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Mini review

The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens

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Abstract

Successful colonisation of plants by pathogens requires efficient utilisation of nutrient resources available in host tissues. Several bacterial and fungal genes are specifically induced during pathogenesis and under nitrogen-limiting conditions *in vitro*. This suggests that a nitrogen-limiting environment may be one of the cues for disease symptom development during growth of the pathogens *in planta*. Here we review recent literature on the effect of nitrogen and nitrogen-regulated genes on disease development, caused by phytopathogenic bacteria and fungi. Furthermore, the potential influence of nitrogen-limitation or general nutrient limitation on several *in planta*-induced bacterial and fungal pathogenicity, virulence and avirulence genes will be discussed.

Introduction

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.

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

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

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 remobilisation 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 β-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 limitation 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 *h*ypersensitive *r*esponse and *p*athogenicity) 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 co-activator. 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

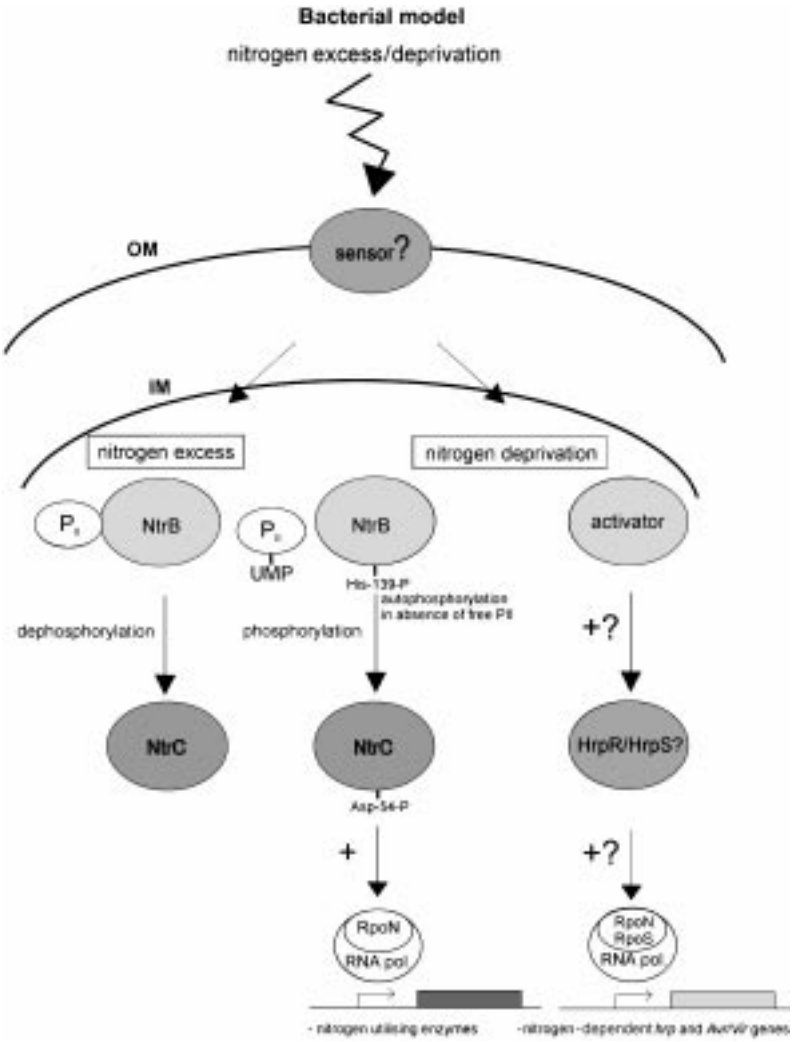

Figure 1A. Model for nitrogen-sensing and induction of nitrogen-dependent *hrp* and *Avr*/*Vir* genes of bacterial pathogens. The encoding genes are depicted as grey boxes. Under conditions of nitrogen excess the P_{II} protein binds to NtrB to activate its phosphatase activity. When this occurs, NtrB dephosphorylates NtrC so it cannot bind to enhancer sequences to increase transcription. However, P_{II}-UMP, which is present during nitrogen deprivation, cannot 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 *Avr*/*Vir* and HR and pathogenicity (*hrp*) genes is hypothesised (for further details see text); OM: outer membrane; IM: inner membrane; P: phospate; UMP: 2p-deoxyuridine 5p-monophosphate.

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 p*lasmid-associated *v*irulence) 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 established yet, conclusively.

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. Figure 1A shows the 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.

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

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, soyabean	*hrp* genes	Secretion of avirulence factors		Rhame et al., 1992; Xiao et al., 1994
	Tomato, soyabean	*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 and Mortensen, 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

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. NR and NiR) 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* (Pérez-García et al., unpubl.), 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, 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 are*A-homologous gene (*Nrf*1) has been cloned (Pérez-García et al., unpubl.) 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 dehydrogenase 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 and Whiteford, 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 *are*A-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 *n*itrogen *p*athogenicity *r*egulation genes 1 and 2), were identified and mutation of either of these genes resulted in an *are*A-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 limitation 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 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; 1998).

In *N. crassa*, mutation of the *nmr* (for *n*itrogen *m*etabolic *r*egulation) 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., unpubl.). 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	*Avr*9	Avirulence factor	*Nrf*1*	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*	GS	n.i.	Stephenson et al., 1997
		*cgDN*3	Suppressor of plant defences		Stephenson et al., 1998
Metarhizium anisopliae	Insects	*pr*1A	Protease	*nrr*1	St. Leger, 1995; Smithson et al. 1995
		*pr*2	Protease		
Arthrobotrys oligospora	Nematodes	*p*II	Protease	n.i.	Ahman et al., 1996

*Isolation of the *Nrf*1 gene from *C. fulvum* has not been published yet; 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 *Path* and *Avr*/*Vir* genes in fungal pathogens is shown.

Conclusions

Supply of additional nitrogen can increase disease development for various plant–pathogen interactions. When nitrogen is not limiting, pathogens can easily acquire nitrogen and will cause more disease on these plants than on host plants where nitrogen is limiting. However, different results have been reported by McElhaney et al. (1998) for the interaction between cabbage and *Xanthomonas campestris* pv. *campestris*.

Several bacterial and fungal genes, envisaged to be involved in pathogenicity, are induced during growth under nutrient-limiting conditions, *in vitro*. We speculate that in most plants, pathogens encounter an environment were nutrients are limiting and that a lack of nitrogen might be one of the environmental factors *in planta* that are able to induce several *Path* and *Avr*/*Vir* genes. The observation that some of the identified *in planta*-induced fungal genes contain sequences in their promoters that represent specific binding sites for major nitrogen regulatory AREA-like transcription factors, supports this hypothesis. However, it has to be emphasised that the phytopathogenic fungi described in this review show large differences in infection strategies, *C. fulvum* does not produce haustoria which is different from *M. grisea* and *C. gloeosporioides*. The formation of haustoria and, in particular, intracellular invasion, suggest that a different environment is encountered by the latter two fungi during pathogenesis. Although several genes coding for AREA-like transcription factors have been cloned from fungal pathogens, their role in expression of *Path* and *Avr*/*Vir* genes still needs further study.

It has to be mentioned that starvation-stress is known to affect a number of morphogenetic processes in fungi. In *Saccharomyces cerevisiae* nitrogen limitation causes a pseudohyphal, invasive growth pattern (Gimeno et al., 1992), whereas for *A. nidulans* it has been shown that carbon and nitrogen starvation induce *Brl*A, the central regulator of sporulation (Skromme et al., 1995). In addition, a large number of insect immunity genes contain a GATA motif in their regulatory region. For the *Drosophila CecA1* gene, coding for an antimicrobial peptide, it was shown that the GATA motif is required for expression in the larval fat body. Overexpression of the gene coding for a *Drosophila* GATA factor, designated Serpent, increased transcription of *CecA1* (Petersen et al., 1999). The authors propose that

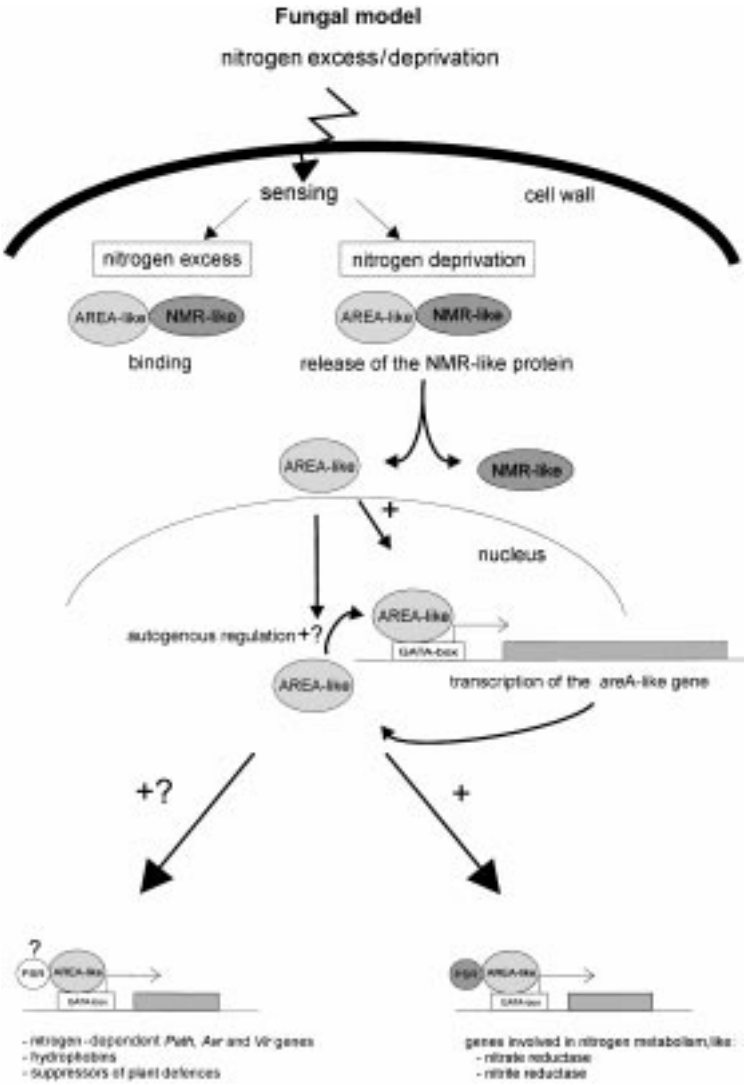

Figure 1B. Model for nitrogen-sensing and induction of nitrogen-dependent *Path* and *Avr*/*Vir* genes of fungal pathogens. The encoding genes are depicted as grey boxes, GATA sequences are depicted as white boxes in the promoter regions. When primary nitrogen sources are available, the negative regulatory (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 NR and NiR; 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 positive effect of the AREA-like proteins on expression of nitrogen-dependent *Avr*/*Vir* genes in fungal pathogens is shown.

Serpent plays a key-role in tissue-specific expression of immunity genes in response to infection.

Based on the data described in this review, 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. The factor(s) that link the sensing of nutrient limitation and the induction of *Path* and *Avr*/*Vir* genes of the pathogen *in planta*, are most probably

major players in communication between pathogen and host plant.

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