The starvation-stress response (SSR) of *Salmonella*. Adv Microb Physiol 40:233-279

Staskawicz BJ, Dahlbeck D and Keen NT (1984) Cloned avirulence gene *Pseudomonas syringae* pv. *glycinea* determines race specific incompatibility on *Glycine max* (L) Merr. Proc Natl Acad Sci USA 81:6024-6028

Stephenson SA, Green JR, Manners JM and Maclean DJ (1997) Cloning and characterisation of glutamine synthetase from *Colletotrichum gloeosporioides* and demonstration of elevated expression during pathogenesis on *Stylosanthes guianensis*. Curr Genet 31:447-54

Stephenson, SA, Maclean DJ and Manners JM (1998) Disruption of the essential pathogenicity gene *CgDN*3 of *Colletotrichum gloeosporioides* results in a hypersensitive response in the host *Stylosanthes guianensis*. 7th International Congress of Plant Pathology. Edinburgh (Scotland). Abstract 1.8.6S

Stevens MA and Rick CM (1988) Genetics and breeding. In: The Tomato Crop (Roberts EH, ed.). pp 35-109, Chapman and Hall, London (UK)

St. Leger RJ, Frank DC, Roberts DW and Staples RC (1992) Molecular cloning and regulatory analysis of cuticle-degrading-protease structural gene from the entomopathogenic fungus *Metarhizium anisopliae*.
Eur J Biochem 204:991-1001

St. Leger RJ (1995) The role of cuticle-degrading proteases in fungal pathogenesis of insects. Can J Bot 73:S1119-S1125

Svaren J and Hörz W (1995) Interplay between nucleosomes and transcription factors at the yeast *PHO5* promoter. Cell Biology 6:177-183

Swords KMM, Dahlbeck D, Kearny B, Roy M and Staskawicz BJ (1996) Spontaneous and induced mutations in a single open reading frame alter both virulence and avirulence in *Xanthomonas campestris* pv. *vesicatoria avr*Bs2.
J Bacteriol 178:4661-4669

Talbot NJ, Ebbole DJ and Hamer JE (1993) Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. Plant Cell 5:1575-1590

Talbot NJ (1995) Having a blast: exploring the pathogenicity of *Magnaporthe grisea*. Trends Microbiol 3:9-16

Talbot NJ, McCafferty HRK, Ma M, Moore K and Hamer JE (1997) Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression.
Physiol Mol Plant Pathol 50:179-195

Tatsumi H, Murakami S, Tsuji R, Ishida Y, Murakami K, Masaki A, Kawabe H, Arimura H, Nakano E and Motai H (1991) Cloning and expression in yeast of a cDNA clone encoding *Aspergillus oryzae* neutral protease II, a unique metalloprotease. Mol Gen Genet 228:97-103

Teng PS (1994) The epidemiological basis for blast management. In: The Rice Blast Disease. (Ziegler RS, Leong SA and Teng PS, eds). pp 409-433, CAB International, Oxford (UK)

Thijs H, Garde J, Goosen T, Tomsett B, Swart K, Heyting C and Van den Broek HWJ (1995)
Polarity of meiotic gene conversion is 5' to 3' within the *nia*D gene of *Aspergillus nidulans*. Mol Gen Genet 247:343-350

Thomas CM, Jones DA, Parniske M, Harrison K, Balint-Kurti PJ, Hatzixanthis K and Jones JDG (1997) Characterisation of the tomato *Cf-4* gene for resistance to *Cladosporium fulvum* identifies sequences which determine recognitional specificity in *Cf-4* and *Cf-9*. Plant Cell 9:2209-2224

Timberlake WE and Marshall MA (1989) Genetic engineering of filamentous fungi. Science 244:1313-1317

Tomsett AB, Dunn-Coleman NS and Garrett RH (1981) The regulation of nitrate assimilation in *Neurospora crassa*: the isolation and genetic analysis of *nmr*-1 mutants. Mol Gen Genet 182:229-233

Tudzynski B, Homann V, Feng B and Marzluf GA (1999) Isolation, characterization and disruption of the *are*A nitrogen regulatory gene of *Gibberella fujikuroi*. Mol Gen Genet 261:106-114

Turner JG and Debbage JM (1982) Tabtoxin-induced symptoms are associated with accumulation of ammonia formed during photorespiration. Physiol Plant Pathol 20:223-233

Unkles SE, Campbell EI, Carrez D, Grieve C, Contreras R, Fiers W, Van den Hondel CAMJJ and Kinghorn JR (1989a) Transformation of *Aspergillus niger* with the homologous nitrate reductase gene. Gene 78:157-166

Unkles SE, Campbell EI, De Ruiter-Jacobs YMJT, Broekhuijsen M, Macro JA, Carrez D, Contreras R, Van den Hondel CAMJJ and Kinghorn JR (1989b) The development of a homologous transformation system for *Aspergillus oryzae* based on the nitrate assimilation pathway: a convenient and general selection system for filamentous fungal transformation. Mol Gen Genet 218:99-104

Valent B and Chumly FG (1991) Molecular and genetic analysis of the rice blast fungus *Magnaporthe grisea.* Annu Rev Phytopathol 29:443-467

Van den Ackerveken GFJM, Van Kan JAL and De Wit PJGM (1992) Molecular analysis of the avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. Plant J 2:359-366

Van den Ackerveken GFJM, Vossen P and De Wit PJGM (1993) The AVR9 race-specific elicitor of *Cladosporium fulvum* is processed by endogenous and plant proteases. Plant Physiol 103:91-96

Van den Ackerveken GFJM, Dunn RM, Cozijnsen TJ, Vossen P, Van den Broek HWJ and De Wit PJGM (1994) Nitrogen limitation induces expression of the avirulence gene *Avr*9 in the tomato pathogen *Cladosporium fulvum.* Mol Gen Genet 243: 277-285

Van Gorcom RFM and Van den Hondel CAMJJ (1988) Expression analysis vectors for *Aspergillus niger.* Nucleic Acids Res 16:9053

Van Gorcom RFM, Pouwels PH, Goosen T, Visser J, Van den Broek HWJ, Hamer JE, Timberlake WE and Van den Hondel CAMJJ (1985) Expression of an *Escherichia coli* ß-galactosidase fusion gene in *Aspergillus nidulans.* Gene 40:99-106

Van Kan JAL, Van den Ackerveken, GFJM and De Wit PJGM (1991) Cloning and characterisation of cDNA of avirulence gene *Avr*9 of the fungal tomato pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. Mol Plant-Microbe Interact 4:52-59

Van Kan JAL, Joosten MHAJ, Wagemakers CAM, Van den Berg-Velthuis GCM and De Wit PJGM (1992) Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum.* Plant Mol Biol 20:513- 527

Vervoort J, Van den Hooven HW, Berg A, Vossen P, Vogelsang R, Joosten MHAJ and De Wit PJGM (1997) The race-specific elicitor AVR9 of the tomato pathogen *Cladosporium fulvum*: a cystine knot protein. FEBS Lett 404:153-158

Vivian A and Gibbon M J (1997) Avirulence genes in plant-pathogenic bacteria: signals or weapons? Microbiol 143:693-704

Völksch B and Weingart H (1998) Toxin production by pathovars of *Pseudomonas syringae* and their antagonistic activities against epiphytic microorganisms. J Basic Microbiol 2:135-145

Ward M, Wilson LJ, Carmona CL and Turner G (1988) The *oli*C3 gene of *Aspergillus niger*: isolation and use as a selectable marker for transformation. Curr Genet 14:37-42

Ward M, Wilkinson B and Turner G (1986) Transformation of *Aspergillus nidulans* with a cloned, oligomycin resistant ATP synthase subunit 9 gene. Mol Gen Genet 202:265-270

Wei ZM, Sneath BJ and Beer S (1992) Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. J Bacteriol 174: 1875-1882

Wei YD and Mortensen CN (1992) Bioassay of toxins as a diagnostic method for *Pseudomonas syringae* pathovars. J Phytopathol 134:110-116

Wernars K, Goosen T, Wennekes LM, Visser J, Bos CJ, Van den Broek HWJ, Van Gorcom RF, Van de Hondel CAMJJ and Pouwels PH (1985) Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amd*S gene. Curr Genet 9:361-368

Wessels JG (1997) Hydrophobins: proteins that change the nature of the fungal surface. Adv Microb Physiol 38:1-45

Whitehead MP, Unkles SE, Ramsden M, Campbell EI, Gurr SJ, Spence D, Van den Hondel CAMJJ, Contreras R and Kinghorn JR (1989) Transformation of a nitrate reductase deficient mutant of *Penicillium chrysogenum* with the corresponding *Aspergillus niger* and *A. nidulans nia*D genes. Mol Gen Genet 216:408-411

Wiame JM, Grenson M and Arst HN Jr (1985) Nitrogen catabolite repression in yeast and filamentous fungi. Adv Microbiol Physiol. 26:1-87

Wilhelm S (1950) The inoculum potential of *Verticillum albo-atrum* as affected by soil amendments. Phytopathol 40:970-974

Williams FJ (1965) Antecedent nitrogen sources affecting virulence of *Colletotrichum phomoides*. Phytopathol 55: 333-335

Wubben JP, Eijkelboom CA and De Wit PJGM (1994) Accumulation of pathogenesis-related proteins in epidermis of tomato leaves infected by *Cladosporium fulvum*. Neth J Plant Pathol 41:23-32

Wubben JP, Joosten MHAJ and De Wit PJGM (1994) Expression and localization of two *in planta* induced extracellular proteins of the fungal tomato pathogen *Cladosporium fulvum*. Mol Plant-Microbe Interact 7:516-524

Xiao XD and Marzluf GA (1993) Amino-acid substitutions in the zinc finger of NIT2, the nitrogen regulatory protein of *Neurospora crassa*, alter promoter element recognition. Curr Genet 24:212-218

Xiao Y, Heu S, Yi J, Lu Y and Hutcheson SW (1994) Identification of a putative alternate sigma factor and characterization of a multicomponent regulatory cascade controlling the expression of *Pseudomonas syringae* pv. *syringae* Pss61 *hrp* and *hrm*A genes. J Bacteriol 4:1025-1036

Xiao Y and Hutcheson SW (1994) A single promoter sequence recognized by a newly identified alternate sigma factor directs expression of pathogenicity and host range determinants in *Pseudomonas syringae*. J Bacteriol 10:3089-3091

Xiao XD, Fu YH and Marzluf GA (1995) The negative-acting NMR regulatory protein of *Neurospora crassa* binds to and inhibits the DNA-binding activity of the positive-acting nitrogen regulatory protein NIT2. Biochemistry 34:8861-8868

Xing T, Higgins VJ and Blumwald E (1997) Race-specific elicitors of *Cladosporium fulvum* promote translocation of cytosolic components of NADPH oxidase to the plasma membrane of tomato cells. Plant Cell 9:249-256

Yelton MM, Hamer JE and Timberlake WE (1984) Transformation of *Aspergillus nidulans* by using a *trp*C plasmid. Proc Natl Acad Sci USA 81:1470-1474

Young JL, Jaral G, Fu YH and Marzluf GA (1990) Nucleotide sequence and analysis of *nmr*, a negative-acting regulatory gene in the nitrogen circuit of *Neurospora crassa*. Mol Gen Genet 222:120-128

Yuan, GF, Fu, YH and Marluf GA (1991) *nit*-4, a pathway-specific regulatory gene of *Neurospora crassa*, encodes a protein with a putative binculear zinc finger DNA-binding domain. Mol Cell Biol 11:5735-5745

Yucel I, Xiao YX and Hutcheson SW (1989) Influence of *Pseudomonas syringae* culture conditions on initiation of the hypersensitive response of culture tobacco cells. Appl Environ Microbiol 7:1724-1729

Summary

During growth of a pathogen in host tissue, pathogenicity genes are usually highly expressed. A detailed understanding of how these pathogenicity genes are regulated is required to gain a better insight in the molecular communication between pathogen and host. **Chapter one** describes several bacterial and fungal genes, which are envisaged to be involved in pathogenicity and are induced *in vitro* during growth under nutrient-limiting conditions. Based on the data described in this chapter, we speculate that in plants, pathogens encounter an environment in which nutrients are limiting. Lack of nitrogen might be one of the key factors that induce these pathogenicity genes.

The interaction between the fungus *Cladosporium fulvum* and its only host, tomato, is used as a model system to study plant-pathogen interactions. This interaction is a typical gene-for-gene relationship, that states that for each avirulence (*Avr*) gene in the pathogen there is a corresponding resistance (*R*) gene in the plant. Direct or indirect interaction between the products of *Avr* and *R* genes leads to incompatibility.

The object of the research performed in this thesis was to obtain a better understanding of the factor(s) involved in regulation of the *C. fulvum* avirulence gene *Avr9*, which is highly expressed *in planta* during colonisation of the intercellular spaces of tomato leaves. The product of this gene is specifically recognised by tomato plants carrying matching resistance gene *Cf-9*. After recognition, the plant mounts a hypersensitive response (HR) that eventually leads to resistance against the fungus.

Before the study was initiated it was known that the *Avr9* gene is induced under conditions of nitrogen starvation *in vitro*. Furthermore, several (TA)GATA sequences were found to be present in the *Avr9* promoter. These sequences had earlier been identified as the binding sites for a wide-domain GATA-type regulator (AREA in *Aspergillus nidulans* and NIT2 in *Neurospora crassa*), involved in nitrogen utilisation. Both observations made it likely to hypothesise that a similar regulator would be involved in induction of *Avr9* expression in *C. fulvum* and that nitrogen-limitation in the apoplast is the environmental factor that induces *Avr9* expression *in planta*.

Chapter two describes the *Avr9* promoter activity in *A. nidulans* transformants, containing

a single copy of an *Avr*9 promoter-*uid*A (GUS) reporter gene fusion in different *are*A backgrounds (*are*A wild-type, *are*A minus, *are*A constitutive), targeted at the *arg*B locus, following nitrogen starvation. Induction of the *Avr*9 promoter was found to be similarly regulated in *A. nidulans* and *C. fulvum*, indicating that the AREA protein of *A. nidulans* is able to induce the *Avr*9 promoter and that *C. fulvum* contains an AREA-like regulator that can bind to (TA)GATA sequences. **Chapter three** describes a mutational analysis of these (TA)GATA sequences which reveals that two TAGATA-boxes, located most proximal to the start codon, both containing two invertedly orientated TAGATA sequences, are crucial for inducibility of *Avr*9 promoter activity in *A. nidulans*.

Mutated *Avr*9 promoter fragments which did not show any inducibility in *A. nidulans* were fused to the *Avr*9 coding region and introduced (not targeted) into strains of *C. fulvum* lacking *Avr*9. However, in *C. fulvum* transformants the *Avr*9 gene was induced when they were grown in rich, liquid media, a condition which normally suppresses *Avr*9 gene expression. We have no Southern data on the transformants but it could be that multiple integrations have caused the loss of nitrogen-dependent *Avr*9 regulation both *in vitro* and *in planta*. This result emphasises that for reliable promoter studies in *C. fulvum* a gene-targeting system is required.

The development of such a system for *C. fulvum* is described in **Chapter four**. For this purpose, the *C. fulvum pyr*1 gene was isolated. The *pyr*1 gene codes for the enzyme orotidine-5'-monophosphate decarboxylase, which is involved in the pyrimidine biosynthetic pathway and is considered to be a versatile selection marker for filamentous fungi. The isolation of the *C. fulvum pyr*1 gene was based on complementation of an *A. nidulans pyr*G-minus mutant strain which was simultaneously transformed with digested genomic DNA of *C. fulvum* containing the wild-type *pyr*1 gene and an autonomously-replicating plasmid.

*C. fulvum pyr*1$^+$ transformants were obtained by introducing a vector, containing the *C. fulvum pyr*1 gene with a defined mutation, into a *C. fulvum pyr*1-mutant strain. Southern blot analysis of these transformants showed that site-directed integration of this vector at the *pyr*1 locus had occurred. Thus, targeting of constructs of interest to the *pyr*1 locus of *C. fulvum* is feasible.

Isolation of the *are*A/*nit*-2 homologue of *C. fulvum*, designated *Nrf*1 (for nitrogen response factor 1), is described in **Chapter five**. The gene encodes a protein which contains a putative zinc finger DNA-binding domain that is 98% identical to the zinc finger domain present

in the AREA and NIT2 proteins. Function equivalence of *Nrf*1 to *are*A was demonstrated by complementation of an *A. nidulans are*A-minus mutant with *Nrf*1. Expression analysis in liquid media revealed that, in contrast to what occurs in wild-type *C. fulvum* strains, in *Nrf*1-deficient strains the *Avr9* gene is not induced under conditions of nitrogen starvation. However, *Nrf*1-deficient strains were still avirulent on tomato plants containing the *Cf-9* resistance gene, indicating that *in planta* still sufficient quantities of the AVR9 elicitor are produced. It appears that, although NRF1 is a major regulator of the *Avr9* gene expression, *in planta* at least one additional positive regulator of *Avr9* gene expression is active.

In **Chapter six** we studied the effect of elevated nitrogen levels on expression of *Avr9* in *C. fulvum* grown *in planta*. We observed that tomato plants containing both the *Cf-9* gene and elevated levels of nitrate in the apoplast show partial resistance against strains of *C. fulvum* containing the *Avr9* gene. This implies that the elevated level of nitrate in the apoplast represses *Avr9* expression.

In **Chapter seven** the data obtained in this research project are discussed in relation to other known avirulence genes. It is still unknown why, in their host, pathogens would produce proteins that betray them. A possible role for the AVR9 elicitor as a kind of "survival protein" for the fungus during infection is discussed. Although, it appears that regulation of the *Avr9* gene is associated with nitrogen circuits in *C. fulvum*, regulation of *Avr9* by NRF1 *in vitro* and *in planta* is not similar. The isolation of additional plant factor(s) which are able to induce *Avr9* is a challenge for future research.

Samenvatting

Bij de interactie tussen een pathogeen en een plant worden er in het pathogeen diverse genen geïnduceerd welke niet tot expressie komen wanneer het pathogeen onder laboratoriumcondities groeit (*in vitro*). Een aantal van deze specifiek *in planta* geïnduceerde genen zijn avirulentiegenen welke coderen voor peptiden/eiwitten die herkend worden door de plant, mits deze het corresponderende resistentiegen heeft. Na herkenning vindt er een typische overgevoeligheidsreactie plaats waarbij enkele plantencellen afsterven en daarmee ook het pathogeen ten gronde gaat. Dit heeft tot gevolg dat de eventuele groei van het pathogene organisme ingeperkt blijft tot de infectieplaats. Een interactie zoals deze is meestal het gevolg van een gen-om-gen relatie. Dit houdt in dat voor een avirulentiegen (*Avr*) in de schimmel er een corresponderend resistentiegen (*R*) in de plant aanwezig is waarbij interactie tussen de producten van deze genen direct of indirect verantwoordelijk is voor de inductie van de overgevoeligheidsreactie.

Identificatie van de factoren verantwoordelijk voor de specifieke inductie van deze genen van het pathogeen in de plant kan een belangrijke bijdrage leveren aan een beter inzicht in de moleculaire communicatie tussen plant en pathogeen tijdens het ziekteproces (pathogenese). **Hoofdstuk een** beschrijft verschillende bacteriële- en schimmelgenen die, naast specifieke expressie in de plant, ook geïnduceerd kunnen worden wanneer de bacteriën of schimmels *in vitro* worden gekweekt in media waarin de hoeveelheid essentiële nutriënten, zoals bijvoorbeeld stikstof, limiterend is. Er wordt dan ook verondersteld dat deze genen coderen voor eiwitten die voor het pathogen nodig zijn om te overleven in de gastheer. Sommige van deze eiwitten zouden een rol kunnen spelen bij het ontrekken van voedingsstoffen uit de plant. Aan de andere kant zouden ze betrokken kunnen zijn bij een snelle anticipatie van het pathogeen op de veranderde omstandigheden, met betrekking tot de beschikbaarheid van nutriënten in de gastheer.

Onze onderzoeksgroep werkt met het model *Cladosporium fulvum* als pathogene schimmel en zijn enige waardplant, tomaat. Uit voorgaande studies is gebleken dat in *C. fulvum* diverse avirulentiegenen worden geïnduceerd wanneer deze een tomatenplant infecteert. Een van deze avirulentiegenen is het goed gekarakteriseerde gen *Avr9*. Het product van dit gen, AVR9, wordt herkend door tomatenplanten die het corresponderende resistentiegen, *Cf-9*, bevatten en zijn als gevolg hiervan, resistent tegen de schimmel. Het doel van het onderzoek zoals beschreven is in dit proefschrift bestond eruit om de regulatie van het

*Avr*9 gen beter te begrijpen. In het algemeen is er nog slechts zeer weinig bekend over hoe avirulentiegenen, afkomstig uit fytopathogene schimmels, worden gereguleerd. Het in dit proefschrift beschreven werk kan dan ook worden beschouwd als een van de eerste gedetailleerde onderzoeken naar de regulatie van dergelijke schimmelgenen.

Het *Avr*9 gen komt hoog tot expressie wanneer de schimmel groeit in de bladeren van een vatbare tomatenplant. Het *Avr*9 gen kan echter ook *in vitro* geïnduceerd worden wanneer de stikstof in het medium limiterend wordt. De promoter van het *Avr*9 gen bevat specifieke (TA)GATA sequenties waaraan, in promotoren van genen afkomstig uit andere filamenteuze schimmels, transcriptiefactoren van het GATA-type (AREA in *Aspergillus nidulans* en NIT2 in *Neurospora crassa*) aan kunnen binden. Deze transcriptiefactoren kunnen een breed scala aan genen induceren welke betrokken zijn bij het stikstofmetabolisme van de schimmel. Bovenstaande bevindingen hebben geleid tot de hypothese dat er in *C. fulvum* een soortgelijke AREA/NIT2 transcriptiefactor aanwezig is, welke verantwoordelijk is voor de regulatie van het *Avr*9 gen. Verder zouden de bovenstaande waarnemingen erop kunnen wijzen dat stikstoflimitering in de apoplast van de tomatenbladeren, waar de schimmel groeit, het signaal is voor de inductie van *Avr*9 expressie.

De resultaten gevonden in **Hoofdstuk twee** steunen de eerste veronderstelling. In dit hoofdstuk wordt aangetoond dat de *Avr*9 promoter in de laboratoriumschimmel *A. nidulans* een overeenkomstig inductiepatroon vertoont als eerder gevonden voor deze promoter in *C. fulvum*. Hiertoe werd de *Avr*9 promoter gefuseerd aan het *uid*A (GUS) "reporter" gen en vervolgens getransformeerd naar verschillende *A. nidulans are*A mutanten (*are*A minus, *are*A constitutief) en het *are*A wild-type. *A. nidulans* transformanten met een enkele kopie van het construct, gericht geïntegreerd op een locatie in het genoom, werden vervolgens gekweekt in medium met en zonder stikstof, waarna de GUS activiteit werd bepaald. De resultaten wijzen erop dat er inderdaad ook in *C. fulvum* een AREA/NIT2 transcriptiefactor aanwezig moet zijn die kan binden aan de (TA)GATA sequenties en expressie van *Avr*9 kan induceren.

Om te bepalen of deze (TA)GATA sequenties inderdaad een rol spelen bij de inductie van *Avr*9 expressie werden specifieke puntmutaties aangebracht in deze mogelijke bindings sequenties (beschreven in **Hoofdstuk drie**). Studies met gemuteerde *Avr*9 promoter-GUS fusies toonden aan dat twee blokken van twee tegenovergesteld georiënteerde TAGATA sequenties, welke vlak voor het startcodon gelegen zijn, essentiëel zijn voor de induceerbaarheid van *Avr*9 expressie in *A. nidulans*. De gemuteerde *Avr*9 promotoren, welke niet konden worden geïnduceerd in *A. nidulans*, werden gefuseerd aan het coderende gebied van het *Avr*9 gen. Vervolgens werden deze constructen getransformeerd naar *C. fulvum*

stammen die het *Avr*9 gen niet bevatten, met de bedoeling, de transformanten te testen op virulentie op Cf9-planten. Vreemd genoeg bleken transformanten waarin dergelijke constructen waren geïntroduceerd toch avirulent te zijn op tomatenplanten met het *Cf-9* gen en blijkbaar dus nog voldoende AVR9 produceren. Expressie studies toonden echter aan dat in deze transformanten, ook wanneer ze gekweekt worden in rijk medium *in vitro Avr*9 werd geïnduceerd. Mogelijk is dit het gevolg van de aanwezigheid van meerdere kopieën van het construct, op diverse locaties geïntegreerd in het genoom van *C. fulvum*. De chromosomale omgeving lijkt dus een grote invloed op de expressie van *Avr*9 te hebben. Een goede vergelijking van de *Avr*9 expressie in deze transformanten is dan ook niet te maken. Gerichte transformatie van *C. fulvum* ("gene-targeting") waarbij constructen door middel van homologe recombinatie op een specifiek locus in het genoom kunnen worden geïntegreerd, is dus essentiëel. De ontwikkeling van een dergelijk systeem voor *C. fulvum* wordt beschreven in **Hoofdstuk vier**. Voor dit doel werd het *C. fulvum pyr*1 gen geïsoleerd. Dit gen codeert voor het enzym orotidine-5'-monofosfaat decarboxylase, dat betrokken is bij de pyrimidine biosynthese. De methode ter isolatie van het *C. fulvum pyr*1 gen was gebaseerd op complementatie van een *pyr*G mutante stam van *A. nidulans*. Hiertoe werd deze *A. nidulans* stam gelijktijdig getransformeerd met gedigesteerd genomisch DNA van *C. fulvum* met het wild-type *pyr*1 gen en een autonoom replicerende plasmide.

DNA gel-blot analyse van genomisch DNA van *C. fulvum pyr*1$^+$ transformanten, die werden verkregen na transformatie met een construct met een specifieke mutatie in het *pyr*1 gen, naar een *C. fulvum pyr*1-mutant, toonde aan dat het mogelijk is om een enkele kopie gericht te laten integreren in het *C. fulvum pyr*1 locus. Op basis van deze resultaten moet het mogelijk zijn om in de toekomst gebruik te maken van dit systeem om onder andere betrouwbare promoteranalyses uit te voeren.

Hoofdstuk vijf beschrijft de isolatie van het *C. fulvum areA/nit*-2-homologe gen, *Nrf*1 (nitrogen response factor 1). Het *Nrf*1 gen codeert voor een eiwit met een mogelijk DNA-bindend "zinc finger" domein, hetgeen karakteristiek is voor een GATA-factor. Dit domein bleek op aminozuurniveau voor 98% identiek aan het "zinc finger" domein van respectievelijk AREA en NIT2. Transformatie van het *Nrf*1 gen naar een *A. nidulans areA*-min stam resulteerde in functionele complementatie voor het *A. nidulans areA* gen. RNA gel-blot analyse liet zien dat, er bij stikstoflimitatie *in vitro*, geen inductie van *Avr*9 expressie plaatsvond in *C. fulvum* transformanten waarin het *Nrf*1 gen was uitgeschakeld. Desondanks bleken *Nrf*1-deficiënte transformanten van *C. fulvum* nog steeds avirulent te zijn op tomatenplanten die het corresponderende *Cf-9* gen bevatten. Blijkbaar wordt er nog steeds

voldoende AVR9 geproduceerd door de *Nrf*1-deficiënte transformanten. NRF1 is dus wel een belangrijke regulator van het *Avr*9 gen, maar tijdens de kolonisatie van de plant door de schimmel zijn waarschijnlijk additionele (planten)factoren aanwezig die in staat zijn *Avr*9 expressie te induceren.

Om te bepalen of een stikstof-limiterende omgeving in de apoplast van tomatenbladeren het signaal is voor *Avr*9 inductie, hebben we in **Hoofdstuk zes** gebruik gemaakt van tomatenplanten die zowel het *Cf-9* resistentiegen bevatten als een verhoogde nitraatconcentratie in de apoplast. Wanneer deze planten werden geïnoculeerd met een *Avr*9-bevattende *C. fulvum* stam, bleek dat deze planten niet een volledige, maar een partiële resistentie vertoonden tegen deze schimmelstam. Dit resultaat impliceert dat expressie van het *Avr*9 gen inderdaad onderdrukt wordt door een verhoogde nitraatconcentratie en dat voornamelijk tijdens de eerste fase van de infectie, stikstoflimiterende condities in de apoplast een signaal kunnen zijn voor de inductie van *Avr*9.

In **Hoofdstuk zeven** worden alle waarnemingen nog een keer naast elkaar gezet en besproken. De gevonden resultaten worden in een breder perspectief geplaatst en vergeleken met expressie van andere bekende avirulentiegenen. Het blijft nog steeds een vraag waarom pathogenen tijdens de pathogenese eiwitten/peptiden produceren die hun aanwezigheid verraden. In dit verband wordt er een mogelijke rol voor AVR9 als "overlevingseiwit" gesuggereerd.

Aan de hand van de gevonden resultaten in dit proefschrift kunnen we concluderen dat we iets meer weten over communicatie tussen pathogeen en plant tijdens het infectieproces. Stikstof heeft een effect op de *Avr*9 regulatie in *C. fulvum*. Vooral de rol van NRF1 lijkt zeer belangrijk. Aan de andere kant is gebleken dat de mechanismen ter regulatie van *Avr*9 *in vitro* en in de plant, naast enig overlap ook verschillen hebben. Er zijn nog additionele factor(en) in de plant die in staat zijn om *Avr*9 te induceren. Daarnaast zijn er, ook na de bevindingen beschreven in dit proefschrift, nog geen duidelijke aanwijzingen voor de rol van AVR9 voor *C. fulvum* tijdens de infectie van tomaat.

Curriculum Vitae

Sandor Sebastiaan Snoeijers werd op 21 maart 1968 geboren te Andijk. Na het behalen van het VWO-diploma aan de Rijksscholengemeenschap te Enkhuizen begon hij in 1987 aan de studie Biologie aan de Universiteit van Amsterdam. De doctoraalfase omvatte twee praktische stageperiodes bij de secties Moleculaire Biologie en Fytopathologie aan de Universiteit van Amsterdam. In september 1993 sloot hij zijn studie met goed gevolg af. Van november 1994 tot juni 2000 verichtte hij promotieonderzoek als onderzoeker in opleiding (OIO) aan de Laboratoria van Moleculaire Genetica en Fytopathologie te Wageningen. Vanaf augustus 2000 is hij werkzaam bij de firma Kreatech Diagnostics te Amsterdam.