A Nitrogen Response Pathway Regulates Virulence Functions in *Fusarium oxysporum* via the Protein Kinase TOR and the bZIP Protein MeaB

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During infection, fungal pathogens activate virulence mechanisms, such as host adhesion, penetration and invasive growth. In the vascular wilt fungus *Fusarium oxysporum*, the mitogen-activated protein kinase Fmk1 is required for plant infection and controls processes such as cell omnipause penetration, vegetative hyphal fusion, or root adhesion. Here, we show that these virulence-related functions are repressed by the preferred nitrogen source ammonium and restored by treatment with L-methionine sulfoximine or rapamycin, two specific inhibitors of Gln synthetase and the protein kinase TOR, respectively. Deletion of the bZIP protein MeaB also resulted in nitrogen source-independent activation of virulence mechanisms. Activation of these functions did not require the global nitrogen regulator AreA, suggesting that MeaB-mediated repression of virulence functions does not act through inhibition of AreA. Tomato plants (*Solanum lycopersicum*) supplied with ammonium rather than nitrate showed a significant reduction in vascular wilt symptoms when infected with the wild type but not with the ΔmeaB strain. Nitrogen source also affected invasive growth in the rice blast fungus *Magnaporthe oryzae* and the wheat head blight pathogen *Fusarium graminearum*. We propose that a conserved nitrogen-responsive pathway might operate via TOR and MeaB to control virulence in plant pathogenic fungi.

INTRODUCTION

Plant pathogenic fungi have evolved mechanisms to invade their hosts, overcome their defenses, and colonize their tissues, thereby causing disease. Expression of these virulence functions is controlled by a network of cellular pathways that respond to signals encountered during host infection. A conserved mitogen-activated protein kinase (MAPK) cascade homologous to the yeast mating and filamentation cascade is essential for pathogenicity in a wide range of plant pathogens (Xu and Hamer, 1996; Zhao et al., 2007). The orthologous MAPK Fmk1 is required for plant infection in the vascular wilt fungus *Fusarium oxysporum*, a soil-borne pathogen that attacks a wide range of economically important crops (Di Pietro et al., 2001). *F. oxysporum* mutants lacking *Fmk1* are impaired in multiple virulence-related processes, such as root adhesion, host penetration, and invasive growth on the living plant tissue (Di Pietro et al., 2001). Fmk1 is also required for vegetative hyphal fusion, a ubiquitous developmental process in filamentous fungi (Prados Rosales and Di Pietro, 2008). The cellular mechanisms through which this conserved MAPK cascade controls such a wide array of pathogenicity functions are largely unknown.

Nitrogen limitation has been proposed as a key signal for activating the expression of virulence genes in plant pathogens (Sneijers et al., 2000). Early studies established that in planta-induced genes, such as *Mpg1* encoding a hydrophobin required for pathogenicity of the rice blast fungus *Magnaporthe oryzae*, or the avirulence gene *Avr9* of the tomato pathogen *Cladosporium fulvum*, are strongly upregulated under conditions of nitrogen limitation (Talbot et al., 1993; Van den Ackerveken et al., 1994). In agreement with these findings, many genes identified in screens for nitrogen starvation-induced transcripts are also upregulated during plant infection (Coleman et al., 1997; Stephenson et al., 1997; Divon et al., 2005; Donofrio et al., 2006). Thus, nitrogen source seems to act as a metabolic switch to trigger expression of infection-related genes in plant pathogenic fungi.

Nitrogen utilization is a highly regulated process. Although fungi are able to use a wide variety of nitrogen sources, readily assimilated compounds, such as ammonium or Glu, are used preferentially over more complex sources (Marzluf, 1997). This is accomplished by a process known as nitrogen catabolite repression, whereby genes required for exogenous nitrogen source utilization are downregulated in the presence of the preferred source (Marzluf, 1997; Wong et al., 2008). Transcriptional regulation of nitrogen catabolic genes is mediated, in part, by the wide-domain regulators AreA in *Aspergillus nidulans* (Arst and Cove, 1973), Nit-2 in *Neurospora crassa* (Fu and Marzluf, 1987), and Gln3/Gat1 in *Saccharomyces cerevisiae* (Beck and Hall, 1999). These global nitrogen regulators are members of the GATA binding family of transcription factors and function as transcriptional activators of genes encoding pathway-specific permeases and enzymes enabling uptake and catabolism of secondary nitrogen sources (Caddick et al., 1986; Fu and Marzluf,
As a consequence, loss-of-function mutants in AreA or Nit-2 are unable to use nitrogen sources other than Gln or ammonium (Arst and Cove, 1973; Marzluf, 1997). AreA orthologs have been identified in a number of filamentous ascomycetes and appear to be both structurally and functionally conserved (Wong et al., 2008).

Since AreA regulates gene expression under nitrogen-limited conditions similar to those encountered in planta, its role in expression of virulence genes and in disease development has been examined in several plant pathogens. The AreA ortholog from C. fulvum, NRF1, partly controlled expression of Avr9, but nrf1 mutants still produced low levels of Avr9 transcript in planta and were fully virulent (Perez-Garcia et al., 2001). Similarly, NUT1, the AreA ortholog from M. oryzae, was required for full induction of MPG1 expression (Soanes et al., 2002) but not for pathogenicity on rice (Oryza sativa) plants (Froeliger and Carpenter, 1996). In other plant pathogens, such as Colletotrichum lindemuthianum or F. oxysporum, inactivation of AreA orthologs decreased virulence to different degrees (Pellier et al., 2003; Divon et al., 2006). Collectively, these studies suggest that AreA contributes to, but is not essential for, fungal virulence on plants.

Interestingly, a genetic screen for nitrogen nonutilizing mutants in M. oryzae identified mutations in two genes that were nonallelic to NUT1 and dramatically affected pathogenicity. These mutants failed to use a range of nitrogen sources and were nonpathogenic on rice (Oryza sativa) plants (Lau and Hamer, 1996), suggesting a major role of nitrogen regulation in plant infection that appears to be largely independent of AreA. The identity of these nitrogen-responsive virulence regulators has not been elucidated so far.

The conserved Ser/Thr kinase TOR (for Target of Rapamycin) orchestrates cell growth in eukaryotes in response to nutrient availability (De Virgilio and Loewith, 2006; Rohde et al., 2008). In S. cerevisiae, inhibition of TOR by rapamycin causes a nutrient stress response, including inhibition of translation initiation, arrest in the G1 phase of the cell cycle, glycerol accumulation, downregulation of glycolysis, and autophagy (Crespo and Hall, 2002). Nitrogen is a particularly important nutrient in TOR signaling. Both nitrogen starvation and rapamycin resulted in rapid dephosphorylation and nuclear accumulation of Gln3, followed by expression of genes involved in the assimilation of alternative nitrogen sources (Beck and Hall, 1999; Crespo and Hall, 2002). Depletion of the preferred nitrogen source Gln with the Gln synthetase inhibitor L-methionine sulfoximine (MSX) also caused nuclear localization of Gln3, suggesting that TOR responds to Gln levels (Crespo et al., 2002). In the filamentous fungus Fusarium fujikuroi, TOR inhibition by rapamycin affected expression of AreA-controlled genes involved in secondary metabolism, translation control, ribosome biogenesis, carbon metabolism, and autophagy (Teichert et al., 2006). The role of TOR in nutrient regulation of fungal virulence on plants has not been examined so far.

This study was initiated with the aim to explore possible crosstalk between nitrogen signaling and the Fmk1 MAPK cascade in the control of virulence functions of F. oxysporum, based on the previous finding in S. cerevisiae that invasive pseudohyphal growth requires both nitrogen limitation and the orthologous Kss1 MAPK cascade (Gimeno et al., 1992; Madhani and Fink, 1997). We previously noted that cellophane penetration, a virulence-related process with analogies to agar invasion in yeast, requires the MAPK Fmk1 and the downstream homeodomain transcription factor Ste12 (Prados Rosales and Di Pietro, 2008; Rispail and Di Pietro, 2009). Here, we show that the preferred nitrogen source ammonium represses cellophane penetration in F. oxysporum and other plant pathogenic fungi and that this repression is reversed by inhibition of Gln synthetase or TOR by MSX and rapamycin, respectively. Nitrogen source and TOR control additional virulence-related functions, such as vegetative hyphal fusion and root adhesion. We further establish that repression of virulence functions by ammonium requires the bZIP protein MeaB. Finally, we show that nitrogen source and MeaB control expression of virulence-related genes during F. oxysporum infection and production of vascular wilt disease on tomato (Solanum lycopersicum) plants. We propose the existence of a conserved nitrogen response pathway that operates via TOR and MeaB to negatively regulate virulence functions in plant pathogenic fungi.

RESULTS

Nitrogen Source Regulates Cellophane Penetration in Plant Pathogenic Fungi

We previously found that the ability of F. oxysporum to cross cellophane membranes defines a major virulence function that requires the Fmk1 MAPK cascade (Prados Rosales and Di Pietro, 2008; Rispail and Di Pietro, 2009). To investigate the role of nitrogen regulation in this process, we determined cellophane penetration ability of the F. oxysporum wild-type strain on minimal medium supplemented with different nitrogen sources. Efficient penetration was detected in the presence of sodium nitrate, but not ammonium nitrate, ammonium sulfate, or ammonium tartrate, suggesting an inhibitory effect of the preferred nitrogen source ammonium (Figure 1A). Penetration on nitrate was largely independent of carbon source, since it occurred both on glycerol and on the preferred carbon source glucose at concentrations as high as 5% (w/v) (see Supplementary Figure 1A online). However, glucose limitation (0.2%) partially relieved inhibition of cellophane penetration by ammonium (see Supplementary Figure 1B online).

We investigated whether the repressing effect of ammonium was functional in other plant pathogenic fungi by testing cellophane penetration in two ascomycete pathogens that differ from F. oxysporum in host range and mode of infection. The head blight pathogen Fusarium graminearum infects developing spikes of wheat (Triticum aestivum) and barley (Hordeum vulgare) (Jansen et al., 2005), while the rice blast fungus M. oryzae penetrates rice leaves by means of specialized dome shaped appressoria (Balbot, 2003). F. graminearum isolate PH-1 and M. oryzae isolate Guy-11 both were able to cross cellophane membranes when grown on sodium nitrate, but not on ammonium nitrate (Figure 1B). These results indicate that inhibition of cellophane penetration by ammonium is conserved in these plant pathogenic ascomycetes.
Nitrogen Regulates Virulence in Fusarium

Figure 1. Nitrogen Source Regulates Cellophane Penetration in Plant Pathogenic Fungi.
(A) Effect of nitrogen source on penetration of cellophane membranes by F. oxysporum. Fungal colonies were grown for 4 d at 28°C on top of cellophane membranes on plates with minimal medium containing 50 mM of the indicated nitrogen source (Before). The cellophane with the fungal colony was removed and plates were incubated for an additional day to determine the presence of mycelial growth on the plate, indicating penetration of the cellophane (After).
(B) Cellophane penetration of F. graminearum and M. oryzae on the indicated nitrogen source was determined as in (A).

Nitrogen Repression of Invasive Growth Requires Gln Synthetase and TOR

Among the different amino acids tested as nitrogen sources, only Gln partially repressed cellophane penetration (Table 1). Repression by Gln was less efficient than by ammonium, possibly due to the inherent instability or inefficient uptake of this amino acid (Figure 2B). We therefore used MSX, an irreversible and highly specific inhibitor of Gln synthetase (Crespo et al., 2002; Figure 2A), to further investigate the role of Gln in nitrogen repression. MSX fully restored cellophane penetration of F. oxysporum on ammonium (Figure 2B). Thus, Gln synthetase activity is strictly required for ammonium-mediated inhibition of cellophane penetration, suggesting that Gln rather than ammonium acts as a signal for nitrogen repression.

In S. cerevisiae, intracellular Gln levels regulate the activity of the conserved protein kinase TOR (Crespo and Hall, 2002; Crespo et al., 2002) (Figure 2A). In contrast with S. cerevisiae, which has two TOR proteins, only a single TOR ortholog was identified in filamentous ascomycetes such as A. nidulans, F. fujikuroi, and Podospora anserina (Demethon et al., 2003; Fitzgibbon et al., 2005; Teichert et al., 2006). Unexpectedly, a BLASTp analysis of the Fusarium comparative genome database detected two TOR gene homologs in F. oxysporum, FOXG_02818 and FOXG_15946, compared with a single homolog in the closely related species F. graminearum and Fusarium verticillioides. The FOXG_02818 gene is located on chromosome 8 and encodes a full-length TOR protein (named TOR1a), while FOXG_15946 is on chromosome 2b and encodes a truncated TOR ortholog (TOR1b), which lacks the 633 N-terminal amino acid residues and has 93% amino acid identity to FOXG_02818 in the remaining part of the protein. Approximately 8 kb upstream of FOXG_15946, a short open reading frame FOXG_15944 encodes 399 N-terminal amino acids of TOR and is separated from FOXG_15946 by a gene with homology to helitron-like transposons (Poulter et al., 2003). The predicted TOR1b gene product contains three of the four N-terminal HEAT (huntingtin, elongation factor 3, regulatory subunit A of PP2A, TOR1) repeats as well as the other key domains of TOR proteins, including FAT (FRAP, ATM, TTRAP), FRB (FKBP12-rapamycin binding), catalytic kinase, and C-terminal FATC domains. RT-PCR analysis with primer pairs specific for each of the TOR genes detected abundant transcript levels of TOR1a and a very faint signal corresponding to the TOR1b transcript (see Supplemental Figure 2 online). This result suggests that the two TOR genes of F. oxysporum are expressed at different levels. However, the functionality of the truncated TORb protein remains unclear.

To ask whether TOR mediates nitrogen repression of invasive growth, we added rapamycin, a highly specific inhibitor of TOR. Rapamycin treatment restored cellophane penetration of F. oxysporum in the presence of repressing concentrations of ammonium (Figure 2B). Among different stress-inducing compounds tested, only rapamycin and, to a lesser extent, caffeine, which has also been reported as an inhibitor of TOR (Reinke et al., 2006), restored cellophane penetration on ammonium (see Supplemental Figure 3 online). This indicates that the derepressing effect of rapamycin is associated with TOR inactivation and is not a consequence of a general stress response.

Starvation of S. cerevisiae for single amino acids triggers a regulatory pathway known as the general amino acid control

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<th>Nitrogen Source (50 mM)</th>
<th>Cellophane Penetration</th>
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<tr>
<td>NaNO₃</td>
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<tr>
<td>NH₄NO₃</td>
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<tr>
<td>(NH₄)₂SO₄</td>
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<td>(NH₄)₂C₂H₄O₆</td>
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Fungal colonies were grown for 4 d at 28°C on top of cellophane membranes on plates with minimal medium containing the indicated nitrogen source. The cellophane with the fungal colony was removed, plates were incubated for an additional day, and presence (+) or absence (−) of fungal mycelium on the underlying medium was scored.
system, which results in activation of invasive growth at ammonium concentrations that normally inhibit this response (Braus et al., 2003). We tested whether starvation for a single amino acid could also restore cellophane penetration of *F. oxysporum* in the presence of ammonium by adding 3-amino-triazole, a histidine analog that induces histidine starvation (Hilton et al., 1965). In contrast with rapamycin, 3-amino-triazole did not override the repressing effect of ammonium (see Supplemental Figure 4 online), suggesting that the general amino acid control system does not play a major role in cellophane penetration. Collectively, these results indicate that (1) Gln is a primary signal for nitrogen repression of cellophane invasion in *F. oxysporum*, and (2) transmission of the repressing signal requires the conserved protein kinase TOR.

**Nitrogen Repression of Invasive Growth Is Controlled by the bZIP Protein MeaB and the Wide Domain Regulator AreA**

The *A. nidulans* MeaB gene was identified by mutations conferring resistance to toxic amino acid analogs and methylammonium, resulting in derepression of nitrogen-regulated genes (Arist and Cove, 1973; Polley and Caddick, 1996). MeaB encodes a protein with a bZIP DNA binding motif that mediates nitrogen metabolite repression in *A. nidulans* (Wong et al., 2007, 2008) (Figure 3A). A BLASTp search of the *F. oxysporum* genome database identified a single MeaB ortholog, FOXG_02277 (see Supplemental Figure 5 online). We took advantage of the availability of a *F. oxysporum* ΔmeaB mutant from a previous insertional mutagenesis screen (López-Berges et al., 2009) to investigate the role of this bZIP factor in nitrogen regulation of cellophane penetration. The ΔmeaB strain showed reduced...
growth on secondary nitrogen sources such as nitrate or nitrite as well as resistance to DL-p-fluorophenylalanine and increased sensitivity to the toxic nitrate analog chlorate (Figure 3B). While these phenotypes are very similar to those reported for the meaB mutant of A. nidulans (Polley and Caddick, 1996; Wong et al., 2007), we noted that the ΔmeaB strain grew very poorly on ammonium as the sole nitrogen source (Figure 3B, compare growth on ammonium nitrate with ammonium sulfate or ammonium tartrate). Introduction of the MeaB gene from F. oxysporum (Fo MeaB) or from A. nidulans (An MeaB) into the ΔmeaB mutant (see Supplemental Figure 8 online) restored wild-type growth on all nitrogen sources tested. We conclude that the role of MeaB in nitrogen utilization is highly conserved between F. oxysporum and A. nidulans.

A primary function of MeaB in nitrogen catabolite repression of A. nidulans is to inhibit the global regulator AreA via transcriptional activation of its corepressor NmrA (Wong et al., 2007). To explore the role of AreA in nitrogen repression of cellophane penetration by F. oxysporum, a ΔareA null mutant was generated by replacing the entire coding region of the AreA gene (FOXG_03165) with the hygromycin resistance cassette (see Supplemental Figure 7A online). PCR analysis of hygromycin-resistant transformants with different combinations of gene-specific primers identified one ΔareA strain showing homologous integration-mediated gene replacement (see Supplemental Figures 7B to 7D online). Phenotypic analysis of nitrogen utilization in the ΔareA mutant showed that it could use only Gln and, to a much lesser extent, ammonium as nitrogen sources and that it was highly resistant to chlorate (Figure 3B). Again, these phenotypes are similar to those reported for areA mutants of A. nidulans (Wilson and Arst, 1998) and N. crassa (Fu and Marzluf, 1987), although the growth phenotype on ammonium was more severe than in A. nidulans. The wild-type growth pattern was completely restored by complementation of the mutant with the wild-type AreA gene (Figure 3B) (see Supplemental Figure 7E online). In S. cerevisiae and A. nidulans, mutations in downstream components of the TOR pathway confer increased resistance to rapamycin (Beck and Hall, 1999; Jacinto et al., 2001; Crespo and Hall, 2002; Fitzgibbon et al., 2005). We found that the ΔmeaB and ΔareA mutants, but not the complemented strains, were slightly more resistant to rapamycin than the wild type (Figure 3B). This suggests that MeaB and AreA may function downstream of TOR.

The ΔmeaB strain was able to cross cellophane membranes even in the presence of repressing concentrations of ammonium, in contrast with the wild type or the strains complemented with either Fo MeaB or An MeaB (Figure 4A). Thus, MeaB is required for ammonium-mediated repression of cellophane invasion in F. oxysporum. Interestingly, addition of rapamycin consistently enhanced penetration of the ΔmeaB mutant on ammonium (Figure 4B), suggesting that MeaB and TOR have independent and additive roles in nitrogen repression of invasive growth.

We next tested the hypothesis that MeaB mediates nitrogen repression of cellophane penetration by inhibiting AreA. This would imply that AreA acts as a positive regulator of this virulence-associated process. Comparative cellophane penetration assays between the wild-type strain and the ΔareA mutant were conducted on minimal medium supplemented with diluted (1:20) potato dextrose broth due to the inability of the ΔareA strain to

Figure 4. Ammonium Repression of Invasive Growth Is Controlled by the bZIP Protein MeaB and the Wide Domain Regulator AreA.

(A) Deletion of MeaB causes invasive growth in the presence of 50 mM of the repressing nitrogen source NH₄NO₃. The F. oxysporum meaB deletion mutant (ΔmeaB) and this mutant complemented with MeaB from either F. oxysporum (Fo) or A. nidulans (An) were grown for 4 d before removing the cellophane membrane.

(B) MeaB and TOR have additive functions in ammonium-mediated repression of invasive growth. Fungal colonies were grown for 3 d before removing the cellophane membrane to quantitatively compare invasion rate. Bar = 5 mm.

(C) AreA is not required for invasive growth and contributes to ammonium-mediated repression of this process. Cellophane penetration of the ΔareA mutant was determined after 4 d of incubation on plates containing PDA or PDB diluted 1:25 with water and supplemented with 50 mM NH₄NO₃ or NH₄NO₃ + 150 ng mL⁻¹ rapamycin.
grow on nitrate as the sole nitrogen source. In contrast with our initial hypothesis, the ΔareA strain was still able to penetrate cellophane membranes. We conclude that AreA is not required for cellophane penetration in *F. oxysporum*. Unexpectedly, the ΔareA mutant retained some penetration ability on ammonium, albeit to a lesser extent than the ΔmeaB strain. Cellophane penetration of the ΔareA strain on ammonium was further enhanced by rapamycin (Figure 4C). These results suggest that the repressing role of MeaB on cellophane invasion is not mediated by inhibition of AreA. Rather, AreA itself seems to act as an ammonium-responsive repressor of cellophane penetration.

**TOR and MeaB Have Partially Independent Roles in Nitrogen Catabolite Repression**

To further dissect the role of TOR, MeaB, and AreA in nitrogen regulation of *F. oxysporum*, we measured transcript levels of three genes functioning in nitrogen catabolism: *Nit1* encoding nitrate reductase (FOXG_04181), *Nii1* encoding nitrite reductase (FOXG_03192), and *MepB* encoding an ammonium permease (FOXG_08912) (Teichert et al., 2008). Quantitative RT-PCR was performed on total RNA obtained from mycelia of the wild type, ΔmeaB, ΔareA, as well as the complemented strains that were grown first on the derepressing source sodium nitrate and transferred for 3 h to sodium nitrate, ammonium nitrate, or ammonium nitrate plus rapamycin. Transcript levels of *Nit1*, *Nii1*, and *MepB* in the wild-type strain were strongly decreased on ammonium, confirming that these genes are subject to nitrogen catabolite repression (Figure 5). Downregulation by ammonium was abolished in the ΔmeaB mutant and restored in the complemented ΔmeaB+MeaB strain, indicating that this process requires MeaB. Induction of *Nit1*, *Nii1*, and *MepB* by nitrate was not detected in the ΔareA mutant, suggesting that it requires AreA.

Addition of rapamycin partially reversed ammonium repression of nitrogen catabolic genes in the wild-type strain, directly implicating TOR in this process (Figure 5). Importantly, rapamycin treatment also increased transcript levels in the ΔmeaB mutant. Thus, the role of TOR in nitrogen repression is at least partly independent of MeaB. By contrast, rapamycin failed to upregulate transcript levels of nitrogen catabolic genes in the ΔareA mutant. This suggests that TOR mediates nitrogen catabolite repression through inhibition of AreA.

We also measured transcript levels of the regulatory genes *Nmr1*, *AreA*, and *MeaB* in the different strains. Consistent with the postulated role of Nmr1 as a MeaB-dependent corepressor of AreA (Wong et al., 2007, 2008), *Nmr1* transcripts were upregulated on ammonium in the wild-type strain but not in the ΔmeaB mutant, indicating that MeaB is required for transcriptional upregulation of *Nmr1* on ammonium (Figure 5). Transcript levels of *AreA* on ammonium were inversely related to those of *Nmr1*: downregulated in the wild type but unaffected in the ΔmeaB strain. Rapamycin caused a significant increase in *AreA* transcript levels on ammonium, both in the wild type and the ΔmeaB mutant, further supporting the idea that TOR negatively regulates AreA through a MeaB-independent mechanism. Transcript levels of *MeaB* were not influenced by nitrogen source. We noted that the complemented ΔmeaB+MeaB strain had higher *MeaB* transcript levels than the wild type, possibly due to multiple copies of the complementing construct or to positional effects at the ectopic insertion site. Interestingly, *MeaB* expression was upregulated in the ΔareA strain, and this increase was completely abolished by rapamycin. This result indicates that AreA and TOR function as negative and positive regulators, respectively, of *MeaB* expression. Collectively, the data from the transcriptional analysis suggest that (1) TOR and MeaB have partially independent roles in nitrogen catabolite repression of *F. oxysporum* and (2) both regulate transcript levels of *AreA*.

**Inactivation of TOR or MeaB Does Not Bypass the Requirement of the Fmk1 MAPK Cascade for Invasive Growth**

*F. oxysporum* mutants lacking the MAPK Fmk1 or its downstream component, the homeodomain transcription factor Ste12, fail to penetrate cellophane membranes even on permissive nitrogen sources such as sodium nitrate (Prados Rosales and Di Pietro, 2008; Rispaíl and Di Pietro, 2009). To explore the possible cross-talk between the activating MAPK cascade and the nitrogen repression pathway, a combination of pharmacological and genetic approaches was used. We first asked whether inactivation of TOR restored cellophane penetration in the Δfmk1 and Δste12 mutants. However, neither of these mutants was able to cross cellophane membranes in the presence of rapamycin (Figure 7A). Second, we tested whether deletion of MeaB could rescue cellophane penetration in a Δfmk1 background. A Δfmk1ΔmeaB double mutant obtained by targeted gene knockout (see Methods for details) was still unable to penetrate cellophane membranes (Figure 6B). Thus, inactivation of either TOR or MeaB does not obviate the requirement of the MAPK Fmk1 for activation of invasive growth.

These results suggest that the nitrogen repression pathway controls cellophane penetration either independently or upstream of the Fmk1 MAPK cascade. To test whether MeaB and Fmk1 function in the same nitrogen regulation pathway, we compared the nitrogen utilization phenotypes of the Δfmk1 and ΔmeaB single mutants with that of the Δfmk1ΔmeaB double mutant. All the strains showed similar growth as the wild type on rich nitrogen sources such as potato dextrose agar (PDA) or Gin (see Supplemental Figure 8 online). By contrast, the Δfmk1 and ΔmeaB strains showed reduced growth on the nonpreferred source, nitrate. Importantly, growth of the Δfmk1ΔmeaB double mutant on nitrate was even more reduced than in each single mutant, suggesting that loss of the two genes has an additive effect. This result supports the idea that MeaB and Fmk1 have separate roles in nitrogen regulation.

We next determined the effect of nitrogen source, *meaB* or *areA* deletion, and rapamycin on transcript levels of *Ste12*. As previously reported for *C. lindemuthianum* (Hoi et al., 2007), the *Ste12* gene of *F. oxysporum* produces two differentially spliced transcripts, one (*Ste12*) containing the five predicted exons and the other (*Ste12ΔE4*) lacking the fourth exon, which encompasses part of the zinc finger (Rispaíl and Di Pietro, 2009). In the wild-type strain grown on ammonium, levels of both *Ste12* transcripts were ~75% lower than those on nitrate (Figure 6C). Ammonium-mediated repression of *Ste12* was abolished in the ΔmeaB and
the ΔareA mutants and fully restored in the complemented strains. By contrast, rapamycin had no effect on Ste12 repression, suggesting that this process is not mediated by TOR. We conclude that ammonium downregulates expression of Ste12 in a MeaB- and AreA-dependent manner.

**Ammonium Negatively Regulates Vegetative Hyphal Fusion and Root Adhesion through TOR, MeaB, and AreA**

Besides cellophane penetration, the Fmk1 MAPK cascade controls additional virulence-related functions, such as vegetative hyphal fusion, a ubiquitous process in filamentous fungi that mediates efficient adhesion of *F. oxysporum* to the roots of its host plant tomato (Prados Rosales and Di Pietro, 2008). To explore the role of nitrogen source in regulation of vegetative hyphal fusion, we determined the frequency of fusion events in *F. oxysporum* germlings grown on different nitrogen sources. The wild-type strain showed a dramatic decrease of hyphal fusion events on ammonium nitrate compared with sodium nitrate (Figure 7A). The repressing effect of ammonium was completely reversed by addition of rapamycin. By contrast, fusion frequency of the ΔmeaB strain on ammonium versus nitrate was increased rather than decreased. Fusion frequency of the ΔareA strain was as high as in the wild type but was not reduced by ammonium. Thus, MeaB and AreA are required for repression of hyphal fusion by ammonium.

Vegetative hyphal fusion in liquid culture results in the formation of macroscopically visible mycelial aggregates (Prados

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**Figure 5. Effect of Nitrogen Source, meaB or areA Deletion, and Rapamycin on Transcript Levels of Nitrogen Catabolic and Regulatory Genes.**

Quantitative real-time RT-PCR analysis was performed in the indicated strains germinated 14 h in PDB diluted 1:25 with water supplemented with 25 mM NaNO₃ and then washed with water and transferred for 3 h to 25 mM NaNO₃ (black columns), 25 mM NH₄NO₃ (dark gray columns), or 25 mM NH₄NO₃ + 150 ng mL⁻¹ rapamycin (light-gray columns) as the sole nitrogen source. Transcript levels of nitrogen catabolic genes *Nit1* (nitrate reductase), *Nii1* (nitrite reductase), and *MepB* (ammonium permease), as well as nitrogen regulatory genes *Nmr1* (corepressor), *AreA*, and *MeaB* are expressed relative to transcript levels of the wild-type (wt) strain on NaNO₃. Bars represent SE from three independent experiments with three replicates each.
Rosales and Di Pietro, 2008). When wild-type microconidia were germinated on sodium nitrate, they formed dense hyphal networks that could not be disrupted by vigorous vortexing (Figure 7B). By contrast, these hyphal aggregates did not form on ammonium nitrate, most likely as a consequence of reduced hyphal fusion. Importantly, aggregate formation on ammonium was restored by rapamycin. The \textit{DmeaB} mutant produced hyphal aggregates independently of the nitrogen source. Complementation with the \textit{meaB} gene from either \textit{F. oxysporum} or \textit{A. nidulans} restored ammonium repression (see Supplemental Figure 9 online). The \textit{DareA} mutant generally formed hyphal aggregates less efficiently than the wild-type strain, probably due to its reduced growth. However, similar to the \textit{DmeaB} strain, aggregation of the \textit{DareA} mutant was not repressed by ammonium (Figure 7B).

Root adhesion occurs during early stages of plant infection and contributes to virulence of \textit{F. oxysporum} (Prados Rosales and Di Pietro, 2008). On sodium nitrate, the wild-type strain adhered efficiently to tomato roots, covering the root surface with a dense mycelium (Figure 7C). By contrast, root adhesion on ammonium nitrate was dramatically reduced, and this reduction was reversed by rapamycin treatment. Ammonium failed to repress root adhesion in the \textit{DmeaB} and \textit{DareA} strains. Taken together, these results suggest that distinct virulence-related functions are regulated by nitrogen status via the protein kinase TOR and the transcription factor MeaB. The global regulator AreA is not required for these functions and plays an active role in ammonium-mediated repression.

**Nitrogen Source and MeaB Control Infection of Tomato Plants**

We next asked whether nitrogen source directly affected the ability of \textit{F. oxysporum} to infect tomato plants. Roots of tomato seedlings were dip-inoculated with microconidia of the wild type, \textit{DmeaB} mutant, or \textit{DmeaB}+\textit{Fo MeaB} complemented strain.
Seedlings were transferred to individual pots containing vermiculite and supplied either with 25 mM sodium nitrate or 25 mM ammonium nitrate. Disease symptoms in plants inoculated with the wild-type strain and supplied with sodium nitrate increased steadily throughout the experiment, and most plants were dead 16 d after inoculation (Figure 8A). By contrast, plants supplied with ammonium nitrate showed a significant delay in symptom development and were still alive 23 d after inoculation. In plants infected with the ΔmeaB strain, disease progression was slightly delayed, but in contrast with the wild type, nitrogen source had no significant effect on the severity of disease symptoms. The complemented ΔmeaB + MeaB strain showed a similar virulence pattern as the wild type. We conclude that ammonium negatively regulates plant infection by *F. oxysporum* in a MeaB-dependent manner.

Disruption of the AreA ortholog *Fnr1* in *F. oxysporum* f. sp *lycopersici* was previously shown to result in a delay of infection rate (Divon et al., 2006). We determined virulence of the ΔareA null mutant on tomato plants supplied either with sodium nitrate or ammonium nitrate. On sodium nitrate, the ΔareA strain showed a significant delay in the development of vascular wilt symptoms compared with the wild type (see Supplemental Figure 10 online). However, in contrast with the wild-type strain, disease severity of the ΔareA mutant was not further reduced in the presence of ammonium. These results confirm previous work showing that AreA contributes to virulence of *F. oxysporum* (Divon et al., 2006). However, they also suggest that AreA contributes to ammonium-mediated repression of plant infection.

To explore the effect of nitrogen source on expression of virulence-related genes, we monitored transcript levels of the *Six1* gene, which is specifically induced in planta (van der Does et al., 2008) using real-time quantitative RT-PCR of total RNA obtained 48 h after inoculation of microconidia on tomato roots supplied either with sodium nitrate or ammonium nitrate. At this early stage, *F. oxysporum* develops infection hyphae that penetrate the root surface and grow inter- and intracellularly within the root cortex (Figure 8B). Transcript levels of *Six1* during infectious growth of the wild-type strain were significantly reduced in the presence of ammonium compared with nitrate, but this decrease was not observed in the ΔmeaB and ΔareA mutants (Figure 8C). Rapamycin treatment dramatically increased *Six1* transcript levels in the three strains. This suggests that MeaB and TOR have independent functions in nitrogen regulation of the in planta–expressed *Six1* gene.

**Figure 7.** Nitrogen Source Regulates Vegetative Hyphal Fusion, Hyphal Aggregation, and Root Adhesion via TOR and MeaB.

**(A)** Frequency of vegetative hyphal fusion of the indicated strains after 18 h of conidial germination in PDB diluted 1:25 with water and supplemented with 25 mM of the indicated nitrogen source was determined microscopically and expressed as percentage of fused germlings versus total number of germlings. Bars represent SE from three independent experiments with three replicates each.

**(B)** Hyphal aggregates forming 36 h after conidial germination under conditions described in (A). Cultures were vortexed to dissociate weakly adhered hyphae and observed in a binocular microscope.

**(C)** Root adhesion assay. Roots of tomato seedlings were immersed for 36 h in microconidial suspensions of the indicated strains in PDB diluted 1:25 with water and supplemented with 25 mM of the indicated nitrogen source and then washed by vigorous shaking in water and observed in a binocular microscope. Adhering fungal mycelium is visible as a white mass covering the roots.

wt, wild type.
DISCUSSION

Fungi respond to the quantity and quality of nitrogen source by switching between distinct developmental programs, allowing them to maximize their potential for proliferation and survival (Schneper et al., 2004). In S. cerevisiae, nitrogen availability determines the initiation of filamentous and invasive growth (Gimeno et al., 1992). Nitrogen status was also proposed to act as a regulatory switch for activating infectious development in plant pathogens (Snoeijers et al., 2000). Here, we show that the preferred nitrogen source ammonium inhibits infection-related functions in the tomato vascular wilt pathogen F. oxysporum. Transmission of the nitrogen repression signal requires independent inputs from the conserved protein kinase TOR and the bZIP protein MeaB, two components that have not been associated previously with fungal pathogenicity on plants. A proposed
model for nitrogen regulation of virulence-related functions in *F. oxysporum* is shown in Figure 9. Repression of invasive growth by ammonium was conserved in two other plant pathogens, *F. graminearum* and *M. oryzae*, suggesting a general role for this nitrogen response pathway in fungal pathogenicity on plants.

Control of Virulence Functions by Nitrogen Source

The preferred nitrogen source ammonium caused repression of cellophane invasion, vegetative hyphal fusion, and root adhesion in *F. oxysporum*. We exploited the cellophane penetration assay to dissect the mechanism of nitrogen repression using a combination of genetic and pharmacological approaches. This simple assay bears similarities with the invasive growth assay in yeast and displays a high level of correlation with the virulence phenotype of *F. oxysporum* on tomato plants (Prados-Rosales and Di Pietro, 2008). So far, mutations in three distinct genes, encoding the MAPK Fmk1, the transcription factor Ste12, and the chitin synthase ChsV, were found to impair cellophane penetration in *F. oxysporum*. Strikingly, these mutations also abolish or severely reduce virulence on tomato plants (Madrid et al., 2003; Prados Rosales and Di Pietro, 2008; Rispail and Di Pietro, 2009). Thus, the ability to cross cellophane membranes defines a major pathogenicity function in *F. oxysporum*.

Cellophane invasion was inhibited by different sources of ammonium, including ammonium sulfate, ammonium tartrate, and ammonium nitrate, but not by sodium nitrate. This suggests a repressing effect of the preferred nitrogen source ammonium rather than an activating effect of the poor source nitrate. Among additional nitrogen sources tested, only Gln was able to partially repress invasive growth. Both ammonium and Gln are readily assimilated by the cell and activate nitrogen catabolite repression in fungi (Marzluf, 1997; Caddick, 2004; Wong et al., 2008). In *S. cerevisiae* and *A. nidulans*, Gln rather than ammonium was proposed to function as the major signal for nitrogen catabolite repression (Margelis et al., 2001; Crespo et al., 2002). We noted that the Gln synthetase inhibitor MSX abolished the repressing effect of ammonium on cellophane penetration, supporting the idea that Gln is the main nitrogen signal for repression of virulence functions in *F. oxysporum* (Figure 9). However, a Gln-independent effect of ammonium in nitrogen signaling cannot be ruled out. In *S. cerevisiae*, ammonium was shown to signal directly through the high affinity ammonium permease and sensor Mep2 to control filamentous growth and agar invasion (Lorenz and Heitman, 1998).

Role of TOR and MeaB in Nitrogen Repression

Rapamycin reversed the repressing effect of ammonium on virulence functions, implicating TOR as a negative regulator of at least three infection-related processes: invasive growth, cell fusion, and fungus-host adhesion. Recently, rapamycin was found to induce expression of adhesin genes and cellular aggregation in the human pathogen *Candida albicans* (Bastidas et al., 2009). Collectively, this suggests a broadly conserved role of TOR in the control of infection-related processes in human and plant pathogens.

Besides TOR, genetic evidence also implicates the conserved bZIP protein MeaB in ammonium-mediated repression of virulence functions. In contrast with the wild-type strain, the ∆meaB mutant displayed nitrogen source-independent activation of invasive growth, hyphal fusion, and root adhesion. Moreover, MeaB was required for ammonium-mediated reduction of vascular wilt disease symptoms on tomato plants. These findings highlight a role of MeaB as a repressor of infection-related processes in *F. oxysporum* (Figure 9).

Both TOR and MeaB have been associated with nitrogen regulation in fungi. While TOR is a key player in nitrogen catabolite repression of *S. cerevisiae* (Beck and Hall, 1999; Crespo and Hall, 2002), its role in filamentous fungi remains controversial. TOR was suggested to have only a minor function in nitrogen metabolism of *A. nidulans* since mutations in TOR pathway genes did not have significant effects on the nitrogen utilization phenotype (Fitzgibbon et al., 2005). The discrepancy could partly be explained by the fact that several components of the TOR and nitrogen regulatory pathways, such as Ure2, MeaB, or Nmr1, are

![Figure 9. Model for Nitrogen Control of Virulence Functions in F. oxysporum through TOR and MeaB.](image)
not conserved between S. cerevisiae and filamentous fungi. Here, we showed that rapamycin treatment increases transcript levels of genes involved in nitrogen catabolism, such as Nit1, supporting a role of TOR in nitrogen catabolite repression of *F. oxysporum*. This result is in agreement with a previous study showing that rapamycin activates expression of nitrogen metabolism-related genes, such as ammonium permease and uricase in *F. fujikuroi* (Teichert et al., 2006).

How do MeaB and TOR control ammonium-mediated repression of nitrogen catabolism and virulence functions? We noted that MeaB is required for ammonium-induced upregulation of Nmr1 in *F. oxysporum*, as previously reported in *A. nidulans* (Wong et al., 2007). Moreover, the defects in nitrogen utilization of *F. oxysporum* and *A. nidulans* meaB mutants were very similar, and the ΔmeaB phenotype in *F. oxysporum* was successfully complemented by the An MeaB gene. In both species, derepression of nitrogen catabolite genes in ΔmeaB was strictly dependent on AreA. Collectively, these shared features suggest a similar mode of action of MeaB in nitrogen catabolite repression of *F. oxysporum* to that proposed in *A. nidulans* (Wong et al., 2007, 2008), whereby MeaB mediates transcriptional activation of the corepressor Nmr1, which inhibits the wide domain regulator AreA (Figure 9).

A central question concerning the role of MeaB in nitrogen repression is how its activity is controlled by the nitrogen signal. An unexpected phenotype of the *F. oxysporum* ΔmeaB mutant was its poor growth on ammonium as the sole nitrogen source (e.g., ammonium sulfate or ammonium tartrate). This phenotype was relieved in the presence of an additional nitrogen source (ammonium nitrate) and fully complemented by the An MeaB gene, suggesting that MeaB has a conserved and previously uncharacterized role in ammonium utilization. The inability to use ammonium as a nitrogen source provides a straightforward explanation for the lack of ammonium-mediated repression in the ΔmeaB strain. Further work is needed to elucidate the exact function of MeaB in ammonium utilization.

Our results, as well as those from *A. nidulans* (Wong et al., 2007), show that MeaB transcript levels are unaffected by nitrogen source. In *F. fujikuroi*, two MeaB transcript sizes were detected, one of which was induced by nitrogen starvation (Schonig et al., 2008). While these results suggest that MeaB activity is regulated predominantly at the posttranscriptional or the posttranslational level, we found that MeaB transcript levels were upregulated in the ΔareA mutant in a TOR-dependent manner. Thus, AreA and TOR may function as negative and positive regulators, respectively, of MeaB expression.

In our study, meaB deletion or rapamycin treatment completely abolished ammonium-induced upregulation of Nmr1. The lack of an additive effect of rapamycin in the ΔmeaB mutant suggests that the ability of MeaB to activate Nmr1 may be controlled either directly or indirectly by TOR (Figure 9). Inspection of the amino acid sequences of fungal MeaB proteins revealed the presence of several highly conserved Ser and Thr residues followed by a Pro, which resemble the rapamycin-sensitive phosphorylation sites in the TOR-regulated yeast protein kinases Sch9 (Urban et al., 2007) and Npr1 (Gander et al., 2008). Moreover, deletion of MeaB resulted in a weak increase in rapamycin resistance. Further studies are needed to address the hypothesis that TOR controls MeaB activity in response to nitrogen source.

Besides the possible link between TOR and MeaB, two lines of evidence suggest that TOR also has MeaB-independent roles in nitrogen regulation. First, rapamycin-induced expression of nitrogen catabolic genes was detected both in the wild type and in the ΔmeaB mutant. Second, cellophane penetration of the ΔmeaB strain in the presence of ammonium was further increased by rapamycin treatment. These results support a model in which TOR has both MeaB-dependent and independent roles in ammonium-mediated repression of nitrogen catabolism and virulence functions (Figure 9).

**Dual Role for AreA as Activator of Nitrogen Catabolic Genes and Repressor of Virulence Functions**

The orthologous GATA factors Gln3 and AreA are strictly required for transcriptional activation of nitrogen catabolic genes in *S. cerevisiae* and *A. nidulans*, respectively (Crespo and Hall, 2002; Wong et al., 2008). Deletion of areA abolished nitrate-induced upregulation of Nit1, Nii1, and MepB, suggesting that AreA functions as an activator of nitrogen catabolic genes in *F. oxysporum*.

Importantly, rapamycin-mediated upregulation of these genes was also abolished in the ΔareA mutant. Similarly, in *F. fujikuroi*, rapamycin was shown to activate AreA-regulated genes in the wild type but not in the ΔareA background (Teichert et al., 2006). Moreover, a gln3 gat1 double mutant of *S. cerevisiae* was blocked in rapamycin-mediated induction of the nitrogen-regulated genes MEP2 and GLN1 and was weakly resistant against rapamycin (Beck and Hall, 1999). Interestingly, the *F. oxysporum* ΔareA strain also showed a slight but consistent increase in rapamycin resistance. Taken together, these results indicate that TOR may mediate nitrogen catabolite repression in *F. oxysporum* by inhibiting the activity of the transcriptional activator AreA.

We initially hypothesized that AreA would function as a MeaB-controlled activator of virulence-related functions in *F. oxysporum*. However, two lines of evidence strongly argue against this hypothesis. First, the ΔareA mutant, although severely affected in growth due to its inability to use nitrogen sources other than ammonium or Gin, was still able to perform cellophane penetration, hyphal fusion, and root adhesion. This rules out a major activating role of AreA in these processes and suggests that MeaB-mediated repression of virulence functions is independent of AreA (Figure 9). Generation of deletion and overexpression alleles of the cognate corepressor Nmr1 should provide a rigorous test for this model.

Second, ammonium-mediated repression of virulence functions was partially relieved in the ΔareA mutant. The latter result was unexpected and suggests that AreA acts as a repressor of nitrogen-regulated virulence functions, in contrast with its well-described role as an activator of nitrogen catabolite genes (Figure 9). Previous evidence for a negative role of AreA in gene expression comes from a transcriptomic analysis in *F. fujikuroi*, which identified subsets of genes that were either down- or upregulated in the ΔareA mutant. The group of genes repressed by AreA included the glyoxylate cycle enzyme isocitrate lyase, which links carbon to nitrogen metabolism, the autophagy-specific
transcription factor IDI4 and the NADP⁺-dependent Glu dehydrogenase (Schonig et al., 2008). It remains to be determined whether the inhibitory role of AreA on virulence functions is mediated directly by transcriptional repression of target genes or indirectly through transcriptional activation of downstream repressors.

**Nitrogen Response Pathway and Fmk1 MAPK Cascade Have Opposing Roles in the Regulation of Virulence**

This study was initiated with the aim of exploring possible cross-talk between nitrogen signaling and the Fmk1 MAPK cascade in the control of virulence functions of *F. oxysporum*. Fmk1 is strictly required for invasive growth and pathogenicity on tomato plants, as well as for vegetative hyphal fusion and root adhesion (Di Pietro et al., 2001; Prados Rosales and Di Pietro, 2008). Here, we show that these infection-related processes are negatively controlled by the preferred nitrogen source ammonium. We previously noted that cellophane penetration by *F. oxysporum* displays certain analogies to agar invasion in *S. cerevisiae*, since both processes require the orthologous MAPKs Fmk1 and Kss1, respectively, as well as the homeodomain transcription factor Ste12 (Madhani et al., 1997; Rispail and Di Pietro, 2009). This study reveals another common feature, repression by ammonium (Gimeno et al., 1992). In addition to invasive growth, two other virulence-related functions of *F. oxysporum*, hyphal fusion and root adhesion, were also repressed by ammonium.

Both MeaB and TOR were required for ammonium-mediated repression of invasive growth. The inhibitory role of TOR detected in this work contrasts with a previous report that suggested a positive effect of TOR on pseudohyphal growth and agar invasion in yeast (Cutler et al., 2001). However, several lines of evidence favor a role for TOR as a repressor. First, invasive growth is generally stimulated by nitrogen limitation (Gimeno et al., 1992), a condition that inhibits TOR activity (Crespo and Hall, 2002; Crespo et al., 2002). Second, TOR inhibition by rapamycin activates expression of Mep2, an ammonium permease required for invasive growth (Lorenz and Heitman, 1998; Hardwick et al., 1999). Third, rapamycin treatment increased expression of hyphae-specific genes and promoted cell–cell adhesion in *C. albicans* (Bastidas et al., 2009). We therefore propose that the nitrogen-responsive TOR pathway and the filamentation/pathogenicity MAPK cascade, while acting on the same targets, have opposing roles in regulation of virulence-related functions.

A central question is how this shared yet opposing control is exerted. Pharmacological and genetic evidence indicate that the two pathways function independently, since neither rapamycin treatment nor deletion of *meaB* restored invasive growth in the ΔmeaB mutant. Moreover, the ΔmeaBΔfmk1 double mutant displayed a more severe nitrogen regulation phenotype than the single mutants. Previous studies highlighted the pivotal role of the homeodomain transcription factor Ste12 in regulating invasive growth and plant pathogenicity downstream of the MAPK cascade (Park et al., 2004; Rispail and Di Pietro, 2009). Interestingly, Ste12 transcript levels were strongly downregulated on ammonium compared with nitrate, suggesting that Ste12 expression is controlled by nitrogen source (Figure 9). Transcriptional repression of Ste12 in *F. oxysporum* required both MeaB and AreA, consistent with the phenotypic effects of ΔmeaB and ΔareA mutations of reversing nitrogen repression of virulence functions and further supporting the idea that AreA acts as a repressor of virulence-related genes, such as Ste12. Ammonium-mediated downregulation of Ste12 was not reversed by rapamycin, reinforcing the idea that, at least in part, MeaB and TOR function independently. However, it is still possible that TOR controls activity of Ste12 at the posttranscriptional level. We are currently addressing this hypothesis.

A second key issue concerns the common downstream targets regulated by the nitrogen response and MAPK pathways and their role during invasive growth, adhesion, and plant infection. Some of these genes are likely to encode extracellular or surface proteins. *Six1*, for example, encodes a small secreted protein that is specifically induced during infection of tomato plants and contributes to virulence of *F. oxysporum* (Rep et al., 2004; van der Does et al., 2008). Expression of *Six1* was dramatically repressed by ammonium in a manner dependent on MeaB and TOR. Full expression of the surface hydrophobin gene MPG1, which is essential for pathogenicity of *M. oryzae*, also required nitrogen limitation and the Fmk1 MAPK (Lau and Hamer, 1996; Soanes et al., 2002). Other cellular responses associated with nitrogen starvation, such as autophagy or generation of reactive oxygen species, have recently been linked to fungal virulence on plants (Veneault-Fourrey et al., 2006; Egan et al., 2007; Brown et al., 2008). The possible role of the pathogenicity MAPK cascade in regulation of these processes has not been studied. More work is required to extend our understanding of how nitrogen and MAPK signaling interact to control infectious growth in plant pathogenic fungi.

**METHODS**

**Fungal Isolates and Culture Conditions**

*Fusarium oxysporum f. sp. lycopersici* race 2 wild-type isolate 4287 (FGSC 9935) was used in all experiments. Generation and molecular characterization of the *F. oxysporum* Δfmk1, Δste12, and ΔmeaB mutants were described previously (Di Pietro et al., 2001; López-Berges et al., 2009; Rispail and Di Pietro, 2009). Fusarium graminearum isolate PH-1 and *Magnaporthe oryzae* isolate Guy-11 were used as negative controls. All fungal strains were stored as microconidial suspensions at −80°C with 30% glycerol. For extraction of genomic DNA and microconidia production, cultures were grown in potato dextrose broth (PDB; Difco) at 28°C with shaking at 170 rpm (Di Pietro and Roncero, 1998). For analysis of gene expression, freshly obtained microconidia were germinated for 14 h in PDB diluted 1:25 with water, supplemented with 25 mM NaNO₃. Mycelia were harvested by filtration, washed three times in sterile water, and transferred for 3 h to liquid minimal medium (MM) (Puhalla, 1985) containing either 25 mM NaNO₃ or 25 mM NH₄NO₃ as the sole nitrogen source. Invasion assays on cellophane membranes (colorless; Manipulos Margok) were performed as described (Prados Rosales and Di Pietro, 2008) using solid MM supplemented with 50 mM of the indicated nitrogen source. Amino acids were added after autoclaving. Rapamycin, L-methionine sulfoximine, caffeine, 3-amino-triazole, menadione, and calcofluor white (all from Sigma-Aldrich) were added after autoclaving to the desired final concentrations from stock solutions prepared following suppliers instructions. For macro- and microscopic analysis of hyphal fusion and agglutination, fungal strains were grown 18 h in PDB diluted...
1:25 with water and supplemented with 25 mM of the indicated nitrogen source and observed in a Leica DMR microscope using the Nomarsky technique or in a Leica binocular microscope. Photographs were recorded with a Leica DC 300F digital camera. For determination of colony growth, 2 × 10^6 microconidia were spotted onto PDA (Difco) with or without 2% (w/v) potassium chlorate, 250 μg mL⁻¹ dl-p-fluorophenylalanine, or 150 to 200 ng mL⁻¹ rapamycin, or MM agar containing either sodium Glu, Gln, NaNO₃, NH₄NO₃, (NH₄)₂C₂H₄O₂, (NH₄)₂SO₄, or NaNO₂ (all 25 mM) as nitrogen source. Plates were incubated at 28°C for the indicated time periods. All experiments included two replicate plates and were performed at least three times with similar results.

Nucleic Acid Manipulations

Total RNA and genomic DNA was extracted from F. oxysporum mycelia following previously reported protocols (Raeder and Broda, 1985; Chomczynski and Sacchi, 1987). Quality and quantity of extracted nucleic acids were determined by running aliquots in ethidium bromide-stained agarose gels and by spectrophotometric analysis in a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies), respectively. Routine nucleic acid manipulations were performed as described in standard protocols (Sambrook and Russell, 2001). DNA and protein sequence databases were searched using the BLAST algorithm (Altschul et al., 1990).

Quantitative Real-Time RT-PCR Analysis

Total RNA was treated with DNase I (Fermentas) and reverse transcribed into first-strand cDNA with ribonuclease inhibitor RNasin Plus RNase inhibitor (Promega) and M-MLV reverse transcriptase (Invitrogen) using a poly-dT antisense primer. Gene-specific primers (see Supplemental Table 1 online) were designed to flank an intron if possible. Quantitative RT-PCR products were obtained using IQ SYBR Green Supermix (Bio-Rad) and an iCycler iQ real-time PCR System (Bio-Rad). Transcript levels were calculated by comparative ΔCt and normalized to Act1. Expression values are presented as values relative to the expression in the wild-type strain under nonrepressing conditions (NaNO₃).

Targeted Gene Knockout

Targeted replacement of the F. oxysporum MeaB gene in the Δfmk1 background was performed using a fusion PCR construct and protocol described previously (López-Berges et al., 2009). Targeted replacement of the entire coding region of the F. oxysporum AreA gene with the hygromycin resistance cassette under control of the A. nidulans GpdA promoter (PpgdA) and TrpC terminator (TrpC) was performed using the split-marker method (Cattell et al., 2003) following the protocol previously described (Rispail and Di Pietro, 2009) (see Supplemental Figure 7 online). PCR reactions were routinely performed with the High Fidelity Template PCR system (Roche Diagnostics) using a MJ Mini Bio-Rad personal thermal cycler. DNA fragments flanking the AreA coding region were amplified from genomic DNA of F. oxysporum with primer pairs areA-1 + areA-M13-1 and areA-2 + areA-M13-2, respectively (see Supplemental Table 1 and Supplemental Figure 5 online) and PCR fused with partially overlapping truncated versions of the hygromycin B resistance cassette (Hygr) using primer combinations areA-1n + Hyy-G and areA-2n + Hyg-Y, respectively. The obtained split-marker fragments were used to cotransform protoplasts of the F. oxysporum wild-type strain to hygromycin resistance, and transformants were purified by monoclonal isolation as described (Di Pietro and Roncero, 1998). Transformants showing homologous insertion of the construct were detected by PCR of genomic DNA with primer pairs areA-3 + hyg-6, areA-1n + areA-2n, and areA-4 + areA-5 (see Supplemental Figures 5B to 5D online).

Complementation of meaB and areA Deletion Mutants

To generate a construct for complementing the meaB deletion mutants, a 4334-bp fragment, spanning from 1981 bp upstream of the wild-type F. oxysporum MeaB translation initiation codon to 832 bp downstream of the translation termination codon, was amplified by PCR using the High Fidelity Template PCR system (Roche Diagnostics) with primer pairs FmeaB-For + FmeaB-Rev. For complementing with the heterologous Aspergillus nidulans MeaB gene, a 4486-bp fragment, spanning from 1934 bp upstream of the wild-type A. nidulans meaB translation initiation codon to 911 bp downstream of the translation termination codon, was amplified with primer pairs AnmeaB-For + AnmeaB-Rev. Both amplified fragments were used to cotransform protoplasts of the meaB deletion mutant with the phleomycin resistance cassette. Three out of 25 phleomycin resistance cotransformants were selected in both complementation experiments for their wild-type growth phenotype in the presence of the toxic nitrate analog chlorate and analyzed for the presence of a functional MeaB allele by PCR with gene-specific primer pairs G5S-5 + G5S-6 and AnmeaB-1 + AnmeaB-2, for homologous and heterologous complementation, respectively. Amplification products, 346- and 287 bp, were detected in the selected phleomycin-resistant cotransformants but not in the ΔmeaB strain (see Supplemental Figure 6 online). We concluded that these transformants, designated ΔmeaB + Fo MeaB and ΔmeaB + An MeaB, had integrated an intact copy of the F. oxysporum and the A. nidulans MeaB gene into the genome, respectively.

For the complementation of the areA deletion mutant, a 6463-bp fragment, spanning from 1931 bp upstream of the wild-type AreA translation initiation codon to 1572 bp downstream of the translation termination codon, was amplified with primer pairs areA-3 + areA-2. The amplified fragment was used to cotransform protoplasts of the areA deletion mutant with the phleomycin resistance cassette. Three out of 15 phleomycin resistance cotransformants were selected for their wild-type growth phenotype in the presence of nitrate as the sole nitrogen source and analyzed for the presence of a functional AreA allele by PCR with gene-specific primers areA-4 + areA-5. A 438-bp amplification product was detected in the selected phleomycin-resistant cotransformants but not in the ΔareA strain (see Supplemental Figure 7 online). We concluded that these transformants, designated ΔareA + ΔareA, had integrated an intact copy of the F. oxysporum AreA gene into the genome.

Virulence and Root Adhesion Assays

Tomato plant inoculation assays were performed in a growth chamber as described (Di Pietro and Roncero, 1998). Plants were supplied either with 25 mM NaNO₃ or 25 mM NH₄NO₃ solutions in water. At different times after inoculation, severity of disease symptoms was recorded using an index ranging from 1 (healthy plant) to 5 (dead plant). Ten plants were used for each treatment. Inoculations for expression analysis in planta and root adhesion assays were performed as described (Di Pietro et al., 2001). Virulence and root adhesion experiments were performed at least three times with similar results.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL database or in the Fusarium Genome database under the following accession numbers: Fo MeaB, FOXG_0277; An MeaB, X98065; AreA, FOXG_03165; Nmr1, FOXG_05080; MepB, FOXG_04437; Nil1, FOXG_04181; Nii1, FOXG_03192; TOR1a, FOXG_02818; TOR1b, FOXG_15946; Fmk1, AF286533; Ste12, FJ609797; Act1, FOXG_01569; Six1, G226894; pAN7.1 (PpgdA-Hygr-TmpC), Z32698.1; F. graminearum MeaB, XP_3836840; Fusarium verticilloides MeaB, FVEG_02542; Magnaporthe grisea MeaB, XP_359471; Neurospora crassa MeaB, XP_365694; and Aspergillus nidulans MeaB, CAAT4053.
Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure 1. Effect of Carbon Source on Cellophane Penetration by Fusarium oxysporum.

Supplemental Figure 2. Expression of Fusarium oxysporum TOR1a and TOR1b Genes.

Supplemental Figure 3. Cellophane Penetration on Ammonium Is Specifically Induced by TOR Inhibition, but Not by Salt, Oxidative, or Cell Wall Stress.

Supplemental Figure 4. Nitrogen Regulation of Invasive Growth Is Not Mediated by the General Amino Acid Control System.

Supplemental Figure 5. Amino Acid Sequence Alignment of Fungal Orthologs of the bZIP Protein MeaB.

Supplemental Figure 6. Complementation of the Fusarium oxysporum meaB Deletion Mutant with Fo MeaB or An MeaB.

Supplemental Figure 7. Targeted Deletion of the Fusarium oxysporum AreA Gene.

Supplemental Figure 8. Nitrogen Utilization Phenotypes Caused by Single and Double Deletion of MeaB and Fmk1.

Supplemental Figure 9. Nitrogen Source Regulates Hyphal Aggregation via TOR and MeaB.

Supplemental Figure 10. AreA Contributes to Virulence of Fusarium oxysporum on Tomato Plants Independently of Nitrogen Source.

Supplemental Table 1. List of Primers Used in This Study.

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A Nitrogen Response Pathway Regulates Virulence Functions in *Fusarium oxysporum* via the Protein Kinase TOR and the bZIP Protein MeaB
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