Expression of the Avirulence Gene *Avr9* of the Fungal Tomato Pathogen *Cladosporium fulvum* Is Regulated by the Global Nitrogen Response Factor NRF1

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Here we describe the role of the *Cladosporium fulvum* nitrogen response factor 1 (*Nrf1*) gene in regulation of the expression of avirulence gene *Avr9* and virulence on tomato. The *Nrf1* gene, which was isolated by a polymerase chain reaction-based strategy, is predicted to encode a protein of 918 amino acid residues. The protein contains a putative zinc finger DNA-binding domain that shares 98% amino acid identity with the zinc finger of the major nitrogen regulatory proteins AREA and NIT2 of *Aspergillus nidulans* and *Neurospora crassa*, respectively. Functional equivalence of *Nrf1* to *areA* was demonstrated by complementation of an *A. nidulans areA* loss-of-function mutant with *Nrf1*. *Nrf1*-deficient transformants of *C. fulvum* obtained by homologous recombination were unable to utilize nitrate and nitrite as a nitrogen source. In contrast to what was observed in the *C. fulvum* wild-type, the *Avr9* gene was no longer induced under nitrogen-starvation conditions in *Nrf1*-deficient strains. On susceptible tomato plants, the *Nrf1*-deficient strains were as virulent as wild-type strains of *C. fulvum*, although the expression of the *Avr9* gene was strongly reduced. In addition, *Nrf1*-deficient strains were still avirulent on tomato plants containing the functional *Cf-9* resistance gene, indicating that in planta, apparently sufficient quantities of stable AVR9 elicitor are produced. Our results suggest that the NRF1 protein is a major regulator of the *Avr9* gene.

Additional keywords: *Avr9* regulation, GATA factor, GATA sequences.

Plant pathogenic bacteria and fungi generally have limited host ranges and are often confined to members of a single plant species or genus. Products of avirulence (*Avr*) gene(s) present in the pathogen play a major role in restricting its host range. *Avr* gene products are recognized 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 localized 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 recognize the pathogen, HR is not triggered, and the host will become diseased (Laugé and De Wit 1998; Vivian and Gibbon 1997).

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* is induced in vitro by culturing bacteria on minimal media (Bogdanove et al. 1998; Gaudriault et al. 1997; Lorang and Keen 1995; Shen and Keen 1993). The expression of *P. syringae Avr* and HR and pathogenicity (*hrp*) 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).

Very little is known of the regulation of fungal *Avr* genes. One exception is *Avr9* of the fungal pathogen *Cladosporium fulvum*, which encodes a 28 amino acid peptide (Van den Ackerveken et al. 1992; Van Kan et al. 1991). 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. Second, in the promoter of *Avr9*, several copies of the sequence (TA)GATA are present, which in *Aspergillus nidulans* and *Neurospora crassa*, 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* (Snoeijsers et al. 1999).

These nitrogen regulatory proteins are members of the GATA family of transcription factors and contain a remarka-

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bly 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 *A. nidulans* and *N. crassa*, these GATA-type transcription factors activate expression of many genes whose products are required for the uptake and utilization 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 (Christensen et al. 1998; Ellis 1996; Fu and Marzluf 1990; Gente et al. 1999; Haas et al. 1995; Hensel et al. 1998; Kudla et al. 1990; MacCabe et al. 1998). 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) contain AREA–NIT2-like proteins.

Some of these genes also have a role in expression of secondary metabolites. The NRE protein of *Penicillium 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 nitrogen responsive factor 1 (*Nrf1*), and report on its role in regulation of *Avr9* expression in vitro and in planta. We found that induction of *Avr9* expression, normally observed during growth conditions of nitrogen starvation in vitro, was now absent in *Nrf1*-deficient transformants and was strongly repressed on plants normally susceptible to the parent *C. fulvum* strains. *Nrf1*-deficient transformants, however, did not show significant reduction in virulence nor did they lose avirulence on *Cf-9* containing plants.

Taken these data together, we conclude that the NRF1 nitrogen factor is a major regulator of *Avr9* expression but, during growth in planta, at least one additional positive regulator of *Avr9* is active.

RESULTS

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

To isolate the *C. fulvum* *areA*–*nit-2*-homologous gene, a polymerase chain reaction (PCR)-based strategy was followed. Initially, PCR with degenerate oligonucleotide primers based on the zinc finger domain of the *areA* and *nit-2* gene of *A. nidulans* and *N. crassa*, respectively (see below), was performed (Haas et al. 1995). Amplification by PCR on genomic DNA 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 below) 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 obtained with two additional specific primers (see below) was used as a 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* homolog of *C. fulvum*. All six clones had a similar restriction pattern and showed the same hybridization pattern on Southern blot analysis with the genomic 1.2-kb PCR product as probe (results not shown).

Functional analysis of the *C. fulvum* *areA*–*nit-2* homolog.

To determine whether the *C. fulvum* genomic clones contain a functional *areA*–*nit-2* homolog, clones 1 to 6 were transformed into the *A. nidulans* *areA* loss-of-function mutant *areA18* (Arst et al. 1989). In the case of *A. nidulans*, a functional AREA protein is required for nitrate assimilation (Caddick et al. 1994) and selection was performed by determining whether growth of the transformants occurred on minimal medium with nitrate as the sole nitrogen source. As a positive control, the wild-type *A. nidulans* *areA* gene, which is present on the pAR4-322-1 plasmid (Small et al. 1999), also was transformed. All *C. fulvum* genomic clones resulted in *A. nidulans* transformants that were able to grow on this nitrate-selective medium.

Twenty independent *A. nidulans* transformants containing *C. fulvum* genomic clones 1 or 3 were chosen for further analysis 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) pAR4-322-1 transformants. Therefore, we could conclude to have isolated the *C. fulvum* *areA*–*nit-2* homolog, which we designated *Nrf1*.

Sequence analysis of the *C. fulvum* *Nrf1* gene.

Genomic clone 1 of *C. fulvum* contains an insert of 5,206 bp. DNA sequence analysis revealed that this insert comprises 1,171 bp upstream of the predicted ATG, 2,754 bp of coding sequence, and 1,217 bp of downstream noncoding sequence, a 64-bp intron. Clone 3 contains a shorter 5′ noncoding region (approximately 500 bp), indicating that, at least in *A. nidulans*, this fragment contains sufficient *Nrf1* promoter sequence to allow *Nrf1* expression and utilization of secondary nitrogen sources.

The *Nrf1* 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 *G. fujikuroi*, *A. nidulans*, *N. crassa*, and *M. grisea* reveals significant homology at the amino acid level (Fig. 1).

Within the zinc finger domain of the predicted NRF1 protein (amino acid position 671 to 725) (Fig. 1), 53 out of 550 amino acids are identical among all AREA–NIT2-like proteins analyzed so far. The last ten C-terminal amino acids of the predicted NRF1 protein also are highly conserved. In *N. crassa*, this C-terminal region interacts with the nitrogen

metabolic repression (NMR) protein, which is involved in nitrogen metabolite repression (Marzluf 1997).

Disruption of the *C. fulvum* *Nrf1* gene.

To inactivate the *Nrf1* gene-by-gene replacement following homologous recombination, a linear *Nrf1*-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 *Nrf1* zinc finger (Fig. 2A).

Approximately 900 hygromycin B-resistant transformants were generated following transformation of the *C. fulvum* wild-type strains races 4 and 5 with pNRF1KO. In the case of nitrate assimilation, a functional NRF1 protein is expected to be required. Therefore, all hygromycin B-resistant transformants were tested for growth on *C. fulvum* minimal medium with sodium nitrate as the sole nitrogen source. Thirty-seven hygromycin B-resistant transformants were unable to grow on this nitrate-selective medium. Subsequently, these transformants were analyzed by PCR with *Nrf1*- (p9 and p10; see below) and *hph* (p11; see below)-specific primers on their genomic DNA.

Three out of the 37 transformants gave a PCR-product, which is indicative of a disrupted *Nrf1* gene. The three transformants, two originating from race 4 (4.94 and 4.125) and one from race 5 (5.224), were analyzed by Southern blotting for presence, location, and copy number of the introduced construct. Transformant 4.125 contained one additional inte-

gration of pNRF1KO in the genome and was not used for further analyses (results 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 *Nrf1* gene (Fig. 2B). *Nrf1* disruptants 4.94 and 5.224 were used for further study.

To characterize the *C. fulvum* *Nrf1* disruptants, growth properties of disruptants 4.94 and 5.224 on minimal medium supplemented with various nitrogen sources were compared with those of the wild-type races 4 and 5 (Table 2). As shown in Table 2, *C. fulvum* races 4 and 5 displayed strong growth on most of the various nitrogen sources. In contrast, the *Nrf1* disruptants were unable to utilize nitrate and nitrite but still used glutamine and, to a lesser extent, other nitrogen compounds such as ammonia, glutamate, and asparagine.

Avr9 expression in *Nrf1*-disruptants of *C. fulvum* is undetectable during nitrogen starvation in vitro.

Avr9 expression can be induced under conditions of nitrogen limitation in vitro. In the *Avr9* gene promoter, 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 the expression of the *Avr9* gene is fully dependent on the presence of AREA (Snøeijers et al. 1999). Northern blot analysis was performed in order to determine whether the *Avr9* gene is induced in the *Nrf1* disruptants

Table 1. Growth properties of *Aspergillus nidulans* strains and transformants of this fungus on minimal medium supplemented with various nitrogen sources

Supplemented nitrogen source (5 mM)	<i>A. nidulans</i> strains		<i>A. nidulans</i> areA18 strain transformation source ^a		
	A4	areA18	pAR4-322-1	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; *Cladosporium fulvum* clones 1 and 3 contain the putative *areA*-*nit-2* homolog of *C. fulvum* (see text). Growth properties of the *A. nidulans* wild-type (A4), *areA* loss-of-function mutant (*areA18*), and the *areA18* mutant transformants are shown.

^b +++: strong growth; ++: good growth; +: growth; ±: weak growth; —: no growth.

^c For growth tests, 20 independent *A. nidulans* *areA18* transformants were analyzed.

Table 2. Growth properties of *Cladosporium fulvum* wild-type strains races 4 and 5 and the corresponding *Nrf1* disruptants 4.94 and 5.224, respectively

Supplemented nitrogen source (5 mM)	<i>C. fulvum</i> wild-type strains		Corresponding <i>Nrf1</i> 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.

In addition, Northern blot analysis of total RNA isolated from leaves infected by the wild-type strains and corresponding *Nrf1*-disruptants was performed to investigate whether the lower concentrations of AVR9 produced by the *Nrf1* disrupt-

ants in planta are the result of reduced *Avr9* expression. Figure 5 shows that *Avr9* mRNA is abundantly present at 20 days postinoculation when tomato leaves were infected by *C. fulvum* wild-type strains. Although significantly reduced when

A

B

Fig. 2. Disruption by replacement of the *Nrf1* gene in *Cladosporium fulvum*. **A**, The 2.8-kb blunted *Hind*III–*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 a black box) of the genomic 1.2-kb *Nrf1* polymerase chain reaction-product (depicted as a dashed box) that was cloned in the TA cloning vector pCR 2.1, resulting in pNRF1KO. Single-copy integration of pNRF1KO at the *Nrf1* locus will result in a 5.8-kb *Xho*I fragment hybridizing with the *hph* cassette or the *Nrf1* gene. **B**, Southern blot analysis of *Xho*I-digested genomic DNA isolated from *C. fulvum* wild-type strains (races 4 and 5) and the *Nrf1* disruptants 4.94 and 5.224 with the 2.8-kb *hph* cassette or the 1.2-kb *Nrf1* gene as probe showed that in 4.94 and 5.224, the 2.8-kb *hph* cassette had replaced the *Nrf1* gene.

compared to the wild-type strains, some *Avr9* expression was detected in the *Nrf1*-disruptants.

DISCUSSION

A better understanding of the function and regulation of avirulence (*Avr*) genes of pathogens is essential because it is not likely that the induction of an active plant defense is the

primary function of their products. Our data suggest that regulation of *Avr* genes is subject to environmental stimuli and nutritional signals. *Avr9* of *C. fulvum* and *infl* of *Phytophthora infestans* are induced in vitro under nitrogen- and carbon-limiting conditions, respectively (Kamoun et al. 1997; Van den Ackerveken et al. 1994).

During nitrogen metabolite repression, preferential utilization of favored nitrogen sources occurs as a result of the re-

Fig. 3. Analysis of *Avr9* expression in vitro of the *Cladosporium fulvum* wild-type strains (races 4 and 5) and the corresponding *Nrf1* disruptants (4.94 and 5.224, respectively). The Northern blot shows *Avr9* 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 hybridized with *Avr9* cDNA (*Avr9*) (Van Kan et al. 1991) and the *C. fulvum* actin (*act*) gene. The *act* gene is constitutively expressed in *C. fulvum* and is used as RNA loading control.

Fig. 4. Hypersensitive response induced in leaves of MM-Cf9 plants upon injection with undiluted and 4x diluted apoplastic fluids (AFs) isolated from leaves of MM-Cf0 plants inoculated with *C. fulvum* wild-type strains races 4 and 5 and the corresponding *Nrf1* disruptants 4.94 and 5.224, respectively. **A**, Necrosis that developed after injection of undiluted AFs isolated from MM-Cf0 plants inoculated with the *C. fulvum* parent strains and the corresponding *Nrf1* disruptants. **B**, Necrosis that developed after injection of 4x diluted AFs from the same MM-Cf0 plants inoculated with *C. fulvum* strains and/or disruptants as shown in **A**. AFs were isolated 20 days postinoculation. Photographs were taken 6 days after injection.

pression of enzymes and permeases required for the utilization of nitrogen sources other than ammonia or glutamine (Marzluf 1997; Wiame et al. 1985). Nitrogen metabolite repression controls genes encoding enzymes of primary metabolism and regulates the genes involved in secondary metabolism such as penicillin and gibberellin biosynthesis (Haas and Marzluf 1995; Tudzynski et al. 1999). In the present study, the putative nitrogen regulation of the *Avr9* gene of *C. fulvum* has been examined in detail. We cloned the *areA-nit-2* homolog of *C. fulvum*, designated *Nrf1*, and studied *Avr9* expression in *C. fulvum* *Nrf1* disruptants.

Several lines of evidence suggest that *Nrf1* is the major nitrogen regulatory gene of *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) (Froeliger and Carpenter 1996; Fu and Marzluf 1990; Kudla et al. 1990; Tudzynski et al. 1999). Secondly, *Nrf1* is able to complement an *areA* loss-of-function mutant of *A. nidulans* by restoring wild-type growth of transformants on a variety of nitrogen sources. These results suggest that NRF1 and AREA respond to similar external nutritional signals of nitrogen metabolite repression. Thirdly, *C. fulvum* *Nrf1*-disruptants are unable to utilize a wide range of nitrogen sources. Therefore, we conclude that the *Nrf1* gene of *C. fulvum* encodes a protein with a similar function as the positively acting global nitrogen regulatory proteins reported for the above-mentioned fungi.

Although the *C. fulvum* *Nrf1* disruptants were unable to grow on a variety of nitrogen sources, they were able to utilize, in addition to glutamine, secondary nitrogen sources such as glutamate and asparagine. In *A. nidulans* and *A. fumigatus*, the AREA protein is not required for ammonia utilization (Hensel et al. 1998; Kudla et al. 1990). As a result, it was expected that the *Nrf1* 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, is induced during pathogenesis on its host *Stylosanthes guianensis* as well as

in vitro under nitrogen-limiting conditions (Stephenson et al. 1997). In general, the GS enzyme is required for ammonia assimilation in a wide variety of organisms. Poor growth of the *Nrf1* disruptants on ammonia could be a result of the fact that the *C. fulvum* gene-encoding GS is under control of NRF1. In addition, ammonium permease, which is required for the uptake of ammonium, could be affected in the *C. fulvum* *Nrf1* disruptants, as reported for *A. nidulans* *areA* loss-of-function mutants (Diallinas et al. 1995).

Because *C. fulvum* is a biotrophic pathogen of tomato, we hypothesized that disruption of *Nrf1* in this fungus would have a major effect on the regulation of the *Avr9* gene and disease development. With the use of a standard inoculation assay, however, disease symptoms caused by the *Nrf1* disruptants were similar to those caused by *C. fulvum* wild-type strains. This indicates that the disruption of *Nrf1*, at least under greenhouse conditions, does 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 *nut1* did not result in reduced virulence (Froeliger and Carpenter 1996). The mechanisms employed by *C. fulvum* and *M. grisea* to infect plants, however, are different. In a compatible interaction (i.e., plant susceptible–fungus virulent), *M. grisea* grows intracellularly (Valent and Chumley 1991), whereas *C. fulvum* grows extracellularly in the apoplast (De Wit 1977). Normal colonization of susceptible tomato plants by *C. fulvum* *Nrf1* disruptants suggests that sufficient primary nitrogen sources such as glutamine are available in the apoplast to allow fungal growth. Possibly, *C. fulvum* has other nitrogen control systems that function independently of *Nrf1*, as suggested for *M. grisea*, which has other regulatory genes involved in nitrogen metabolism and virulence (Lau and Hamer 1996).

The nitrogen metabolic repression gene (*nmr*) encodes a repressor of *areA-nit-2*-like genes. *nmr* was first isolated from *N. crassa* (Young et al. 1990) and, recently, from *A. nidulans* (Adrianopoulos et al. 1998). *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 (Adrianopoulos et al. 1998; Tomsett et al. 1981). The NMR protein is supposed to interact with AREA–NIT2, thereby preventing DNA-binding when sufficient primary nitrogen sources such as glutamine are present (Marzluf 1997). A gene with significant homology to the *nmr* gene was isolated recently from *C. fulvum* by our group (A. Pérez-García, S. Snoeijers, T. Goosen, M. Joosten, and P. de Wit, unpublished). We hypothesize that disruption of this gene in *C. fulvum* will lead to derepression of the genes under control of NRF1.

In addition to *areA-nit-2* and *nmr* genes, other genes are involved in the regulation of nitrogen metabolism. The *tamA* gene of *A. nidulans* encodes a protein required for full expression of genes under *areA* control (Davis et al. 1996; Kinghorn and Pateman 1975). 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 suggested 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. 1991; Yuan et al. 1991). In our assays, we could not detect *Avr9* expression in

Fig. 5. *Avr9* expression in planta of the *Cladosporium fulvum* wild-type strains and the corresponding *Nrf1*-disruptants. Total RNA from MM-Cf0 plants infected by the *C. fulvum* wild-type strains races 4 and 5 and the corresponding *Nrf1*-disruptants 4.94 and 5.224, respectively, was isolated 20 days postinoculation. Total RNA (10 µg) was loaded on a 1.5% denaturing formaldehyde-agarose gel, blotted onto a Hybond N⁺ membrane, and hybridized with *Avr9* cDNA (*Avr9*) (Van den Ackerveken et al. 1991) and the *C. fulvum* actin (*act*) gene. The *C. fulvum* *act* gene is constitutively expressed in *C. fulvum* and is used as a marker for fungal colonization. Note the strongly reduced expression of *Avr9* in the *Nrf1* disruptants 4.94 and 5.224 as compared with the corresponding parent strains races 4 and 5, respectively.

Nrf1 disruptants of *C. fulvum* in vitro following nitrogen starvation, suggesting that induction of *Avr9* expression in vitro is fully under control of the NRF1 protein. Upon inoculation of *Nrf1* disruptants on susceptible tomato plants, however, some expression of *Avr9* could be detected and AVR9 elicitor accumulated in the apoplast, albeit to much lower levels than in the wild-type *C. fulvum* strains. This residual expression of *Avr9* was sufficient to render *Avr9*-containing *Nrf1* mutants avirulent on MM-Cf9C plants. Possibly, the residual *Avr9* expression in planta represents this gene's basal transcription level, which remained just below the detection level in vitro. Thus, although the NRF1 protein is a major regulator of *Avr9* expression, at least one additional basal transcription factor is involved in the induction of *Avr9* expression in planta as well as, possibly, in vitro.

MATERIALS AND METHODS

Fungal strains and culture conditions.

The *A. nidulans* strains used were A4 (wild-type) and the *areA* loss-of-function mutant *areA18* (*yA2*, *pabaA1*, *inoB2*, and *areA18*) (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 2×10^8 conidiospores were transferred to 100 ml of supplemented liquid minimal medium containing, per liter, 1.5 g of KH_2PO_4 (pH 6.0), 0.5 g of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.5 g of KCl, 10 g of D-glucose, 0.1 mg of pyridoxine, and 1 g of L-proline (Pontecorvo et al. 1953). Cultures were incubated for approximately 17 h at 37°C in an orbital shaker at 220 rpm.

Strains of *C. fulvum* (races 4 and 5) were grown on potato dextrose agar (PDA) or in liquid B5 medium (Duchefa Biochemie, 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 liquid cultures. *C. fulvum* *Nrf1*-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 4×10^7 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 that were transferred to 25 ml of liquid B5 medium supplemented with 50 mM nitrate, 50 mM glutamine, or without any nitrogen source. After 24 h of incubation at 23°C and shaking at 100 rpm, mycelium was reharvested, 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 also is present in *C. fulvum*. Initially, degenerate oligonucleotide primers p1 (5'-TGTAC NAAYTGYTTYACNCA-3') and p2 (5'-TTCTTPATNACPTC NGTYTT-3'), based on the region coding for the zinc finger domain of the *areA* and the *nit-2* gene of *A. nidulans* and *N. crassa*, respectively, were used (Haas et al. 1995). PCR on genomic DNA resulted in a 141-bp fragment, which was subsequently ligated into the TA cloning vector pCR 2.1

(Invitrogen, Carlsbad, CA, U.S.A.), and sequenced. The DNA sequence of this fragment revealed high homology with the domain that encodes the conserved DNA-binding zinc finger domain of other known AREA-NIT2-like proteins.

On the basis of the DNA sequence of the 141-bp fragment, specific primers were designed to isolate the flanking sequences. The specific zinc finger primers p3 (5'-TGTAATAA TTGTTTTACGCA-3') and p4 (5'-TTCTTGATGACGTCGGT CTT-3') were used in combination with primers annealing to vector sequence p5 (5'-TACCGGGCCCCCCTCGATT-3') and p6 (5'-TCTAGAACTAGTGGATCCCC-3'), respectively. PCR with these specific primers on a cDNA library in a Uni-ZAP XR (Stratagene, La Jolla, CA, U.S.A.) prepared from *C. fulvum* mycelium grown under nitrogen-starvation conditions was performed. Fragments of 1.2 kb (with primers p3 and p5) and 300 bp (with primers p4 and p6) were amplified and ligated into the TA cloning vector pCR 2.1. Subsequently, these fragments were sequenced and, on the basis of the DNA sequences, primers p7 (5'-TGAATACTCCGCAATGGACG-3') and p8 (5'-CAGGGGAAGCTCGTGGGTACG-3') were designed to amplify a genomic 1.2-kb fragment, which was used as homologous probe to screen a λ BlueStar (Novagen, Madison, WI, U.S.A.) *C. fulvum* genomic library.

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 the sole nitrogen source. *A. nidulans* transformants were analyzed for their ability to grow on minimal medium supplemented with various nitrogen sources. As a positive control, the wild-type *A. nidulans areA* gene present on the pAR4-322-1 plasmid (Small et al. 1999) also was transformed. The *C. fulvum* gene that was able to complement an *A. nidulans areA* loss-of-function mutant was designated *Nrf1*.

In order to obtain a *Nrf1*-disruption construct, the 2.8-kb *HindIII-SstI* fragment containing the hygromycin B resistance gene (*hph* cassette) originating from plasmid pAN7-1 (Punt et al. 1987) was blunted and inserted into the *NotI* site of the sequence coding for the zinc finger domain, resulting in pNRF1KO. In pNRF1KO, the *hph* cassette is flanked on both sides by an approximately 0.6-kb *Nrf1* sequence (Fig. 2A). Subsequently, linear pNRF1KO was transformed to the *C. fulvum* wild-type strains races 4 and 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 their inability to grow on *C. fulvum* minimal medium containing, per liter, 0.5 g of KCl, 0.5 g of Mg-glycerophosphate, 1 mg of FeSO_4 , 0.35 g of K_2SO_4 , 15 g of sucrose, and 15 g of agar (pH 6.0) with 0.51 g of sodium nitrate as the sole nitrogen source. Genomic DNA of *C. fulvum* hygromycin B-resistant transformants that did not grow on this nitrate-selective medium was analyzed by PCR with the *Nrf1*-specific primers p9 (5'-GGCTCACCACAGTGGTTTC

AA-3') and p10 (5'-GCACAGAGTTGCCACTTCCG-3'), and the *hph*-specific primer p11 (5'-CATACACCGGGCAAA GCAGG-3') to determine whether the insertion of the *hph* gene had occurred at the *Nrf1* locus. Finally, DNA isolated from the putative *C. fulvum* *Nrf1*-disruptants was analyzed 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 by the fungus was isolated according to Laugé et al. (1997). Total RNA was isolated according to the manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.). Southern and Northern blotting procedures were performed, essentially according to Sambrook et al. (1989). Probes were labeled with the Random Primers Labeling System (Life Technologies, Rockville, MA, U.S.A.), including 20 μ Ci α^{32} P-dATP (Amersham, Buckinghamshire, England).

Plants and inoculations.

Ten-day-old PDA cultures of *C. fulvum* were used to prepare conidiospore suspensions (approximately 5×10^5 conidiospores per 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 containing only the functional *Cf-9* gene (*Hcr9-9C*) (Parniske et al. 1997) and mediating recognition of the AVR9 peptide (transgenic seeds provided by M. Parniske, Sainsbury Laboratory, John Innes Centre, Norwich, England) were used. Five-week-old plants were inoculated with conidiospore suspensions, as described by De Wit (1977).

Isolation and analysis of apoplastic fluids.

For detection of AVR9 in *C. fulvum*-infected tomato leaves, AFs were isolated, as described by De Wit and Spikman (1982), and injected into leaflets of 5-week-old MM-Cf9 plants containing the complete *Cf-9* locus (Parniske et al. 1997). AFs were analyzed on 15% (wt/vol) sodium dodecyl sulfate polyacrylamide gels. Western blot analyses were carried out after electrotransfer of the separated proteins to nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany). The blots were probed with polyclonal antibodies against ECP2, which is a reliable marker for colonization by *C. fulvum* (Laugé et al. 1997; Wubben et al. 1994). GARAP antibodies were obtained from Bio-Rad (Veenendaal, The Netherlands).

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LITERATURE CITED

Adrianopoulos, A., Kourambas, S., Sharp, J. A., Davis, M. A., and Hynes, M. 1998. Characterization of the *Aspergillus nidulans* *nmrA* gene involved in nitrogen metabolite repression. *J. Bacteriol.*

- 180:1973-1977.
- Arst, H. N., Jr., Tollervey, D., and Caddick, M. X. A. 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.
- Bogdanove, A. J., Bauer, D. W., and Beer, S.V. 1998. *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the Hrp (type III secretion) pathway. *J. Bacteriol.* 180:2244-2247.
- Burger, G., Strauss, C., and Franz Lang, B. 1991. *nirA*, the pathway-specific regulatory gene of nitrate assimilation in *Aspergillus nidulans*, encodes a putative GAL4-type zinc finger protein and contains four introns in highly conserved regions. *Mol. Cell Biol.* 11:5746-5755.
- Caddick, M. X., Peters, P., and Platt, A. 1994. Nitrogen regulation in fungi. *Antonie Leeuwenhoek* 65:169-177.
- Christensen, T., Hynes, M. J., and Davis, M. A. 1998. Role of the regulatory gene *areA* of *Aspergillus oryzae* in nitrogen metabolism. *Appl. Environ. Microbiol.* 64:3232-3237.
- Davis, M. A., Small, A. J., Kourambas, S., and Hynes, M. J. 1996. The *tamA* 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, P. J. G. M. 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, P. J. G. M., and Spikman, G. 1982. Evidence for the occurrence of race- and cultivar-specific elicitors of necrosis in intercellular fluids of compatible interactions between *Cladosporium fulvum* and tomato. *Physiol. Plant Pathol.* 2:1-11.
- Diallinas, G., Gorfinkiel, L., Arst, H. N., Jr., Cecchetto, G., and Scazzocchio, C. 1995. Genetic and molecular characterization of a gene encoding a wide specificity purine permease of *Aspergillus nidulans* reveals a novel family of transporters conserved in prokaryotes and eukaryotes. *J. Biol. Chem.* 270:8610-8622.
- Ellis, C. M. 1996. Regulation of polyketide gene expression: The isolation and function of nitrogen regulatory factor NRFA from *Penicillium urticae*. Ph.D. thesis. Biological Sciences, University of Calgary, Canada.
- 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, Y. H., and Marzluf, G. A. 1990. *nit-2*, the major nitrogen regulatory gene in *Neurospora crassa* encodes a protein with a putative zinc finger DNA-binding domain. *Mol. Cell. Biol.* 10:1056-1065.
- Gaudriault, S., Malandrin, L., Paulin, J. P., and Barny, M. A. 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.
- 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.
- Haas, H. B., and Marzluf, G. A. 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.
- Haas, H. B., Bauer, B., Redi, G., and Marzluf, G. A. 1995. Molecular cloning and analysis of *nre*, the major nitrogen regulatory gene of *Penicillium chrysogenum*. *Curr. Genet.* 27:150-158.
- Harling, R., Kenyon, L., Lewis, B. G., Oliver, R. P., Turner, J. G., and Coddington, A. 1988. Conditions for efficient isolation and regeneration of protoplasts from *Fulvia fulva*. *J. Phytopathol.* 122:143-146.
- Hensel, M., Arst, H. N., Jr., Aufaure-Brown, A., and Holden, D. W. 1998. The role of the *Aspergillus fumigatus* *areA* gene in invasive pulmonary aspergillosis. *Mol. Gen. Genet.* 258:553-557.
- Kamoun, S., Van West, P., De Jong, A. J., De Groot, K. E., Vleeshouwers, V. G. A. A., and Govers, F. 1997. A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol. Plant-Microbe Interact.* 10:13-20.
- Kinghorn, J. R., and Pateman, J. A. 1975. Studies of partially repressed mutants at the *tamA* and *areA* loci in *Aspergillus nidulans*. *Mol. Gen. Genet.* 140:137-147.
- Kudla, B., Caddick, M. X., Langdon, T., Martinez-Rossi, N. M.,

- Bennett, C. F., Sibley, S., Davies, R. W., and Arst H. N., Jr. 1990. The regulatory gene *areA* 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.
- Lau, G. W., and Hamer, J. E. 1996. Regulatory genes controlling *mpg1* expression and pathogenicity in the rice blast fungus *Magnaporthe grisea*. *Plant Cell* 8:771-781.
- Laugé, R., and De Wit, P. J. G. M. 1998. Fungal avirulence genes: Structure and possible functions. *Fungal Genet. Biol.* 24:285-297.
- Laugé, R., Joosten, M. H. A. J., Van den Ackerveken, G. F. J. M., Van den Broek, H. W. J., and De Wit, P. J. G. M. 1997. The in planta-produced extracellular proteins ECP1 and ECP2 of *Cladosporium fulvum* are virulence factors. *Mol. Plant-Microbe Interact.* 10:725-734.
- Lorang, J. M., and Keen, N. T. 1995. Characterization of *avrE* from *Pseudomonas syringae* pv. *tomato*: A *hrp*-linked avirulence locus consisting of at least two transcriptional units. *Mol. Plant-Microbe Interact.* 8:49-57.
- MacCabe, A. P., Vanhanen, S., Gelpke, M. D. S., Van de Vondervoort, P. J. I., Arst, H. N., and Visser, J. 1998. Identification, cloning and sequence of the *Aspergillus niger areA* wide domain regulatory gene controlling nitrogen utilisation. *Biochem. Biophys. Acta* 1396:163-168.
- Marzluf, G. A. 1997. Genetic regulation of nitrogen metabolism in the fungi. *Microbiol. Mol. Biol. Rev.* 61:17-32.
- Oliver, R. P., Roberts, I. N., Harling, R., Kenyon, L., Punt, P. J., Dingemans, M. A., and Van den Hondel, C. A. M. J. J. 1987. Transformation of *Fulva fulvia*, a fungal pathogen of tomato, to hygromycin B resistance. *Curr. Genet.* 12:231-233.
- Parniske, M., Hammond-Kosack, K. E., Golstein, C., Thomas, C. W., Jones, D. A., Harrison, K., Wulff, B. B. H., and Jones, J. D. G. 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.
- Pontecorvo, G., Roper, J. A., Hemmonds, L. M., McDonald, K. D., and Bufton, A. W. J. 1953. The genetics of *A. nidulans*. *Adv. Genet.* 5:141-239.
- Punt, P. J., Oliver, R. P., Dingemans, M. A., Pouwels, P. H., and Van den Hondel, C. A. M. J. J. 1987. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. *Gene* 56:117-124.
- Salmeron, J. M., 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, E. F., and Maniatis, T. A. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
- Screen, S., Bailey, A., Charnley, K., Cooper, R., and Clarkson, J. 1998. Isolation of a nitrogen response regulator gene (*nrr1*) from *Metarhizium anisopliae*. *Gene* 221:17-24.
- Shen, H., and Keen, N. T. 1993. Characterization of the promoter of avirulence gene D from *Pseudomonas syringae* pv. *tomato*. *J. Bacteriol.* 175:5916-5924.
- Small, A. J., Hynes, M. J., and Davis, M. A. 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.
- Snoeijsers, S. S., Vossen, P., Goosen, T., Van den Broek, H. W. J., and De Wit, P. J. G. M. 1999. Transcription of the avirulence gene *Avr9* of the fungal tomato pathogen *Cladosporium fulvum* is regulated by a GATA-type transcription factor in *Aspergillus nidulans*. *Mol. Gen. Genet.* 261:653-659.
- Stephenson, S. A., Green, J. R., Manners, J. M., and Maclean, D. J. 1997. Cloning and characterisation of glutamine synthetase from *Colletotrichum gloeosporioides* and demonstration of elevated expression during pathogenesis on *Stylosanthes guianensis*. *Curr. Genet.* 31:447-454.
- Tomsett, A. B., Dunn-Coleman, N. S., and Garret, R. H. 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, G. A. 1999. Isolation, characterization and disruption of the *areA* nitrogen regulatory gene of *Gibberella fujikuroi*. *Mol. Gen. Genet.* 261:106-114.
- Valent, B., and Chumley, F. G. 1991. Molecular genetic analysis of the rice blast fungus *Magnaporthe grisea*. *Annu. Rev. Phytopathol.* 29:443-467.
- Van den Ackerveken, G. F. J. M., Van Kan, J. A. L., and De Wit, P. J. G. M. 1992. Molecular analysis of the avirulence gene *avr9* of *Cladosporium fulvum* fully supports the gene-for-gene hypothesis. *Plant J.* 2:359-366.
- Van den Ackerveken, G. F. J. M., Dunn, R. M., Cozijnsen, T. J., Vossen, P., Van den Broek, H. W. J., and De Wit, P. J. G. M. 1994. Nitrogen limitation induces expression of the avirulence gene *avr9* in *Cladosporium fulvum*. *Mol. Gen. Genet.* 243:277-285.
- Van Kan, J. A. L., Van den Ackerveken, G. F. J. M., and De Wit, P. J. G. M. 1991. Cloning and characterization of cDNA of avirulence gene *avr9* of the fungal pathogen *Cladosporium fulvum*, causal agent of tomato leaf mold. *Mol. Plant-Microbe Interact.* 4:52-59.
- Vivian, A., and Gibbon, M. J. 1997. Avirulence genes in plant-pathogenic bacteria: Signals or weapons? *Microbiology* 143:693-704.
- Wernars, K., Goosen, T., Wennekes, L. M., Visser, J., Bos, C. J., Van den Broek, H. W. J., Van Gorcom, R. F., Van den Hondel, C. A., and Pouwels, P. H. 1985. Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amdS* gene. *Curr. Genet.* 9:361-368.
- Wiame, J. M., Grenson, M., and Arst, H. N., Jr. 1985. Nitrogen catabolite repression in yeast and filamentous fungi. *Adv. Microbiol. Physiol.* 26:1-87.
- Wubben J. P., Joosten M. H. A. J., and De Wit P. J. G. M. 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, Y., and Hutcheson, S. W. 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, Y., Heu, S., Yi, J., Lu, Y., and Hutcheson, S. W. 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 *hrmA* genes. *J. Bacteriol.* 4:1025-1036.
- Young, J. L., Jarai, G., Fu, Y. H., and Marzluf, G. A. 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, G. F., Fu, Y. H., and Marzluf, G. A. 1991. *nit-4*, a pathway-specific regulatory gene of *Neurospora crassa*, encodes a protein with a putative binuclear zinc finger DNA-binding domain. *Mol. Cell. Biol.* 11:5735-5745.