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The global nitrogen regulator, FNR1, regulates fungal nutrition-genes and fitness during *Fusarium oxysporum* pathogenesis

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SUMMARY

Fusarium oxysporum is a soil-borne pathogen that infects plants through the roots and uses the vascular system for host ingress. Specialized for this route of infection, *F. oxysporum* is able to adapt to the scarce nutrient environment in the xylem vessels. Here we report the cloning of the *F. oxysporum* global nitrogen regulator, *Fnr1*, and show that it is one of the determinants for fungal fitness during *in planta* growth. The *Fnr1* gene has a single conserved GATA-type zinc finger domain and is 96% and 48% identical to AREA-GF from *Gibberella fujikuroi*, and NIT2 from *Neurospora crassa*, respectively. *Fnr1* cDNA, expressed under a constitutive promoter, was able to complement functionally an *N. crassa nit-2*^{RIP} mutant, restoring the ability of the mutant to utilize nitrate. *Fnr1* disruption mutants showed high tolerance to chlorate and reduced ability to utilize several secondary nitrogen sources such as amino acids, hypoxanthine and uric acid, whereas growth on favourable nitrogen sources was not affected. *Fnr1* disruption also abolished *in vitro* expression of nutrition genes, normally induced during the early phase of infection. In an infection assay on tomato seedlings, infection rate of disruption mutants was significantly delayed in comparison with the parental strain. Our results indicate that FNR1 mediates adaptation to nitrogen-poor conditions *in planta* through the regulation of secondary nitrogen acquisition, and as such acts as a determinant for fungal fitness during infection.

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INTRODUCTION

In host–pathogen interactions, pathogen nutrition is a prerequisite as well as a goal for successful colonization. Nevertheless, the process of pathogen nutrition during host invasion remains poorly understood. For fungal phytopathogens it has been proposed that nitrogen availability during *in planta* growth may be a limiting factor (Coleman *et al*., 1997; Snoeijers *et al*., 2000; Stephenson *et al*., 1997; Talbot *et al*., 1993; Van den Ackerveken *et al*., 1994). Thus, in accordance with the particular life style, nutritional challenges may require various adaptations from the pathogen. Fungi can utilize a wide range of nitrogen sources. Ammonia and glutamine are preferred, but when these primary sources are absent or present in low concentrations, other secondary sources can be used (Marzluf, 1997). Utilization of these sources is highly regulated and requires *de novo* synthesis of a set of pathway-specific catabolite enzymes and permeases, which otherwise are under nitrogen catabolite repression (NCR). A key component in nitrogen utilization is the global nitrogen regulator, a transcription factor of the GATA-binding zinc finger type (Marzluf, 1997). In *Aspergillus nidulans* and *N. crassa* this factor is represented by AREA and NIT2, respectively (Arst and Cove, 1973; Fu and Marzluf, 1990), and transcription of NCR-regulated genes requires induction by AREA/NIT2, often in combination with a pathway-specific signal which indicates the presence of a specific substrate (Blinder and Magasanik, 1995; Fu and Marzluf, 1987a; Kudla *et al*., 1990; Marzluf, 1997). A typical feature of the GATA family of proteins is the highly conserved pair of vicinal Cys_2–Cys_2 residues composing the zinc finger DNA-binding domain that recognizes the consensus DNA sequence 5′-HGATAR-3′ (Ravagnani *et al*., 1997; Starich *et al*., 1998). In addition, under favourable (repressing) conditions, the conserved carboxyl terminus domain mediates, together with the DNA-binding

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domain, protein–protein interaction with a negative-acting regulator (NMR; Pan *et al*., 1997; Xiao *et al*., 1995).

As the nitrogen status of the cell influences many cellular processes, *AreA/nit-2* orthologues have been cloned and characterized from a wide variety of filamentous fungi. In plant pathogenic fungi, the global nitrogen regulators have been studied under the assumption that nitrogen starvation *in planta* will trigger pathogenicity genes through the action of AREA. The gene coding for the avirulence factor, AVR9, from *Cladosporium fulvum* is induced during nitrogen starvation (Van den Ackerveken *et al*., 1994), and this induction is controlled in part by the nitrogen response factor, NRF1 (Perez-Garcia *et al*., 2001). However, disruption of *Nfr1* did not completely abolish *Avr9* expression *in planta*, and did not affect avirulence nor virulence. By contrast, disruption of *Clnr1* in *Colletotrichum lindemuthianum* had severe effect on pathogenicity, compromising the infection cycle at the formation of secondary hyphae, and almost completely eliminating the formation of anthracnose symptoms (Pellier *et al*., 2003). Conversely, disruption of the *Nut1* gene in the rice blast fungus, *Magnaporthe grisea*, had only a minor effect on virulence (Froeliger and Carpenter, 1996). In *Gibberella fujikuroi AreA-GF* is required for gibberellin production (Tudzynski *et al*., 1999), but the pathogenicity of this mutant has not been described.

Based on the experimental evidence a unifying role for the global nitrogen regulator during pathogenesis is lacking, and its impact might vary according to the particular life style of the pathogen. *F. oxysporum* f. sp. *lycopersici* is a soil-borne facultative parasitic fungus and the causal agent for vascular wilt in tomato. The parasitic phase of *F. oxysporum* infection has been divided into three parts (Beckman, 1987). In the 'Determinative Phase I' the fungus infects tomato plants through the roots, either by direct incursion of penetration hyphae, or via wounds and cracks formed in the emerging lateral roots. The penetration continues through the cortex until the fungus reaches the vascular tissue. In the 'Determinative Phase II' the fungus uses the xylem vessels and the xylem sap stream to colonize the entire plant. During this phase little damage is inflicted on host tissues. The temporal and spatial aspects of Phase II are meant to optimize total fungal growth (Beckman, 1987), yet they force the fungus to be satisfied with the scarce nutrient availability of the xylem vessels. At some point fungal growth expands to adopt a necrotic-like 'Expressive Phase' starting about 10–14 days post infection (dpi). The typical wilt symptoms that have given name to the disease appear as a result of aggressive fungal growth accompanied by the collapse of infected vessels, and clogging due to mycelium and host-derived production of gums and tyloses.

We have recently provided evidence for nutritional adaptation of *F. oxysporum*, demonstrating concerted expression of organic nitrogen acquisition genes during early phases of infection (Divon *et al*., 2005). As nitrogen availability during *in planta* growth is presumed to be limiting, control mechanisms ensuring the availability of nutrients during infection might influence pathogen fitness. Here we report the cloning and characterization of the *Fusarium* nitrogen regulator, *Fnr1*, and show that it serves as a common transcriptional regulator of specific nutrition genes. Through the use of disruption mutants we provide evidence that this factor is a determinant of fungal fitness during *F. oxysporum* infection.

RESULTS

Cloning and sequence analysis of *Fnr1*, the putative nitrogen regulator gene from *F. oxysporum*

During *in planta* growth of *F. oxysporum* f. sp. *lycopersici* the transcription of several genes with putative roles in organic nitrogen acquisition was shown to be up-regulated (Divon *et al*., 2005). As AREA/NIT2 factors have been identified as regulators of nitrogen nutrition genes in filamentous fungi, we sought to isolate the *AreA/nit-2* orthologue from *F. oxysporum* and assess its role during adaptive phases of fungal growth. Based on sequence similarity to the *AreA-GF* gene from *G. fujikuroi* (Tudzynski *et al*., 1999) and a *Fusarium graminearum AreA*-like gene (labelled *Fg-AreA*), a putative *AreA/nit-2* orthologue, designated *Fusarium* nitrogen regulator 1, *Fnr1*, was isolated as described in the Experimental procedures. The genomic region spanned 2961 nt, consisting of three exons separated by two introns, and encoded a predicted open reading frame of 906 amino acids (GenBank accession no. DQ387858). Among the conserved domains was a 51-residue-long GATA-type zinc finger DNA binding domain situated at position 627–678 (Fig. 1A). Also conserved was an 11-residue motif at the carboxy terminus (Fig. 1B), known to be involved in protein–protein interactions with the nitrogen metabolite repressor, NMR, in *N. crassa* and *A. nidulans* (Pan *et al*., 1997; Xiao *et al*., 1995). The putative FNR1 protein shared 96% and 86% identity with the AREA factors from *G. fujikuroi* and *F. graminearum*, respectively, and was 48% identical to the *N. crassa* NIT2 protein. A more recently annotated version of the *F. graminearum AreA* gene (GenBank accession nos EAA72662.1 and XP_388810.1) predicts an additional 69 amino acid residues in the N-terminal part of the protein that share considerable similarity to the N-terminus of AREA-GF from *G. fujikuroi* and NUT1 from *M. grisea*, and moderate similarity to other global nitrogen regulators. The shorter version cloned here may therefore represent a truncated 5′ end, or an alternative 5′ start site of the *Fnr1* gene.

Multiple sequence alignment of FNR1 was performed with the putative *F. graminearum* FG-AREA factor and eight additional factors. Employing the neighbour-joining method (Saitou and Nei, 1987) a phylogenetic tree was obtained (Fig. 2). FNR1 clustered closely together with the orthologous factors of *G. fujikuroi* and *F. graminearum*, consistent with their common origin within the

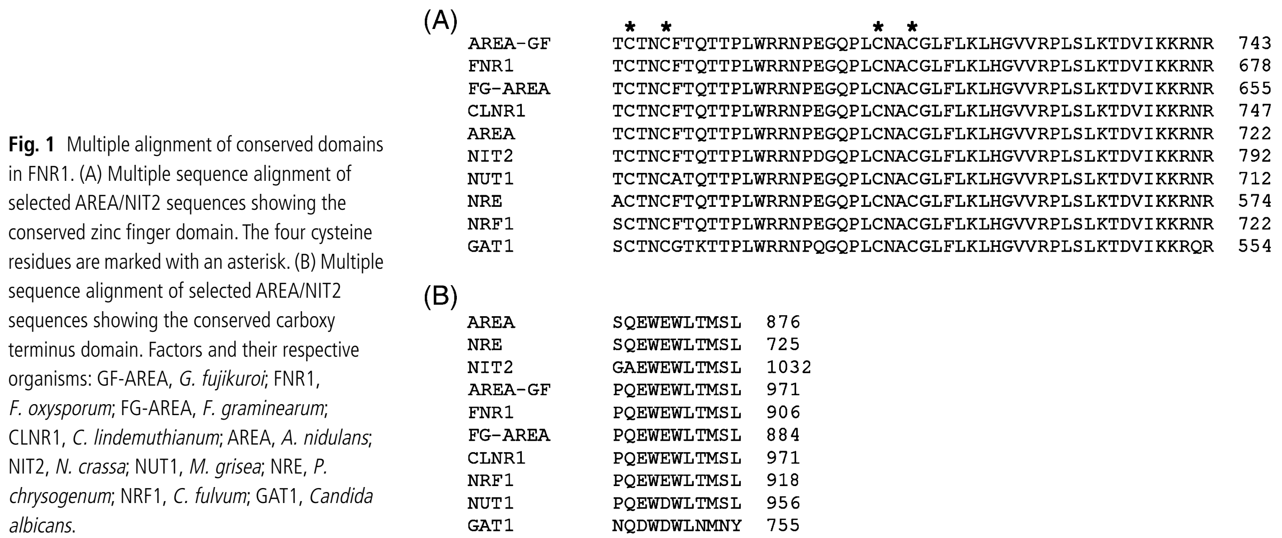

Fig. 1 Multiple alignment of conserved domains in FNR1. (A) Multiple sequence alignment of selected AREA/NIT2 sequences showing the conserved zinc finger domain. The four cysteine residues are marked with an asterisk. (B) Multiple sequence alignment of selected AREA/NIT2 sequences showing the conserved carboxy terminus domain. Factors and their respective organisms: GF-AREA, *G. fujikuroi*; FNR1, *F. oxysporum*; FG-AREA, *F. graminearum*; CLNR1, *C. lindemuthianum*; AREA, *A. nidulans*; NIT2, *N. crassa*; NUT1, *M. grisea*; NRE, *P. chrysogenum*; NRF1, *C. fulvum*; GAT1, *Candida albicans*.

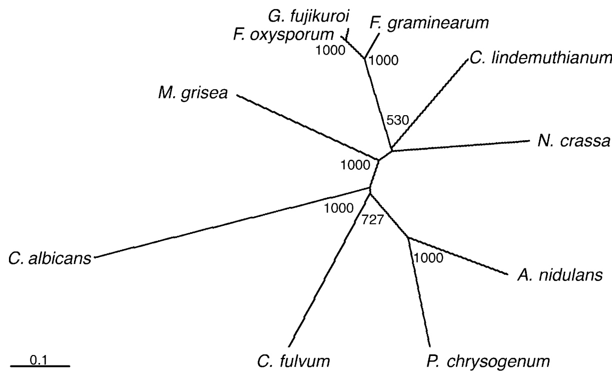

Fig. 2 Phylogenetic analysis of AREA/NIT2 orthologues. The analysis was based on full-length amino acid sequence using the neighbour-joining method. Shown is a radial tree and bootstrap scores based on 1000 reiterations (values indicated on the nodes). Note the highly significant subclade of the factors from the *Fusarium* spp. The GAT1 factor from *C. albicans* was defined as an outgroup. The node to *N. crassa* shows trichotomy. Scale bar indicates number of substitutions per site.

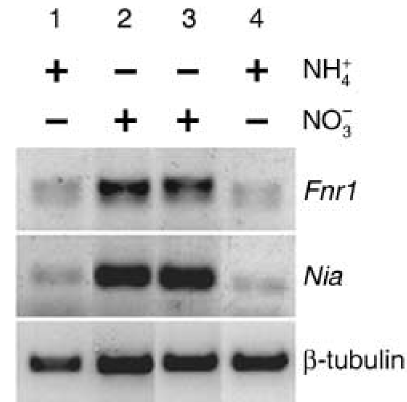

Fig. 3 Coordinate expression of the *Fnr1* and *Nia* genes. Expression of *Fnr1* and *Nia* (accession no. CK615583) was tested using RT-PCR under repressive (25 mM ammonium) and de-repressive (25 mM nitrate) conditions. Fungi were grown in repressive medium for 60 h (lane 1), washed and transferred to de-repressive medium for 3 and 6 h (lanes 2 and 3), washed and transferred back to repressive medium for 50 min (lane 4). Beta-tubulin (accession no. AY641578) served as control. Primers are shown in Table 1: *Fnr1*, primer 76044/76045; *Nia*, primer Fol11H10F/Fol11H10R; β-tubulin, FolbtubFW/FolbtubREV.

order Hypocreales. It was more distantly related to those of *Aspergillus* and *Penicillium.* The tight clade of FNR1 with AREA-GF from *G. fujikuroi* strengthens the evidence for a role as global nitrogen regulator for this protein.

Expression of *Fnr1*

Although highly conserved in sequence, *AreA/nit-2* homologues from various organisms vary in their mode of expression in response to nitrogen sources (Fu and Marzluf, 1987a; Gente *et al.*, 1999; Haas *et al.*, 1995; Platt *et al.*, 1996; Tao and Marzluf, 1999; Tudzynski *et al.*, 1999). Using semiquantitative RT-PCR, we tested the expression of *Fnr1* under repressive and de-repressive conditions mediated by ammonium and by nitrate, respectively. The *Fnr1* gene showed a constitutive, low level of expression during growth with ammonium; however, a marked increase in expression was noted after transfer of mycelia to nitrate-medium (Fig. 3). Upon transfer back to ammonium, transcription of *Fnr1* returned to the basal level within 50 min. This indicates a level of transcriptional control and/or rapid transcript turnover of the *Fnr1* gene. Transcription of the nitrate reductase gene, *Nia*, was correlated with the level of *Fnr1* transcript, which is in agreement with that found for *N. crassa* and *G. fujikuroi* (Fu and Marzluf,

1987b; Johnstone *et al.*, 1990; Okamoto *et al.*, 1991; Tudzynski *et al.*, 1999).

Functional analysis of FNR1

In order to demonstrate the functionality of the *Fnr1* gene product, the coding region of the *Fnr1* gene was fused to a 1.5-kb fragment of modified *cpc-1* constitutive-type promoter (plasmid pHD2; see Experimental procedures). Plasmid pHD2 was used to complement the *N. crassa nit-2* RIP3 mutant, carrying an RIP-induced loss-of-function mutation in the *nit-2* gene. The *nit-2*^{RIP} mutant lacks the ability to grow on nitrate, due to the absence of the NIT2 protein, but grows well on ammonium as nitrogen source (Tao and Marzluf, 1999). Upon co-transformation of the *nit-2*^{RIP} mutant with pHD2 and pMP6 (conferring hygromycin resistance), hygromycin-resistant transformants exhibiting restoration of nitrate utilization were recovered (Fig. 4A). Importantly, this ability correlated with the presence of *Fnr1* DNA and transcript (Fig. 4B,C). None of the hygromycin-resistant control strains, *nit-2*^{RIP} transformed with only pMP6, grew on nitrate. Furthermore, complemented *nit-2*^{RIP} mutants showed a restored sensitivity to chlorate, indicating re-establishment of nitrate reductase (NIT3) activity and reduction of chlorate (CLO_3^-) to toxic chlorite (CLO_2^-) (data not shown). These results show that the *Fnr1* gene encodes a functional orthologue of the *N. crassa* NIT2 protein. The ability to restore transcription of NCR-regulated genes demonstrates the functional conservation of the AREA/NIT2 factor across phylogenetic boundaries.

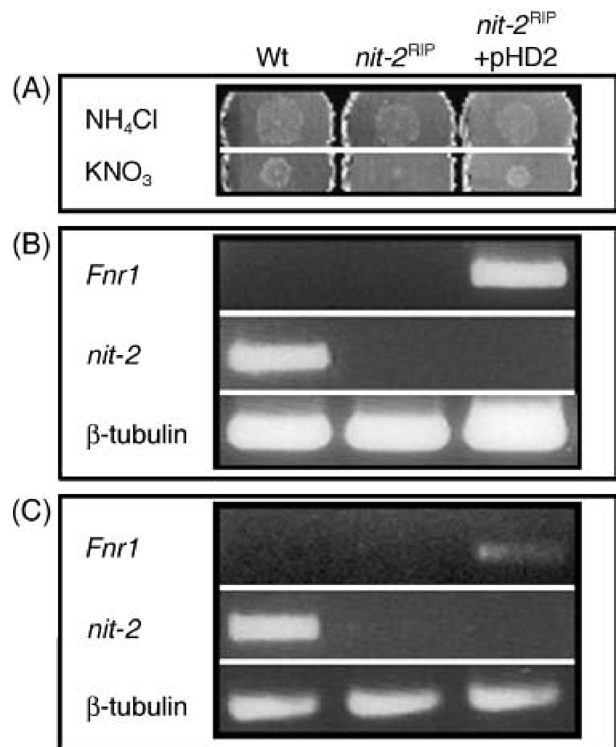

Fig. 4 Complementation of the *N. crassa nit-2*^{RIP} mutant with *Fnr1*. (A) Growth of wild-type, *nit-2*^{RIP} mutant and an *N. crassa nit-2*^{RIP}/*Fnr1* transformant on different media. Spore suspensions were deposited in the centre of a Petri dish (white spot) and the appearance of radial growth was assayed. Plates were cultured at 34 °C on Vogel minimal medium in which NH_4NO_3 was substituted by either 20 mM KNO_3 or NH_4Cl. (B) Presence of *Fnr1*, *nit-2* and β-tubulin DNA in wild-type, the *nit-2*^{RIP} mutant and *N. crassa nit-2*^{RIP}/*Fnr1* transformant, as determined by genomic PCR. (C) Presence of *Fnr1*, *nit-2* and β-tubulin transcript in wild-type, *nit-2*^{RIP} mutant and *N. crassa nit-2*^{RIP}/*Fnr1* transformant, as determined by RT-PCR. Primers are shown in Table 1: *Fnr1*, primer 76044/76045; *nit-2*, nit2–1298L/nit2–1826R; *N. crassa* β-tubulin, btub1304L/btub1862R.

Disruption of the *Fnr1* gene

F. oxysporum Fnr1 disruption mutants were generated by homologous gene replacement in which part of the coding region of the *Fnr1* gene was excised and replaced by a hygromycin cassette (plasmid pHD1; see Experimental procedures). Hygromycin-B-resistant colonies were obtained and screened using three different primers in combination with primer 78192, specific for the 5′ untranslated region of the *Fnr1* gene (Fig. 5A). Primer pair 79906/78192 amplified in wild-type and ectopic integration strains a PCR product of 2293 kb. Disruption of *Fnr1* should cause a net increase of 831 bp in this region, which was confirmed for transformants 1804P-32 (D1) and 2104P-151 (D2; Fig. 5B, panel 1). Using primer pair 73582/78192, a PCR product of 1896 bp containing the 5′ part of the *Fnr1* gene and the inserted hygromycin cassette was obtained exclusively from transformants D1 and D2 (Fig. 5B, panel 2). Finally, primer pair 73583/78192, specific for a 611-bp region of the *Fnr1* gene that was missing in the replacement vector, was designed to yield a PCR product of 1733 bp only in the ectopic-type integration and wild-type strain. PCR confirmed the absence of this region in transformants D1 and D2, whereas two other transformants, 2104-P-122 (ND1) and 2104-P-62 (ND2), represented ectopic-type integrations (Fig. 5B, panel 3). Southern analysis using the *Hph* gene as a probe revealed that transformant D2 had undergone a single insertion event, whereas transformant D1 contained two copies of the *Hph* gene (Fig. 5C). From these results we concluded that transformants D1 and D2 were disrupted in the *Fnr1* gene by homologous recombination, but that mutant D1 also contained an additional insertion of the *Hph* gene.

Growth properties of *fnr1*$^-$ mutants *in vitro*

In order to examine *Fnr1* function we analysed the single integration mutant D2, the integration and ectopic mutant D1, as well as 2104-P-122 (ND1) and 2104-P-62 (ND2), which represent solely ectopic-type integrations. Mutations in the *AreA/nit-2*-like

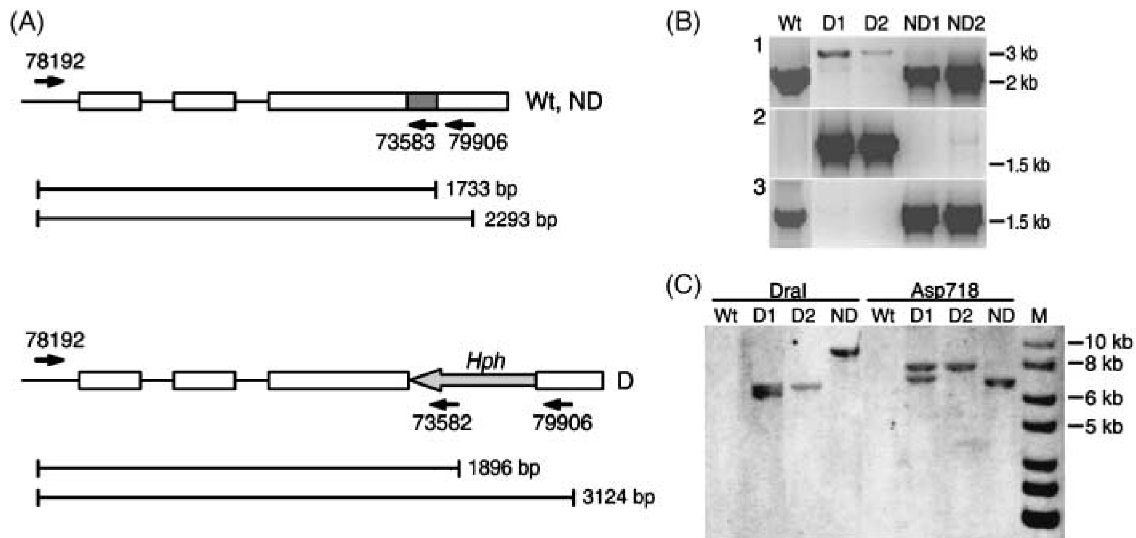

Fig. 5 Examination of *Fnr1* disruption mutants. (A) Schematic presentation of primer sites used for genomic PCR screening to differentiate between ectopic and homologous fungal recombinants. The *Fnr1* gene is presented as would be expected in wild-type and ectopic integration strains (Wt, ND; upper panel), and for disruption mutants (D; lower panel). Small black arrows indicate primers. Empty boxes indicate *Fnr1* exons, lines indicate 5′ UTR and introns, dark grey box in upper panel indicates a fragment of the *Fnr1* gene that is excised from the replacement construct, and grey arrow in lower panel indicates hygromycin cassette insertion. Scale bars indicate expected length of the respective PCR products. Primers are as follows: primer 78192, specific for *Fnr1* 5′ UTR region; primer 79906, specific for the region downstream of expected integration of the hygromycin cassette; primer 73582, specific for the hygromycin cassette; and primer 73583, specific for an excised fragment of the *Fnr1* gene in disruption mutants. Primer sequences are shown in Table 1. (B) Genomic PCR results of wild-type, disruption mutant strains, 1804-P-32 (D1) and 2104-P-151 (D2), and two ectopic integration strains, 2104-P-122 (ND1) and 2104-P-62 (ND2). Shown are PCR products using primer 78192 in combination with: primer 79906 (panel 1), yielding PCR products of 2293 bp for wt and ND1 and 2, and 3124 bp for strain D1 and 2; primer 73582 (panel 2), yielding PCR products of 1896 bp for D1 and 2; and primer 73583 (panel 3), yielding PCR products of 1733 bp for wt and ND1 and 2. (C) Southern blot analysis of wild-type, disruption mutant strains, 1804-P-32 (D1) and 2104-P-151 (D2), and ectopic integration strain, 2104-P-122 (ND). Genomic DNA was digested with either *Dra*I or *Asp*718, which do not digest within the *Fnr1* gene and the replacement construct. Southern blots were probed for the presence of the *Hph* gene using DIG-labelling.

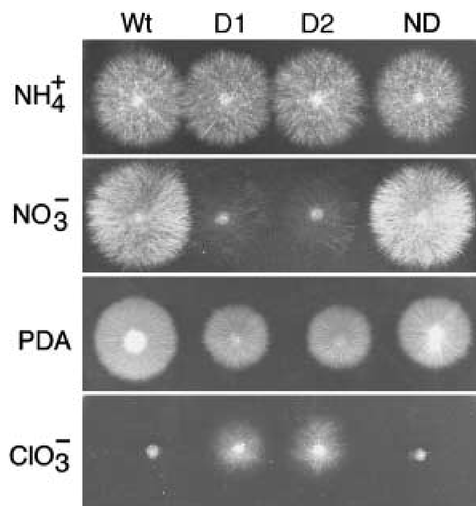

Fig. 6 Growth of *fnr1*$^-$ mutants on media that is selective for nitrate reductase activity. The radial growth of wild-type, disruption mutants 1804-P-32 (D1) and 2104-P-151 (D2), and ectopic integration transformant 2104-P-122 (ND) as tested on solid minimal medium containing 50 mM NH_4^+ or NO_3^-, or PDA medium supplemented with 6% potassium chlorate. Growth was measured after 2 days of incubation.

gene generally result in loss of or reduced ability to utilize a wide range of nitrogen sources including nitrate (Froeliger and Carpenter, 1996; Pellier *et al.*, 2003; Perez-Garcia *et al.*, 2001). The lack of nitrate reductase activity was confirmed for the *fnr1*$^-$ mutants by testing radial growth on solid medium containing nitrate as the sole nitrogen source, and by testing tolerance to chlorate (Fig. 6). Disruption mutants displayed a reduced ability to grow on nitrate as the sole nitrogen source, as opposed to growth on ammonia or on rich medium (Fig. 6, panels NO_3^-, NH_4^+ and PDA). Whereas potato dextrose agar (PDA) supplemented with 6% chlorate completely inhibited fungal growth of wild-type and the ectopic-type integration transformants, the disruption mutants developed medium-sized colonies within 4 days (Fig. 6, panel CLO_3^-).

In order to test the range of secondary nitrogen utilization affected in this mutant, growth on different nitrogen sources was examined. As shown in Fig. 7, the growth of all mutants on glutamine (5 and 50 mM) was indistinguishable from the wild-type. However, incubation for 2 days on minimal medium supplemented with 5 mM asparagine as the sole nitrogen source resulted in intermediate growth of the mutants as compared with wild-type and an ectopic integration transformant. Glutamate, histidine and proline further differentiated between the wild-type/ectopic

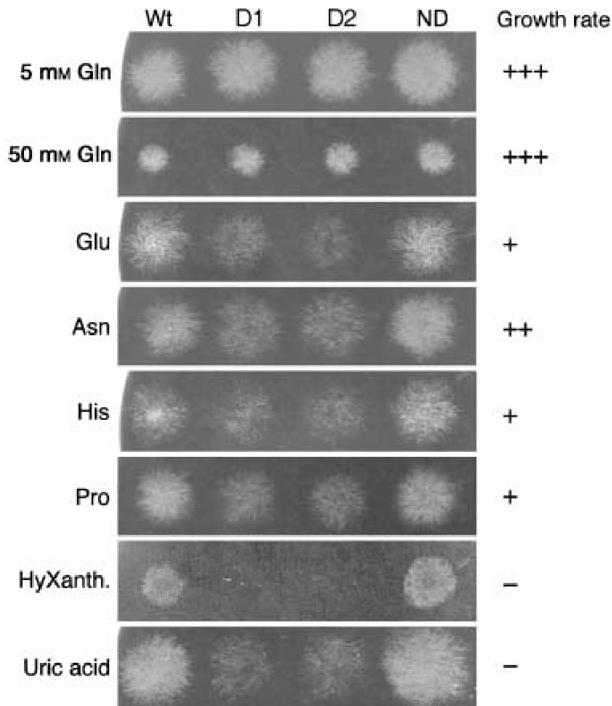

Fig. 7 Reduced growth of *fnr1*⁻ mutants on various secondary nitrogen sources. Radial growth of wild-type, disruption mutants 1804-P-32 (D1) and 2104-P-151 (D2), and ectopic integration transformant 2104-P-122 (ND) as tested on solid minimal medium containing 5 or 50 mM glutamine, or 5 mM of glutamate, asparagine, histidine, proline, hypoxanthine (HyXanth.) or uric acid as the sole nitrogen source. Growth was measured after 2 days of incubation. Growth rate is indicated for mutants (as compared with wild-type) with: +++, strong growth; ++, intermediate growth; +, slow growth; –, very weak or no growth.

and the *fnr1*⁻ mutants. The greatest delay in growth of the mutants was noted on minimal medium supplemented with 5 mM hypoxanthine or uric acid. Apart from the effects noted on nitrogen source utilization, the mutants behaved like wild-type with respect to development and sporulation (data not shown). The results above confirm functionality of FNR1 as a global nitrogen regulator.

The *Fnr1* gene product regulates transcripts identified with nitrogen acquisition

Our previous work has shown common up-regulation of a subgroup of nitrogen acquisition genes under conditions of nitrogen starvation (NS) and *in planta*: general amino acid permease (*Gap1*), peptide transporter (*Mtd1*) and Uricase (Divon *et al.*, 2005). We examined whether FNR1 is responsible for the control of their coordinate expression. To this end, we compared expression of the three *in planta*-induced transcripts during control and NS conditions in *F. oxysporum* wild-type, *fnr1*⁻ mutants, and in

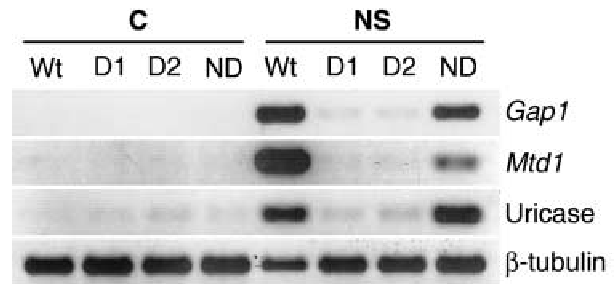

Fig. 8 Transcript accumulation of nutrition genes in wild-type and *fnr1*⁻ mutants during growth in nitrogen-limiting medium. RT-PCR showing expression of *in planta*-induced nutrition genes in wild-type (Wt), disruption mutants 1804-P-32 (D1) and 2104-P-151 (D2), and ectopic integration transformant 2104-P-122 (ND), during growth in control (C) and nitrogen starvation (NS) conditions. The β-tubulin gene (accession no. AY641578) was used as a constitutive control. *Gap1* (accession no. CK615491, CK615492), *Mtd1* (accession no. CK615494), and Uricase (CK615495) are identified in Divon *et al.* (2005). Primers are shown in Table 1; β-tubulin, FolbtubFW/FolbtubREV.

one ectopic integration transformant. As shown in Fig. 8, under NS conditions transcript accumulation of all three genes was abolished or greatly reduced in the *fnr1*⁻ mutants, indicating that the *Fnr1* gene product is the regulator of these genes in *F. oxysporum*. Importantly, the low expression of the general amino acid permease and uricase in the mutants was in accordance with the minimal growth noted on solid medium supplemented with various amino acids, or with hypoxanthine or uric acid.

Fnr1 disruption affects fungal fitness during *in planta* growth

The role of FNR1 in regulation of nutrition genes previously shown to be induced *in planta* (Divon *et al.*, 2005) suggests that it might determine fitness of *F. oxysporum* during infection. To test this, we performed infection assays on 14-day-old tomato seedlings and compared the infection rate of disruption and ectopic-type integration transformants to the wild-type. The two independent insertion transformants D1 and D2 yielded the same growth patterns in all experiments that were carried out in a variety of growth assays. Both mutants showed the expected phenotype based on knowledge from other fungi in which the *AreA* gene was characterized, and thus the mutant phenotype is consistent with disruption of the *Fnr1* gene. We therefore used the single integration mutant D2 in pathogenicity tests and included the D1 mutant for confirmation of the results. One other transformant, 2104-P-122 (ND1) represented ectopic-type integrations. Tomato seedlings (25 plants each) were inoculated by immersing the roots in conidial suspensions (2×10^5 conidia/mL) and thereafter planting them in inert perlite. Disease symptoms

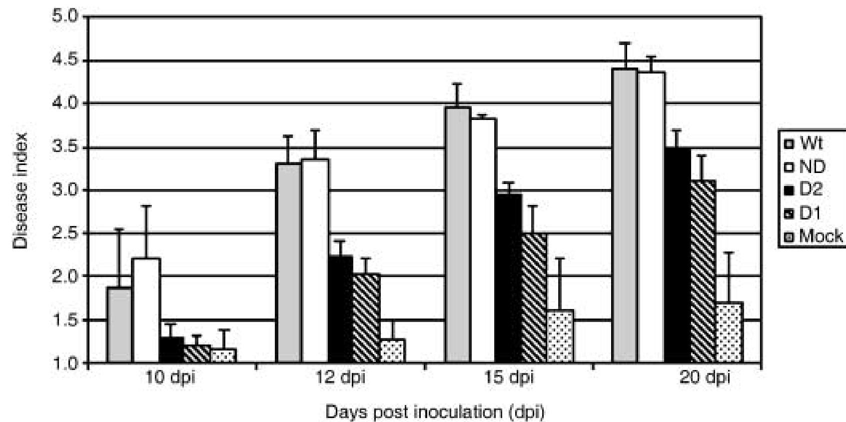

Fig. 9 Progression of wilt disease in tomato seedlings infected with various strains of *F. oxysporum*. Plants of the susceptible cultivar MoneyMaker were inoculated with a spore concentration of 2×10^5 conidia/mL of *F. oxysporum* wild-type, two *fnr1*⁻ mutant strains (1804-P-32, D1; and 2104-P-151, D2), and ectopic integration strain 2104-P-122 (ND). Disease severity was scored at different days post infection (dpi), using an index from: 1, healthy plant; 2, one wilted leaf; 3, two wilted leaves; 4, three or more wilted leaves; 5, dead plant. Infections with wild-type and D2 were repeated three times; infections with D1, ND and mock were repeated twice. Error bars indicate the standard deviations of two or three repetitions.

were scored at different time intervals using a scale ranging from 1 (healthy plant) to 5 (dead plant) according to Di Pietro *et al.* (2001). The results of three independent infection experiments are shown in Fig. 9. The onset of noticeable disease symptoms on plants infected with wild-type fungus was at 10 dpi with an average index of 1.88 for wild-type. At 20 dpi a large number of the plants were dead (average index = 4.40; Fig. 9). When comparing infection of tomato seedlings infected with wild-type fungus to two independent *fnr1*⁻ mutant strains, a clear delay in development of disease symptoms was noted. The delay was obvious even at 10 dpi (average index of mutant strains = 1.30 and 1.20 as opposed to 1.88 for the wild-type). As the infection progressed this difference was maintained. Symptoms in both the wild-type and the mutant strains were accompanied by browning of the vascular tissue. The mock-inoculated control plants did not show any signs of browning (average index at 20 dpi = 1.71). Hygromycin- and chlorate-resistant colonies were recovered from the stem of seedlings infected with the *fnr1*⁻ mutants at 20 dpi, indicating that the *Fnr1* disruption was retained throughout the infection. Fungal avirulence was not affected by the *Fnr1* disruption as determined by normal resistance of the cultivar Motelle containing the I2 gene to this mutant (data not shown). The results indicate that disruption of the *Fnr1* gene affects fungal fitness during *in planta* growth.

DISCUSSION

Efficient acquisition of nutrients from the host is the essence of parasitic interaction, and nutritional adaptability might be expected to affect a pathogen's virulence. Regulatory components of nutrient assimilation, such as the AREA/NIT2 global nitrogen regulator, have been studied in several fungi, but their role in determining pathogenicity has yet to be fully clarified (Froeliger and Carpenter, 1996; Pellier *et al.*, 2003; Perez-Garcia *et al.*, 2001).

In the present work, we have cloned and characterized the global nitrogen regulator gene from *F. oxysporum*, *Fnr1*. As determined by phylogenetic analysis, the *Fnr1* gene encodes a protein closely related to global nitrogen regulators from saprobes and plant pathogenic fungi within the group of Pyrenomycetes (*N. crassa*, *M. grisea*, *G. fujikuroi*, *F. graminearum* and *C. lindemuthianum*), as well as Plectomycetes (*A. nidulans* and *Penicillium chrysogenum*) and Hyphomycetes (*C. fulvum*). Within the predicted protein sequence is a single, conserved, GATA-type zinc finger DNA binding domain. Also conserved are the last 11 amino acid residues at the carboxy terminus of the protein, known to mediate interaction with the negative regulator, NMR (Pan *et al.*, 1997; Xiao *et al.*, 1995). At the transcriptional level, the *Fnr1* gene is regulated in response to nitrogen, but a low basal level of expression is maintained regardless of the nitrogen source. Our results indicate that its transcription is repressed by ammonium and induced by nitrate. Rapid disappearance of *Fnr1* transcript upon transfer to ammonium also indicates that the mRNA is subject to rapid turnover. This is consistent with the behaviour of the *AreA*/*nit-2* orthologues from *G. fujikuroi* and *A. nidulans* (Platt *et al.*, 1996; Tudzynski *et al.*, 1999), whereas the level of *nit-2* mRNA in *N. crassa* appears to be very stable (Tao and Marzluf, 1999). The *Fnr1* cDNA driven by a constitutive promoter was able to complement an *N. crassa nit-2*^{RIP} mutant, restoring its ability to utilize nitrate as the sole nitrogen source. The use of a constitutive promoter did not seem to affect *Fnr1* functionality as a nitrogen regulator and the results are similar to *nit-2* complementation by

the *AreA-GF* gene from *G. fujikuroi*, driven from a native promoter (Tudzynski *et al.*, 1999). Other *AreA/nit-2* orthologues have been shown to complement the more distantly related saprophyte, *A. nidulans* (Froeliger and Carpenter, 1996; Pellier *et al.*, 2003), indicating the high degree of functional conservation within this gene family.

Despite the common function as global nitrogen regulators further subtle differences are indicated by the differential ability of *AreA/nit-2* mutants to utilize secondary nitrogen sources. We found *fnr1*⁻ mutants to be particularly compromised in their utilization of nitrate, hypoxanthine and uric acid. This is commonly found for other *AreA/nit-2* mutants (Froeliger and Carpenter, 1996; Pellier *et al.*, 2003; Perez-Garcia *et al.*, 2001). Asparagine was able to support intermediate growth of *fnr1*⁻ mutants on solid medium, which is similar to what was found in *C. lindemuthianum* (Pellier *et al.*, 2003). Whereas the *fnr1*⁻ mutants exhibited limited growth on proline and glutamate, these sources resulted in full growth in the *nut1*⁻ mutant in *M. grisea* (Froeliger and Carpenter, 1996). Interestingly, inactivation of the *Nrf1* and *Clnr1* genes from *C. fulvum* and *C. lindemuthianum*, respectively, also resulted in reduced ability to grow on favourable nitrogen sources, ammonia in the case of *nrf1*⁻, and both ammonia and glutamine in the case of *clnr1*⁻ (Pellier *et al.*, 2003; Perez-Garcia *et al.*, 2001). We did not find this to be the case in *F. oxysporum.* In summary, it is evident, from this work and others that disruption of the major nitrogen regulatory gene causes drastic changes in the ability to metabolize a wide range of nitrogen sources for the fungus; however, the specific effects of the different sources may vary with each fungus.

F. oxysporum mutants disrupted in the *Fnr1* gene exhibited reduced fitness in an infection assay on susceptible tomato seedlings. This indicates that FNR1, although not essential for pathogenicity, is a determinant for fungal virulence through its impact on fungal fitness. Previous work has shown that fungal nutrition genes (*Gap1*, *Mtd1* and *Uricase*) are jointly up-regulated during nitrogen starvation and *in planta* early growth stages (Divon *et al.*, 2005). Here we show that FNR1 is the common regulator of these genes in agreement with studies conducted in *Saccharomyces cerevisiae*, *N. crassa* and *A. nidulans* (Nahm and Marzluf, 1987; Oestreicher and Scazzocchio, 1993; Stanbrough and Magasanik, 1996). The delayed infection rate of *fnr1*⁻ mutants is probably due to the absence of such nutrition gene products. It is well documented that additional permeases and metabolic enzymes are under the control of AREA/NIT2 (Marzluf, 1997), e.g. proteases (Cohen, 1973; Hanson and Marzluf, 1975). Although not verified in the present study, the same mode of regulation would be expected in *F. oxysporum.* Proteases have received much attention as virulence factors, and it is also conceivable that they might act, possibly in concert with amino acid and peptide transporters, to contribute to nutrition and fungal fitness *in planta*, and hence to the *fnr1*⁻ mutant phenotype. Although *Fnr1* disruption also affected expression of nitrate reductase, it is unlikely that absence of this activity is responsible for the *fnr1*⁻ phenotype *in planta* as we have shown that the nitrate reductase gene, *Nia*, is actually down-regulated during *F. oxysporum* infection (Divon *et al.*, 2005). Additionally, nitrate-non-utilizing mutants of *M. grisea* and *Stagonospora nodorum* retain full pathogenicity (Cutler *et al.*, 1998; Lau and Hamer, 1996).

Although compromised, infection by *fnr1*⁻ mutant strains is not abortive. This can be explained by the fact that disruption of the *Fnr1* gene did not affect utilization of other favourable nitrogen sources such as ammonia, glutamine and, to a lesser extent, asparagine. The major amino acid in the xylem of 46-day-old tomato plants was found to be glutamine, present at a concentration of 0.5 mM (White, 1981). Notably, amino acid levels in tomato leaves infected with *Pseudomonas syringae* indicated that asparagine is the major transported amino acid (Perez-Garcia *et al.*, 1998). Similarly, asparagine and glutamine are involved in remobilization of nitrogen during leaf senescense (Dangl *et al.*, 2000), which probably takes place during *Fusarium* wilt. Although these amino acids are the main nitrogen sources, the results here show that other nitrogen sources are important as well.

Our findings are similar to that shown for the *nut1*⁻ mutant from *M. grisea*, which showed reduction in spot size in a mild infection assay on rice leaves (Froeliger and Carpenter, 1996). In other plant-pathogens, however, disruption of the global nitrogen regulator has caused variable effects. In *C. fulvum* disruption of the nitrogen response factor gene, *Nrf1*, did not affect virulence (Perez-Garcia *et al.*, 2001). As a biotrophic fungus, *C. fulvum* might have developed other ways for coping with scarce *in planta* nutrient availability that include forcing the plant into increasing the apoplastic nitrogen content (Solomon and Oliver, 2001, 2002). Conversely, *Clnr1* disruption in *C. lindemuthianum* drastically compromised the infection cycle on the common bean (Pellier *et al.*, 2003). Cytological analysis revealed that *clnr1*⁻ mutants were impaired at the formation of secondary hyphae during the onset of the necrotrophic phase, indicating that CLNR1 is necessary to support the sudden increase in fungal biomass at this stage. By contrast, *fnr1*⁻ mutants seem to be affected during the determinative phases as the disease development was delayed, yet was not abortive. The lengthy determinative phase in *F. oxysporum* colonization in which it can draw upon available plant resources attenuates the effect of *Fnr1* on fungal infection. Thus, the gradual disease development of *F. oxysporum* and *M. grisea* may make them less susceptible to nitrogen limitation, here manifested by a less strict dependency on the nitrogen regulator than what was found for *C. lindemuthianum*. In addition, the tissue specificity of the pathogen might dictate the differential need for the global nitrogen regulator during infection. This is exemplified by the finding that root infection by *M. grisea* is dependent on the *Nut1* gene product to a greater extent than reported for leaf infection (Dufresne and Osbourn, 2001).

Finally, we cannot rule out the possibility that other developmental or pathogenicity factors, unrelated to nitrogen metabolism, are regulated by FNR1, and might affect fungal virulence. The *Avr9* gene regulated by NRF1 (Perez-Garcia *et al.*, 2001), and the hydrophobin gene *Mpg1* from *M. grisea*, controlled by the additional nitrogen regulators NPR1 and NPR2 (Lau and Hamer, 1996), are examples of such genes. However, screening of a subtractive library of nitrogen-starved fungus failed to identify such genes as induced both during nitrogen starvation and *in planta* growth (Divon *et al.*, 2005).

Plant pathogen nutrition during infection is still poorly understood, and the complex effect of nutrition on cellular processes makes the interpretation of such data difficult. The global nitrogen regulator, FNR1, from *F. oxysporum* is the common activator of nutrition genes that are up-regulated during early stages of infection. *Fnr1* disruption mutants cause delayed disease symptoms on tomato seedlings. FNR1 can thus be regarded as one of the signals accounting for fungal fitness during infection, most probably through the regulation of nitrogen nutrition. Global nitrogen regulators have been studied in several plant pathogenic fungi; however, the demand for this factor during infection varies and needs to be analysed in the context of each particular plant–pathogen interaction. *F. oxysporum* f. sp. *lycopersici*, as a soil-borne and root-infecting pathogen on tomato, adds new perspective to the involvement of this factor during vascular disease.

EXPERIMENTAL PROCEDURES

Fungal strains and culture conditions

Fusarium oxysporum f. sp. *lycopersici* (race 2) was obtained from the laboratory of Y. Katan, The Hebrew University of Jerusalem, Israel (Ori *et al.*, 1997; Sela-Buurlage *et al.*, 2001). The fungal strain was stored at room temperature as conidia dried in sterilized sand. The pathotype of the isolate was periodically confirmed by plant infection assays. *N. crassa* wild-type strain 74-OR23-1A; FGSC 987 was obtained from the Fungal Genetics Stock Center and the *nit-2* RIP3 mutant (Tudzynski *et al.*, 1999) was kindly provided by G. Marzluf. For gene expression analysis in *F. oxysporum*, conidia were obtained from 3–4-day-old potato dextrose broth (PDB) cultures. For nitrate reductase expression conidia at a concentration of 1×10^6 spores/mL were cultured in repressive conditions (25 mM ammonium) for 60 h, then washed and transferred to de-repressive conditions (25 mM nitrate) for 3 and 6 h, then washed again and transferred back to repressive conditions for 50 min. For analyses of nutrition-gene expression during control (C) and nitrogen starvation (NS) conditions, young mycelia from 2-day-old cultures in PDB were filtered, rinsed thoroughly, and cultured as described in Divon *et al.* (2005). The medium was supplemented with 65 μg/mL hygromycin B (Calbiochem, San Diego, CA) where appropriate. Growth assays on solid medium for *fnr1*$^-$ mutants, an ectopic integration transformant and wild-type were performed on Czapek medium (per litre; 2 g $NaNO_3$, 0.5 g KCl, 0.5 g $MgSO_4·7H_2O$, 1 g K_2HPO_4, 0.01 g $FeSO_4$, 30 g sucrose) with 1.5% Noble agar (Difco Laboratories, Detroit, MI) and when required, $NaNO_3$ was substituted with various nitrogen sources (see text). Chlorate resistance was tested on potato dextrose agar (PDA) medium supplemented with 6% potassium chlorate (Sigma, St Louis, MO). Experiments were repeated twice. For re-isolating the fungal strains from plants, seedlings infected with mutant strains were incubated on PDA plates supplemented with 6% potassium chlorate. Seedlings infected with wild-type or an ectopic integration strain were placed on Czapek agar plates. Hygromycin B (65 μg/mL) was added where appropriate. *N. crassa* strains were grown for 20 h at 30 °C in Vogel's-N medium with 2% (w/v) sucrose and 20 mM NH_4Cl. The mycelia were filtered, rinsed and transferred to Vogel's-N medium supplemented with 20 mM KNO_3 for an additional 4 h of incubation. When solid medium was used the Vogel's-N medium was supplemented with 1.5% (w/v) Noble agar (Difco Laboratories). Where appropriate, the media were supplemented with 100 μg/mL hygromycin B (Calbiochem).

Inoculation

Fourteen-day-old tomato seedlings (cv. MoneyMaker or Motelle) were inoculated by cutting the root tips and dipping the roots for 20 min in a suspension containing 2×10^5 conidia/mL water as previously described (Divon *et al.*, 2005). Mock-inoculated plants were immersed in water. Seedlings were planted in plastic boxes containing perlite and grown in the greenhouse at 25 °C under natural light. Infections, 25 plants in each experiment, were repeated three times with wild-type and D2 strains, and twice with D1 and ND strains, and with mock.

Nucleic acid extraction and RT-PCR

Total RNA from *F. oxysporum* was extracted with the RNeasy Plant Mini kit (Qiagen, Hilden, Germany) and DNase treated with RQ1 RNase free DNAse (Promega, Madison, WI) on the columns according to the protocol. *F. oxysporum* DNA was extracted using the DNeasy Plant Mini Kit (Qiagen). *N. crassa* nucleic acid extractions were performed as previously described (Yatzkan *et al.*, 1998). RNA samples were treated with RQ1 RNase free DNAse (Promega) and then purified with the RNeasy plant mini kit (Qiagen) according to the manufacturer's protocol. For gene expression analysis in *F. oxysporum* standard RT-PCR was used. Reverse transcription was done with the Superscript II RNase H$^-$ kit (Invitrogen) using 1 μg of DNA-free total RNA and 70 pmol of poly dT-18 primer. Two microlitres of the RT-reaction were used as template for subsequent PCR. Primers are indicated in the figure legends and Table 1, and b-tubulin (accession no. AY641578) served as a reference. Cycling parameters were essentially as

Table 1 Primers used in this work.

Primer ID	Primer sequence	Primer ID	Primer sequence
69205	ATGGCCACCACCTATTCCCT	81576	GTTGAGTTTGAAGACGGACGTGATTAAG
69207	CGAAGATGGCGGTTGACATAG	81577	ACAAAGCACACAAGTCCCCTTACAATCA
69663	TCAAAGACTCATTGTCAACCATT	btub1304L	CCGTCTCCAC TTCTTCATGG
70459	ATGGCCACCACCTATTCCCTTGCCC	btub1862R	AGCATCCTGG TACTGCTGGT
70460	TCAAAGACTCATTGTCAACCATTCCCATT	Fol11H1OF	TTAGGCAGATCCATGTATTCG
70778	CCCAAGCTTGTTCGTTTTGGAACAC	Fol11H1OF	GTACTCCGCGCTGTTATGC
70779	CGGGATCCTGAAGGCCAACCACTGTG	FolbtubFW	CTCGCTGGTGTACCAATGC
70780	ATAGTTTAGCGGCCGCTCAAAGACTCATTGTCAACCATT	FolbtubREV	AGCTCAACTCCGATCTGCG
72495	TCTGACAATTCTGGCTCGCCTATCAAC	Gap1F	GGCCGAGGTACAACTTCCAG
72860	CTGGTCAGCACGTCATGGTCCA	Gap1R	GCCGAGGTACCATCTTTGAC
73582	ATCCGGAGCTTGCAGGATCGCCGC	hph565F	CGG TGT CGT CCA TCA CAG
73583	CGCTCCCGACTCATCAAGTGATGAAG	hph1139R	TCG CCC TTC CTC CCT TTA TT
76044	GTAACGACACCACCTGCTAC	Mtd1F	GGTACCAGAGGATGATGGG
76045	CATGCTGATCATGCCGGTAG	Mtd1R	TGGAGTGGTGCGCAGGTC
77523	CTCAGGGTCATGAATTCAACTA	nit2-1298L	TTGGCCCCAACAACTCTTCT
78055	GGGGTACCCATATGGCCACCACCTATTCC	nit2-1826R	GACGGATCCCATCCCATAGA
78056	GCTCTAGATCAAAGACTCATTGTCAACC	UricaseF	CGAGGTACACAACTCTTCCT
78192	CTCGTGACGTCTCGGCCAGCTTCA	UricaseR	CATGGTGGCTTGCACACTAG
79906	TCCGTCTTCAAACTCAACGGTCTCAC		

described in Divon *et al.* (2005). Genomic DNA was extracted from lyophilized fungal cultures using the Puregene DNA extraction system (Gentra Systems, Minneapolis, MN). The DNA was further purified by Dneasy plant mini kit (Qiagen, www.qiagen.com). For Southern blot analysis 2 µg of genomic DNA was digested with either *Asp718* or *Dra*I (Roche Applied Science), and fragments were resolved by electrophoresis on 1% agarose gels and blotted to Hybond-N+ nylon membrane (Amersham Biosciences). Hybridization was performed with a Dig-labelled-DNA probe corresponding to 575 bp of the bacterial hygromycin B resistance gene (*hph*, hygromycin phosphotransferase) that was used as a dominant selectable marker for the transformation. The Dig-labelled probe was synthesized by PCR, according to the manufacturer's instructions (Roche Diagnostics, IN) using primers hph565F and hph1139R with pCSN44 plasmid DNA as template (Staben *et al.*, 1989).

Cloning of the *Fnr1* gene

The *Fusarium* nitrogen regulator gene, *Fnr1*, was cloned and sequenced using nested primers based on the sequence of the putative *F. graminearum AreA*/*nit-2* orthologue, which showed high similarity in a BLASTN search using the *AreA-GF* gene from *G. fujikuroi* (Tudzynski *et al.*, 1999; *F. graminearum* contig 1.348; DNA [189595, 193132]). The sequence was, in the Softberry gene prediction database (http://www.softberry.com/berry.phtml; FGENESH), predicted to contain an open reading frame including a translation start site, a stop codon and a poly adenylation signal. The genomic region of *Fnr1* was isolated by amplification of two overlapping fragments using primer pairs 69205/69207 and 72495/69663 (Table 1). Sequencing of these fragments facilitated specific extension of primers 69205 into 70459, and 69663 into 70460, allowing for the amplification of the full-length *Fnr1* cDNA clone. The flanking sequences of *Fnr1* were verified by PCR amplification and sequencing of the PCR product using 5′ primer pair 78192/69207, and 3′ primer pair 81576/81577. The *Fnr1* sequence was submitted to the GenBank under accession no. DQ387858.

Construction of vectors for fungal transformation

A vector, pHD1, designed to favour homologous recombination and disruption of the *Fnr1* gene via a double cross-over event was made as follows. A fragment containing the last 797 bp of the *Fnr1* gene was PCR amplified, inserting restriction sites for *Bam*HI (primer 70779) and *Not*I (primer 70780) at the 5′ and 3′ ends, respectively. The fragment was inserted upstream of the hygromycin B (*hygB*) cassette in vector pAB1004. Plasmid pAB1004 is similar to pCB1004 (Carroll *et al.*, 1994), but in a Bluescript SK-vector. The 5′ part of the *Fnr1* gene from the translation start site (1553 bp) was amplified by PCR using 5′ primer 77523 (including an *Apa*I site) and 3′ primer 70778 (including a *Hin*dIII site), and was inserted downstream of the *hygB* resistance cassette in vector pAB1004. The size of the resulting plasmid (pHD1) was 6670 bp, containing the *Fnr1* genomic sequence in which 626 bp of the coding sequence (including parts of the zinc finger domain) were replaced by the *hygB* resistance gene driven by the *TrpC* promoter. Construction of vector pHD2, for the complementation of the *N. crassa nit-2*^{RIP} mutant with the *Fnr1* gene, was carried out as follows. *Fnr1* cDNA was amplified inserting restriction sites

for *Kpn*I (primer 78055) and *Xba*I (primer 78056) at the 5′ and 3′ ends, respectively, thereby allowing for a replacement with the *Hph* gene and *TrpC* terminator from the pMP6 vector (a construct conferring hygromycin resistance, kindly provided by M. Plamann) with *Fnr1*. The resulting plasmid, pHD2, contained the *Fnr1* coding region fused to 1.5 kb of the modified *N. crassa cpc-1* promoter. All primers are shown in Table 1.

Fungal transformation

Transformation of *F. oxysporum* f. sp. *lycopersici,* race 2, was adapted from a protocol provided by B. Cohen (Cohen *et al.*, 2002). For protoplast preparation fungal spores were obtained from a 4–5-day PDB culture by filtration and centrifugation at 3000 ***g*** for 20 min. Approximately 10^8 spores were germinated overnight (16–17 h) in 100 mL PDB medium at 150 r.p.m. at room temperature. Young mycelia were filtered, washed once with 0.7 M NaCl, and then digested in 25 mL digestion medium [250 mg driselase (Sigma, D-9515), 250 mg glucanex (Sigma, L1412), 1.25 mg chitinase (0.575 units; Sigma, C-6137) in 25 mL 0.7 M NaCl], with slow shaking (70 r.p.m.) at 32 °C for 1–2.5 h to release protoplasts. Digests were filtered through a 50-μm mesh, and centrifuged at 1500 ***g*** for 5 min at 4 °C to collect the protoplasts. The protoplasts were washed once in 10 mL STC buffer (1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) and resuspended in STC buffer with the cell density adjusted to 10^8 protoplasts/mL. For transformation 100 μL (10^7 protoplasts) of protoplast suspension was mixed in 15-mL conical plastic tubes with DNA suspended in maximum 10 μL STC buffer. For transformation 10 μg of circular plasmid was used. The mix was incubated for 30 min on ice. Then 200 μL, 200 μL and 800 μL of PEG/$CaCl_2$ solution [60% (w/v) polyethylene glycol (MW 3500–4000), 50 mM $CaCl_2$ in 10 mM Tris-HCl pH 7.5] were added. The tubes were incubated for an additional 30 min on ice, before 1 mL STC buffer was added. For plating, 200 μL of transformed protoplasts was mixed with 20 mL molten regeneration medium (PDB, 20% sucrose, 1.6% bactoagar; Difco) and poured into Petri dishes. After 3 h plates were overlaid with 10 mL molten regeneration medium containing 195 μg/mL hygromycin B (Calbiochem or Invitrogen). Plates were incubated at 26 °C for 4–7 days for transformed colonies to emerge. *Fnr1*-disrupted mutants were selected on PDA plates supplemented with 65 μg/mL hygromycin B and 6% chlorate. Resistant colonies were screened using genomic PCR with three sets of primers (Table 1): forward primer 78192, and reverse primers 79906, 73582 and 73583 (Fig. 5A). Thirty cycles of PCR were performed as follows: denaturation at 94 °C for 40 s, annealing at 69 °C for 40 s, and extension at 72 °C for 3 min. An initial denaturation step at 94 °C for 2 min was performed. For complementation of the *N. crassa nit-2*^{RIP} mutant, the pHD2 circular construct was cotransformed, by electroporation, according to Margolin *et al.* (1997), into *nit-2*^{RIP} conidia, along with plasmid pMP6, harbouring a cassette conferring hygromycin resistance. Hygromycin-resistant transformants were examined for ability to grow on nitrate as the sole nitrogen source as well as for integration and expression of *Fnr1* with primers 76044 and 76045. The *nit-2* and β-tubulin PCR and RT-PCR controls were obtained using the primers nit2-1298 L/nit2-1826R and btub1304L and btub1862R (Scheffer *et al.*, 2005), respectively (Table 1).

Alignments and phylogenetic analysis

Multiple alignment was performed in ClustalX (1.64b). The alignment was subsequently submitted for phylogenetic tree construction using the neighbour-joining method (Saitou and Nei, 1987) in PHYLIP format (PHYLogeny Inference Package). *C. albicans*, showing overall low similarity to the other operational taxonomic units (OTUs), was defined as an outgroup. The tree was visualized with the TreeView (1.5.3) software. The following AREA/NIT2-orthologues were used: *Aspergillus nidulans*, AREA (X52491); *Candida albicans*, GAT1 (AY293736); *Cladosporium fulvum*, NRF1 (AF312694); *Colletotrichum lindemuthianum*, CLNR1 (AY168017); *Fusarium graminearum*, FG-AREA [contig 1.348; DNA (189595, 193132)]; *Fusarium oxysporum*, FNR1 (DQ387858); *Gibberella fujikuroi*, GF-AREA (Y11006); *Magnaporthe grisea*, NUT1 (U60290); *Neurospora crassa*, NIT2 (M33956); *Penicillium chrysogenum*, NRE (U02612).

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