HapX-Mediated Iron Homeostasis Is Essential for Rhizosphere Competence and Virulence of the Soilborne Pathogen Fusarium oxysporum

Manuel S. López-Berges, Javier Capilla, David Turrà, Lukas Schafferer, Sandra Matthijs, Christoph Jöchl, Pierre Cornelis, Josep Guarro, Hubertus Haas, and Antonio Di Pietro

INTRODUCTION

Soilborne fungal pathogens cause devastating yield losses and are highly persistent and difficult to control. During the infection process, these organisms must cope with limited availability of iron. Here we show that the bZIP protein HapX functions as a key regulator of iron homeostasis and virulence in the vascular wilt fungus Fusarium oxysporum. Deletion of hapX does not affect iron uptake but causes derepression of genes involved in iron-consuming pathways, leading to impaired growth under iron-depleted conditions. F. oxysporum strains lacking HapX are reduced in their capacity to invade and kill tomato (Solanum lycopersicum) plants and immunodepressed mice. The virulence defect of ΔhapX on tomato plants is exacerbated by coinoculation of roots with a biocontrol strain of Pseudomonas putida, but not with a siderophore-deficient mutant, indicating that HapX contributes to iron competition of F. oxysporum in the tomato rhizosphere. These results establish a conserved role for HapX-mediated iron homeostasis in fungal infection of plants and mammals.

Soilborne fungal pathogens are ubiquitous, highly persistent, and extremely difficult to control. They cause root rots, wilts, stunting, and seedling damping-off in a wide range of plant species, leading to devastating losses in field and greenhouse crops both in industrialized and developing countries. Agricultural practices, such as crop rotation, resistance breeding, and application of fungicides, are insufficient to prevent root diseases of important crop plants (Haas and Défago, 2005). One of the most important soilborne pathogens is Fusarium oxysporum, the causal agent of vascular wilt disease in more than 100 different plant species (Armstrong and Armstrong, 1981; Dean et al., 2012). Like other root-infecting fungi, F. oxysporum persists in the soil for extended time periods, either in the form of thick-walled chlamydospores or as a saprophyte on dead organic matter. Compounds exuded by the host plant trigger spore germination, followed by directed hyphal growth and penetration of the root, preferentially through natural openings at the junctions of epidermal cells (Lagopodi et al., 2002; Pérez-Nadales and Di Pietro, 2011). Inside the root, the fungus grows inter- and intracellularly until it reaches the vascular tissue, where it colonizes the xylem vessels, provoking wilting and plant death. Some F. oxysporum isolates also cause opportunistic infections in humans, which range from superficial or locally invasive to disseminated, depending on the immune status of the individual (Nucci and Anaissie, 2007). Previous work established that a single isolate of F. oxysporum f sp lycopersici, FGSC 9935, is able to cause disease both in tomato (Solanum lycopersici) plants and in immunodepressed mice (Ortoneda et al., 2004). The availability of the complete genome sequence (Ma et al., 2010) makes this strain an excellent model for studying the genetic basis of transkingdom pathogenicity in fungi.

During saprophytic and preinfection stages, F. oxysporum competes with other microorganisms in the soil and the plant rhizosphere for limited nutrients and essential elements, such as iron (Simeoni et al., 1987). Ever since the earliest reports on antagonistic disease-suppressing soil microorganisms more than 70 years ago, it has been known that nonpathogenic rhizosphere-colonizing microbes can protect plants against root-infecting pathogens, a mechanism termed biocontrol (Baker, 1968). Fluorescent pseudomonads are effective biocontrol...
agents against plant pathogenic fungi, bacteria, and nematodes (Mercado-Blanco et al., 2001; Haas and Défago, 2005; Weller, 2007). *Pseudomonas* spp owe their fluorescence to an extracellular diffusible pigment called pyoverdine (Pvd), which displays a high affinity for Fe\(^{3+}\) ions and functions as a siderophore (Ravel and Cornelis, 2003). In addition to Pvd, secondary siderophores with lower iron affinity, including pyochelin, pseudomonine, quinolobactin, omnicorugatin (Ocg), and noocardamine, are produced by different *Pseudomonas* strains (Cornelis and Matthijs, 2002; Matthijs et al., 2008). The battery of siderophores enables fluorescent pseudomonads to efficiently compete for limited iron resources in the soil (Ravel and Cornelis, 2003).

Iron is an essential cofactor for a wide range of cellular processes, but its excess is toxic to the cell (Halliwell and Gutteridge, 1984). Iron homeostasis requires fine-tuned mechanisms to maintain the balance between uptake, storage, and consumption of iron. In the saprophytic model fungus *Aspergillus nidulans*, maintenance of iron homeostasis is mediated essentially by two transcription factors, SreA and HapX, which are interconnected in a negative feedback loop (Haas et al., 1999; Hortschansky et al., 2007). During iron starvation, the bZIP protein HapX, initially identified as an interactor of the heterotrimeric CCAAT binding core complex (Tanaka et al., 2002; Hortschansky et al., 2007), downregulates the expression of sreA, a repressor of siderophore biosynthesis and of iron-dependent pathways (Mercier et al., 2006; Hortschansky et al., 2007; Schrettl et al., 2010; Hsu et al., 2011). HapX governs iron homeostasis and virulence in the human pathogens *Aspergillus fumigatus* (Schrettl et al., 2010), *Candida albicans* (Chen et al., 2011; Hsu et al., 2011), and to a lesser extent in *Cryptococcus neoformans* (Jung et al., 2010). HapX is conserved throughout the fungal kingdom, but its function during fungal pathogenicity on plants has not been explored so far.

In this study, we addressed the role of HapX and iron homeostasis in the infection process of *F. oxysporum*. We show that HapX is a major regulator of the transcriptional response to iron limitation and establish its relevance during fungal infection of plants. Moreover, we provide evidence for its function during

---

**Figure 1.** Loss of *hapX* Impairs Growth of *F. oxysporum* under Iron-Limiting Conditions but Not Iron Uptake.

(A) Growth of the indicated fungal strains on solid media differing in availability of iron. Cultures were grown for 3 d at 28°C. wt, wild type.

(B) Relative mycelial growth of the indicated strains on MM with or without Fe was estimated by measuring the area corresponding to fungal colonies on the inverted contrast images in (A), and normalized to the growth of the wild type strain on MM+Fe.

(C) Relative dry biomass of the indicated strains grown in liquid MM with or without Fe for 5 d at 28°C. Bars represent \( \pm \) from three independent experiments with three technical replicates each. Values with the same letter are not significantly different according to Mann-Whitney test (\( P = 0.05 \)).

(D) The indicated strains were grown in iron-depleted MM for 24 h and transferred for 1 h to iron-depleted MM (–Fe) or iron-replete MM (+Fe). Intracellular iron concentration was determined colorimetrically and expressed relative to the wild-type strain under iron-depleted conditions. Bars represent \( \pm \) from three independent experiments with three technical replicates each. Values with the same letter are not significantly different according to Mann-Whitney test (\( P = 0.05 \)).

Bars in (A) = 5 mm.
iron competition of Fusarium oxysporum against siderophore-producing pseudomonads. These results reveal a key role for HapX in iron homeostasis, virulence, and rhizosphere competence of this important fungal pathogen.

RESULTS

Loss of HapX Impairs Fungal Growth under Iron-Limiting Conditions without Affecting Iron Acquisition

A BLASTP search of the F. oxysporum genome database identified a single predicted HapX ortholog, FOXG_07577, which displays 32% overall identity with HapX from A. fumigatus. Alignment of the amino acid sequence revealed the presence of all the characteristic domains of this class of transcription factors (see Supplemental Figure 1 online), including an N-terminal region essential for the interaction with the CCAAT binding complex (Hortschansky et al., 2007), a bZIP domain, and several Cys-rich motifs putatively involved in iron sensing (Hortschansky et al., 2007; Schrettl et al., 2010). To study the role of HapX in F. oxysporum, we replaced the entire FOXG_07577 coding sequence with the hygB resistance cassette to generate several ΔhapX deletion mutants (see Supplemental Figure 2 online). The ΔhapX strains showed no growth defects on rich media, but mycelial growth was markedly reduced under iron-limiting conditions and was almost undetectable in the presence of the iron chelator bathophenanthroline disulfonic acid disodium salt (BPS) (Figures 1A and 1B). Likewise, biomass production of the ΔhapX mutant in liquid culture was similar to the wild-type strain under iron-replete conditions but was reduced by more than 50% in iron-depleted medium (Figure 1C). Reintroduction of the intact hapX allele into the ΔhapX mutant, yielding the complemented strain ΔhapX+hapX (see Supplemental Figure 2 online), fully restored wild-type growth (Figures 1A to 1C).

To test whether impaired growth of ΔhapX under iron-depleted conditions is caused by the inability of the mutant to obtain iron from the environment, we measured intracellular iron content 1 h after a shift from iron-depleted to iron-replete conditions (Tamarit et al., 2006). Levels of iron in the ΔhapX mutant were slightly higher than those detected in the wild type and the complemented strain (Figure 1D), suggesting that HapX is not essential for iron uptake in F. oxysporum.

We next examined the role of iron and HapX in the transcription of known iron regulatory genes. Transcript levels of hapX were significantly upregulated in the wild type during iron starvation, indicating that hapX is an iron-repressed gene (Figure 2). Expression of the srbA gene encoding a sterol regulatory element binding protein that regulates iron acquisition in response to hypoxia (Blatzer et al., 2011a) was also induced by iron depletion, and this process was independent of HapX. However, transcript levels of sreA encoding a negative regulator of siderophore biosynthesis decreased under iron starvation conditions, and this decrease was strictly dependent on HapX (Figure 2).

Deletion of hapX Leads to Deregulation of Siderophore Biosynthesis

With the aim of further characterizing the iron uptake system in F. oxysporum, we interrogated the genome database using BLASTP and found putative structural orthologs of all the key siderophore biosynthetic genes characterized in Aspergillus (Figure 3A; see Supplemental Table 1 online). In addition, we identified a second ortholog of sidC, FOXG_17422, which is specific for the genus Fusarium (see Supplemental Figure 3 online). To explore the role of HapX in the regulation of siderophores, we monitored the expression of key genes involved in iron uptake: sidD (encoding Orn monooxygenase), sidC and sidD (encoding two siderophore nonribosomal peptide synthetases [NRPSs]), sidG (encoding fusaranine C [FsC]-acetyl CoA–N₂-transacylase), and mirB (encoding a putative siderophore transporter). Transcript levels of these genes were sharply increased in mycelia grown under iron starvation as compared with iron-sufficient conditions (Figure 3B). In line with these data,
A chrome azurol S (CAS) assay detected significant amounts of extracellular siderophores in culture supernatants of fungal strains grown under iron starvation conditions (Figure 3C) but not under iron-sufficient conditions. Transcriptional induction of siderophore genes by iron starvation was higher in the ΔhapX mutant (200, 25, 140, 180, and 65%, respectively, compared with the wild type), although total levels of extracellular siderophores did not differ significantly between the two strains. Strikingly, however, intracellular siderophore content in mycelia of the ΔhapX mutant was eight times higher than in the wild type and the ΔhapX+hapX strain (Figure 3C).

Analysis of culture supernatants by a combination of reversed-phase high-performance liquid chromatography (HPLC) and high-resolution electrospray ionization mass spectroscopy (MS) detected two different siderophores, FsC and malonichrome (Figure 4A). Malonichrome (peak 2) is a ferrichrome-type compound consisting of a cyclic hexapeptide with the structure Gly-Ala-Gly-(N<sup>5</sup>-malonyl-N<sup>5</sup>-hydroxyornithine)₃ and was previously described as an extracellular siderophore of Fusarium roseum (Emery, 1980). FsC (peak 4) is a cyclic tripeptide consisting of three N<sup>5</sup>-anhydromevalonyl-N<sup>5</sup>-hydroxyornithine (termed fusarinine) residues linked by ester bonds and was reported...
It is also worth noting that the pH of the ΔhapX culture supernatant (4.7) was much lower than that of the wild type (7.2).

Three intracellular ferrichrome-type siderophores were detected in mycelial extracts: malonichrome, ferricrocin, and ferrichrome C (Figure 4B). Similar to malonichrome, ferricrocin and ferrichrome C are cyclic hexapeptides whose structures are Gly-Ser-Gly-(N\(^\alpha\)-acetyl-N\(^\beta\)-hydroxyornithine)\(_3\) and Gly-Ala-Gly-(N\(^\alpha\)-acetyl-N\(^\beta\)-hydroxyornithine)\(_3\), respectively (Haas et al., 2008). Mycelia of the ΔhapX mutant showed a dramatic increase of ferricrocin and ferrichrome C levels (16- and 11-fold, respectively), confirming the results obtained in the CAS assay.

Deletion of hapX Causes Derepression of Iron Regulated Genes and Accumulation of Protoporphyrin IX under Iron Starvation Conditions

Because hapX deletion had no apparent effect on iron uptake (Figure 1D), we asked whether the growth defects of the ΔhapX mutant under iron-limiting conditions could result from iron misuse. To test this hypothesis, global RNA expression profiles of the wild type and the ΔhapX mutant were compared under iron-sufficient and iron-limiting conditions. To search for genes that are repressed under iron starvation in a HapX-dependent manner, we established two selection criteria: (1) a minimum twofold downregulation under steady state iron starvation versus iron sufficiency in the wild type (genes repressed by iron starvation); (2) a minimum twofold upregulation during steady state iron-starved growth in the ΔhapX mutant relative to the wild type (genes derepressed in ΔhapX under iron starvation). Among the 114 genes identified in the screen, 23 (20%) can be directly assigned to iron-dependent processes, such as respiration, the tricarboxylic acid (TCA) cycle, amino acid metabolism, iron-sulfur-cluster biosynthesis, heme biosynthesis, oxidative stress detoxification, vacuolar iron storage, and iron regulation (see Supplemental Data Set 1 online). A comparison with previous genome-wide transcriptional profiling studies in A. fumigatus (Schrettl et al., 2010), C. albicans (Chen et al., 2011), and Schizosaccharomyces pombe (Mercier et al., 2008) defines a list of gene orthologs that share HapX-dependent repression under iron starvation conditions (Table 1). Notably, in Saccharomyces cerevisiae, which lacks a HapX ortholog, a large proportion of this gene set is posttranscriptionally repressed during iron starvation by Cth1 and Cth2 (Puig et al., 2008).

Real-time RT-PCR was performed to measure transcript levels of representative genes from different iron-consuming pathways: cycA encoding the heme protein cytochrome C (respiration), acoA and lysF encoding the iron-sulfur proteins aconitate and homoaconitase (TCA cycle and Lys biosynthesis, respectively), and hemaA encoding the α-amino-levulinic acid synthase (heme biosynthesis), (Hortschansky et al., 2007; Schrettl et al., 2010). All of these were confirmed to exhibit strong repression under iron-depleted conditions in the wild type but not in the ΔhapX mutant (Figure 5). Inspection of the regulatory regions revealed the presence of multiple CCAAT motifs, representing potential binding sites of the Hap protein complex (see Supplemental Figure 4 online).

Under iron-depleted conditions, hyphae of the ΔhapX mutant exhibited a characteristic red autofluorescence (Figure 6), which
Table 1. Iron Metabolism-Related Genes Repressed by HapX Orthologs under Iron Starvation

<table>
<thead>
<tr>
<th>Gene</th>
<th>Gene Product</th>
<th>Wild Type ( \pm ) Fe, log2</th>
<th>Wild Type/hapX (-) Fe, log2</th>
<th>Af</th>
<th>Ca</th>
<th>Sp</th>
<th>Sc</th>
</tr>
</thead>
<tbody>
<tr>
<td>FOXG_02862</td>
<td>Copper-resistance protein Crd2</td>
<td>3.93</td>
<td>-2.44</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_06292</td>
<td>3-isopropylmalate dehydratase (Ile/Leu/Val biosynthesis)</td>
<td>3.17</td>
<td>-4.09</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_15294</td>
<td>Mycelial catalase Cat2 (heme)</td>
<td>3.10</td>
<td>-1.84</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_04395</td>
<td>Dihydroxy acid dehydratase (Ile/Val biosynthesis)</td>
<td>3.07</td>
<td>-1.56</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_04047</td>
<td>Vacular iron transporter Ccc1</td>
<td>2.74</td>
<td>-2.79</td>
<td>x</td>
<td>x</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_10653</td>
<td>Pyridine nucleotide-disulphide oxidoreductase</td>
<td>2.71</td>
<td>-1.62</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_05207</td>
<td>Aconitate hydratase, mitochondrial</td>
<td>2.54</td>
<td>-2.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_10442</td>
<td>Nitrite/sulfite reductase ferredoxin-like half domain</td>
<td>2.49</td>
<td>-2.37</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_04849</td>
<td>Cytochrome P450 monoxygenase</td>
<td>2.39</td>
<td>-1.61</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_09278</td>
<td>Succinate dehydrogenase iron-sulfur protein; TCA cycle</td>
<td>2.37</td>
<td>-1.21</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_11281</td>
<td>NADH-dependent glutamate synthase (GLT1)</td>
<td>2.32</td>
<td>-2.22</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_12892</td>
<td>lysF, Aconitase family (aconitate hydratase, Lys biosynthesis)</td>
<td>2.30</td>
<td>-1.94</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_12260</td>
<td>Peroxidase; catalase/peroxidase HPI</td>
<td>2.27</td>
<td>-1.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_01544</td>
<td>Succinate dehydrogenase, flavoprotein subunit; TCA cycle</td>
<td>2.24</td>
<td>-1.25</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_01159</td>
<td>Homocitrate synthase (Lys biosynthesis)</td>
<td>2.21</td>
<td>-1.00</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_09472</td>
<td>Cytochrome P450</td>
<td>2.20</td>
<td>-1.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_05118</td>
<td>Acyl-CoA dehydrogenase</td>
<td>1.84</td>
<td>-1.05</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_03713</td>
<td>acoA, Aconitase family (aconitate hydratase), TCA cycle</td>
<td>1.73</td>
<td>-1.41</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_08910</td>
<td>Cytochrome P450 monoxygenase</td>
<td>1.63</td>
<td>-1.97</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_00142</td>
<td>Cytochrome c peroxidase</td>
<td>1.54</td>
<td>-1.33</td>
<td>x</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_06266</td>
<td>Siderophore transcription factor SreA</td>
<td>1.23</td>
<td>-1.60</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_06399</td>
<td>FMN-dependent dehydrogenase</td>
<td>1.17</td>
<td>-2.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FOXG_12815</td>
<td>cycA, Cytochrome c</td>
<td>1.12</td>
<td>-1.49</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>FOXG_01103</td>
<td>Hemerythrin HHE cation binding domain-containing protein</td>
<td>1.06</td>
<td>-1.22</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
strains grown as described in Figure 2. Transcript levels of Quantitative real-time RT-PCR analysis was performed in the indicated Iron Use.

(Figure 5). We found that mortality rates in mice strain 4287 can infect and kill immunodepressed mice persici (Ortoneda et al., 2004). We found that mortality rates in mice responses, and then gradually decreased during the later stages of root infection, consistent with an extreme iron starvation re-

upregulated in F. oxysporum during the early stages of tomato root infection, consistent with an extreme iron starvation re-

besides causing vascular wilt on tomato plants, F. o. lycopersici strain 4287 can infect and kill immunodepressed mice (Ortoneda et al., 2004). We found that mortality rates in mice inoculated with the ΔhapX mutant were significantly lower (P < 0.0003) than in those inoculated with the wild type or the ΔhapX+hapX strain. All of the mice infected with the ΔhapX mutant survived the experiment, whereas the wild type and the complemented strain consistently caused death in 80 to 90% of the animals (Figure 8A). Fungal tissue burden in lungs and kidneys of surviving mice inoculated with the ΔhapX mutant was significantly lower (P < 0.0001) than in animals inoculated with the wild type or ΔhapX+hapX (Figure 8B). We also noted that transfer of F. oxysporum from iron-depleted minimal medium (MM) to human blood triggered a rapid transcriptional upregulation of the hapX, sidA, and srbA genes (Figure 8C). These results establish a key role for HapX in reprogramming of iron-dependent gene expression during infectious growth of F. oxysporum on plant and mammalian hosts.

HapX Mediates Iron Competition of F. oxysporum against Root-Colonizing Pseudomonads

F. oxysporum competes for limited iron with other rhizosphere-inhabiting microorganisms, such as fluorescent pseudomonads (Scher and Baker, 1982; Simeoni et al., 1987). We tested the role of HapX in the interaction of F. oxysporum with two root-colonizing Pseudomonas isolates: Pseudomonas putida KT2440 producing the siderophore Pvd (Matthijs et al., 2009) and Pseudomonas fluorescens SBW25 producing both Pvd and Ocg (Matthijs et al., 2008). In vitro, both Pseudomonas spp displayed an antagonistic effect against F. oxysporum, visible as a halo of mycelial growth inhibition around the bacterial colony (Figure 9). The antagonistic effect was specific for iron-depleted conditions and was dependent on siderophore production by Pseudomonas spp, because it was abolished in the P. putida pvd− and P. fluorescens pvd− ocg− mutants. This finding strongly suggests that growth inhibition of F. oxysporum is linked to competition for iron. Further supporting this idea, the antagonistic effect of the Pseudomonas spp wild-type strains was exacerbated against the F. oxysporum ΔhapX mutant, but this effect was not detected in the P. putida pvd− and P. fluorescens pvd− ocg− mutants (Figure 9). Analogous results were obtained using a different in vitro antagonism assay (see Supplemental Figures 5 and 6 online).

We next tested the role of HapX during plant infection by F. oxysporum in the presence of the rhizosphere-colonizing strain P. putida KT2440. Coinoculation was performed by dipping tomato roots for 2 h in a suspension of 109 bacterial cells · mL−1, followed by normal inoculation with F. oxysporum microconidia. As found in previous experiments (Figure 7), mortality rates of tomato plants inoculated with the ΔhapX mutant were significantly lower than those of plants inoculated with the wild type or ΔhapX+hapX strains (Figures 10A to 10C; see Supplemental Table 2 online). Coinoculation of tomato roots with P. putida KT2440 resulted in a significant delay in plant mortality caused by the different F. oxysporum strains, confirming the previously reported biocontrol activity of this bacterial isolate. Strikingly, the attenuation in virulence of the ΔhapX mutant was exacerbated by coinoculation with the P. putida KT2440 wild-type strain but not with the pvd− mutant (cf. Figures 10A to 10C). This strongly suggests that the inhibitory effect of P. putida KT2440

Figure 5. Deletion of hapX Causes Deregulation of Genes Involved in Iron Use.

Quantitative real-time RT-PCR analysis was performed in the indicated strains grown as described in Figure 2. Transcript levels of cycA, acoA, lysF, and hemA genes are expressed relative to those of the wild-type (wt) strain grown under iron-replete conditions. Bars represent SE from three independent experiments with three technical replicates each. Values with the same letter are not significantly different according to the Mann-Whitney test (P > 0.05).

[See online article for color version of this figure.]
against *F. oxysporum* ΔhapX is partially caused by competition for iron in the tomato rhizosphere.

Tomato plants infected with the ΔhapX mutant contained significantly less fungal biomass than those infected with the wild type or the complemented strain, as determined by real-time quantitative PCR of total DNA extracted from tomato roots at 7 d after inoculation (DAI) (Figure 10D). Concomitant with the reduction in disease severity, we noted a decrease of *F. oxysporum* biomass in plants coinoculated with *P. putida* KT2440. Strikingly, the relative decrease of fungal biomass in plants inoculated with the *F. oxysporum* ΔhapX mutant was 2.5 times stronger after coinoculation with the *P. putida* wild-type strain compared with the *pvd* mutant (Figure 10D). Collectively, these results show that HapX functions in iron competition of *F. oxysporum* against siderophore-producing pseudomonads and that this role has a direct effect on the ability of the fungus to proliferate in the rhizosphere and to cause disease on tomato plants.

**DISCUSSION**

Iron is essential for virtually every organism. Although iron is abundant on earth, its availability is limited because of oxidation to insoluble forms by atmospheric oxygen. For this reason, and also because of its toxicity when present in excess, organisms have developed efficient strategies for iron homeostasis. In fungi, iron starvation increases expression of genes required for siderophore-mediated iron uptake (Mei et al., 1993; Haas et al., 2003; Oide et al., 2006; Greenshields et al., 2007; Schrettl et al., 2007). Meanwhile, iron-consuming pathways are rapidly downregulated to optimize the use of the limited iron resource.

![Figure 6. PpIX Accumulates in the ΔhapX Mutant during Iron Starvation.](image-url)

Fungal strains were grown for 24 h in MM with or without iron and were observed microscopically using the Nomarski technique (DIC) to visualize germlings or a dsRed-fluorescence filter to detect PpIX autofluorescence. wt, wild type. Bars = 20 μm. [See online article for color version of this figure.]

<table>
<thead>
<tr>
<th>Table 2. Putative Virulence-Related Genes Induced by Iron Starvation in a HapX-Dependent Manner</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>FOXG_04016</td>
</tr>
<tr>
<td>FOXG_10677</td>
</tr>
<tr>
<td>FOXG_16469</td>
</tr>
<tr>
<td>FOXG_15833</td>
</tr>
<tr>
<td>FOXG_09759</td>
</tr>
<tr>
<td>FOXG_11325</td>
</tr>
<tr>
<td>FOXG_10097</td>
</tr>
<tr>
<td>FOXG_10728</td>
</tr>
<tr>
<td>FOXG_09366</td>
</tr>
<tr>
<td>FOXG_16838</td>
</tr>
<tr>
<td>FOXG_16398</td>
</tr>
</tbody>
</table>

Putative virulence-related genes of *F. oxysporum* are shown that fulfill the following criteria in microarray-based transcriptional profiling: (1) more than twofold upregulated in the wild type under steady state iron starvation versus iron sufficiency (Wild Type ± Fe) and (2) more than twofold downregulated during steady state iron-starved growth in ΔhapX compared with the wild type (Wild Type/hapX − Fe). Genes are ranked based on the level of downregulation in the Wild Type ± Fe condition. Genes predicted to encode a signal peptide and/or transmembrane domain are marked with an x in the columns SignalP and THMM, respectively.
When initiating this study, we hypothesized that a soilborne pathogen, such as *F. oxysporum*, must face iron limitation both during saprophytic and pathogenic stages of its life cycle. In natural soils, total soluble Fe\(^{3+}\) represents as little as \(10^{-210}\) M at equilibrium with soil iron (Simeoni et al., 1987), resulting in competition for iron among soil-inhabiting microorganisms (Haas and Défago, 2005). The availability of iron may be even more limited in mammalian or plant hosts, both of which have efficient mechanisms to sequester iron from invading microorganisms (Jurkevitch et al., 1993; Skaar, 2010). In support of this idea, loss of siderophores decreases virulence in microbial pathogens of humans (Ratledge and Dover, 2000; Schrettl et al., 2004; Schrettl et al., 2007; Cornelis, 2010) and plants (Mei et al., 1993; Schrettl et al., 2004; Oide et al., 2006; Greenshields et al., 2007; Schrettl et al., 2007). Here we find that the bZIP protein HapX, a conserved regulator of fungal iron homeostasis, is required for adaptation of *F. oxysporum* to iron-limiting conditions. Loss of HapX affects multiple aspects of the fungal life cycle, including saprophytic growth, competition with other microorganisms, as well as virulence on plant and mammalian hosts.

**HapX Mediates Adaptation to Iron Starvation by Downregulating Iron-Consuming Pathways**

Our data suggest that HapX is necessary for efficient growth of *F. oxysporum* under iron-limiting conditions but not under iron sufficiency. This result is similar to those reported in *A. nidulans*, *A. fumigatus*, *C. neoformans*, and *C. albicans* (Hortschansky et al., 2007; Jung et al., 2010; Schrettl et al., 2010; Hsu et al., 2011). The specific role during iron starvation suggests a key function of HapX in iron acquisition and/or use. However, the *F. oxysporum* ΔhapX mutant is not affected in iron uptake. This result is in line with a previous study in the human pathogen *C. albicans* (Hsu et al., 2011). By contrast, global RNA expression profiling revealed that HapX is essential for iron starvation–triggered downregulation of genes from different iron-dependent pathways. Thus, a key role of HapX in iron homeostasis of *F. oxysporum* is to shut down iron-consuming processes, such as respiration, amino acid metabolism, the citric acid cycle, or heme biosynthesis, when iron becomes limiting. Genome-wide transcriptional analyses in *S. pombe*, *A. fumigatus*, *C. neoformans*, and *C. albicans* also suggested that HapX orthologs are required for downregulation of iron-consuming genes during iron starvation (Mercier et al., 2006; Puig et al., 2008; Jung et al., 2010; Schrettl et al., 2010; Chen et al., 2011). This hypothesis is further corroborated by our finding that the *F. oxysporum* ΔhapX mutant has dramatically increased levels of PpIX as a consequence of heme pathway deregulation. Collectively, these results establish a conserved function of HapX in repression of iron-dependent pathways and highlight the essential role of this transcription factor in fungal adaptation to iron starvation conditions.

**HapX Is a Regulator of Siderophore Biosynthesis**

*F. oxysporum* ΔhapX mutants show an increase in transcript levels of siderophore biosynthetic genes under iron limitation.
Accordingly, levels of the intracellular siderophores ferricrocin and ferrichrome C during iron limitation are much higher in the \( \Delta \)hapX mutant than in the wild type. By contrast, loss of HapX in Aspergillus caused a reduction in triacetylfusarinine C (TAFC), an extracellular siderophore derived by \( N_2 \)-acetylation of FsC (Hortschansky et al., 2007; Schrettl et al., 2010). We detected only FsC, but not TAFC, in \( F. \) oxysporum culture supernatants. This result was unexpected, because TAFC has been reported both in Aspergillus spp and Fusarium graminearum (Oide et al., 2006; Schrettl et al., 2007; Blatzer et al., 2011b; Yasmin et al., 2012). The \( F. \) oxysporum genome encodes orthologs of all the known enzymes involved in TAFC biosynthesis, including SidG, which catalyzes \( N_2 \)-acetylation of FsC (Figure 4A; see Supplemental Table 1 online). Expression of sidG was detected by quantitative real-time RT-PCR (Figure 3B); therefore, we speculate that the \( F. \) oxysporum SidG homolog may lack sufficient enzymatic activity for TAFC production under the conditions tested.

Ferricrocin is the major intracellular siderophore in most Ascomycetes analyzed so far (Haas et al., 2008). The \( F. \) oxysporum genome encodes two NRPSs with similarity to ferrichrome-type NRPSs, such as \( A. \) fumigatus SidC, suggesting that at least two of the three intracellular siderophores detected in this study are synthesized by the same NRPS. Loss of HapX resulted in a coordinated increase of the ferricrocin and ferrichrome C contents, suggesting that the two siderophores may be synthesized by the same NRPS. The structure of ferrichrome C closely resembles that of ferricrocin, with the exception of an Ala replacing a Ser. The cellular content of ferricrocin is \( \sim \)15 times higher than that of ferrichrome C; therefore, it is feasible that ferrichrome C is synthesized by the ferricrocin-specific NRPS through relaxed specificity of the Ser-specific adenylation domain (Haas et al., 2008).

HapX is required for downregulation of sreA, encoding a repressor of siderophore biosynthesis, during iron-limiting conditions (this study; Hortschansky et al., 2007; Schrettl et al., 2010). HapX and SreA are interconnected by a negative feedback loop in \( A. \) nidulans, \( A. \) fumigatus, and \( S. \) pombe (Mercier et al., 2006; Hortschansky et al., 2007; Schrettl et al., 2010). The \( \Delta \)hapX mutant was thus expected to display constitutive repression of siderophore genes and a reduction of the intra- and extracellular pool of siderophores during iron starvation. Instead, transcription of sidA, sidC, sidD, sidG, and mirB as well as intracellular siderophore levels were increased in the \( \Delta \)hapX mutant during iron limitation. In \( A. \) nidulans, transcript levels of sidC during iron limitation were also increased in the \( \Delta \)hapX mutant (Hortschansky et al., 2007). By contrast, siderophore biosynthetic genes in the \( A. \) fumigatus \( \Delta \)hapX mutant were downregulated during iron starvation (Schrettl et al., 2010). How siderophore biosynthesis remains activated in the \( \Delta \)hapX mutant...
while the sreA repressor gene is upregulated remains to be determined. It has been suggested that both SreA and HapX may posttranslationally sense iron (Haas et al., 1999; Hortschansky et al., 2007). In *S. pombe*, HapX and SreA orthologs interact with the monothiol glutaredoxin Grx3 for iron regulation (Mercier and Labbé, 2009; Jbel et al., 2011; Kim et al., 2011). Our data further support the idea that the SreA repressor is only functional under iron sufficiency. According to this model, sreA derepression in the *D hapX* mutant during iron limitation does not result in reduced siderophore production, because SreA is present in an inactive state. Further studies are needed to confirm this hypothesis.

**Figure 9.** HapX Is Required for Efficient Iron Competition of *F. oxysporum* with Fluorescent Pseudomonads.

Growth of the indicated *F. oxysporum* strains was determined on solid MM in the presence of the rhizosphere-colonizing bacterial strains *P. putida* KT2440 (wild-type [wt] or *pvd*^2^, strain lacking the siderophore Pvd) or *P. fluorescens* SBW25 (wild-type, *pvd*^2^, or *pvd*^2^ *ocg*^2^ lacking the siderophores Pvd and Ocg). *F. oxysporum* microconidia were evenly spread on top of Glc casamino acids medium with or without iron. Bacterial strains were point-inoculated on the same day, and cultures were incubated for 4 d at 28°C. Mycelial growth is visible as a gray background, whereas growth inhibition appears as a dark halo surrounding the bacterial colony. Bars = 5 mm.

**HapX Contributes to Iron Competition of *F. oxysporum* against Soil- and Rhizosphere-Inhabiting Pseudomonads**

Vascular wilts caused by *F. oxysporum* f spp are among the most common plant diseases in agriculture (Dean et al., 2012). Chemical control of the pathogen is largely unviable, because of regulatory restrictions and its high persistence in the soil (Agrios, 1997). Biological control measures, such as disease-suppressive soils, have been investigated for more than 70 years (Hornby, 1979; Alabouvette et al., 2009). The genus *Pseudomonas* is among the most extensively studied biocontrol agents (Alabouvette et al., 1979; Kloepper et al., 1980; Haas and Défago, 2005) and includes aggressive rhizosphere-colonizing strains that can increase plant growth and improve plant health (Haas and Défago, 2005; Weller, 2007; Cornelis, 2010). Known biocontrol mechanisms include the production of antibiotics, hydrogen cyanide, lytic exoenzymes (Thomashow and Weller, 1996), cyclic lipopeptides (Raaijmakers et al., 2006), competition for nutrient niches (Kamilova et al., 2005), siderophore-mediated iron competition (Thomashow and Weller, 1996), and induced systemic resistance (De Vleesschauwer and Höfte, 2009). The role of siderophores in antagonism of fluorescent pseudomonads against plant pathogenic fungi is well established (Haas and Défago, 2005; Weller, 2007; Cornelis, 2010), although several aspects, such as the presence of low-affinity siderophores besides the high-affinity siderophore Pvd, are not completely understood (Cornelis and Matthijs, 2002).

We provide several lines of evidence supporting a role of extracellular siderophores in the antagonism of *Pseudomonas* against *F. oxysporum*, both in vitro and on the tomato rhizosphere. First, the strongest in vitro growth inhibition effect with two bacterial strains was detected against the *F. oxysporum* D hapX mutant under iron-limiting conditions. Second, the in vitro antagonistic effect was strictly dependent on the production of bacterial siderophores. Third, coinoculation with the rhizosphere-colonizing *P. putida* strain KT2440 resulted in a marked decrease in *F. oxysporum* infection on tomato plants and a significant delay in the development of vascular wilt symptoms. It is important to note that part of the protective effect of *P. putida* was independent of siderophore production, because it was present both in the bacterial wild-type strain and the *pvd*^2^ mutant. This suggests the presence of additional modes of action previously reported for *Pseudomonas* spp, such as antibiosis or competition for nutrients and space in the rhizosphere (Weinberg, 1986; Azegami et al., 1988; Gill and Warren, 1988). Interestingly, plants infected with the *F. oxysporum*
Conserved Role of HapX in Virulence on Plants and Mammals

Iron uptake and metabolism is required for virulence of both bacterial (Expert, 1999; Taguchi et al., 2010) and fungal plant pathogens (Eichhorn et al., 2006; Oide et al., 2006; Greenshields et al., 2007). Although our data suggest that HapX is not required for in vitro iron uptake by *F. oxysporum*, we find that the ΔhapX mutants are significantly attenuated in their capacity to cause vascular wilt symptoms and mortality in tomato plants, thus providing evidence for a role of HapX in virulence of a plant pathogen. Biomass of the ΔhapX mutant in tomato roots was reduced; therefore, we conclude that HapX contributes to survival and proliferation of the fungus on the plant host. Interestingly, *F. oxysporum* strains lacking hapX were also unable to efficiently colonize and kill immunodepressed mice, confirming previous reports in the human pathogens *C. neoformans*, *A. fumigatus*, and *C. albicans* (Jung et al., 2010; Schrettl et al., 2010; Hsu et al., 2011). HapX is the first virulence determinant for which an essential role during plant and animal infection has been demonstrated in the same fungal pathogen. Previous studies in *F. oxysporum* identified either factors that are required for pathogenicity on tomato but not on mice, including the *Fusarium* Mitogen-activated protein Kinase1 (Fmk1), the small G protein Rho1, and the glucansyltransferase Gas1 (Di Pietro et al., 2001; Caracuel et al., 2005; Martinez-Rocha et al., 2008), or vice versa, such as the pH response factor PacC, the light response factor White Collar-1 (Wc-1), or the secreted *Fusarium* Pathogenesis Related-1 (PR-1)-like protein Fpr1 (Caracuel et al., 2003; Ruiz-Roldán et al., 2008; Prados-Rosales et al., 2012). Our results suggest that HapX functions in two alternative genetic programs associated with infectious fungal growth on plants and mammals, most likely involving transcriptional reprogramming under severe iron limitation encountered in both types of hosts (Jurkevitch et al., 1993; Loper and Henkels, 1997; Weinberg, 1999; Weiss, 2002).

Deregulation of genes required for metabolic adaptation to iron deficiency may account for the impaired ability of the *F. oxysporum* ΔhapX mutant to proliferate in the host, as observed in the *in vitro* condition. However, our data suggest an additional...
role for HapX in the transcriptional activation of virulence-related genes. Iron starvation–induced genes, such as srba, sidA, or hapX itself, are dramatically upregulated during growth of *F. oxysporum* in tomato roots or human blood. Microarray analysis identified an entire set of genes whose expression is activated by iron starvation in a HapX-dependent manner. A significant fraction of these genes encodes predicted secreted proteins that have been linked to fungal virulence, including cell wall–degrading enzymes, such as pectate lyase and endoglucanase (Di Pietro et al., 2009), aspartyl proteases (Naglik et al., 2003), or the SIX3 protein (van der Does et al., 2008). Additional virulence-related genes activated by HapX include those encoding integral membrane proteins, such as PTH11-like receptors (De Zwaan et al., 1999) or ABC transporters (Urban et al., 1999; Coleman and Mylonakis, 2009). Unexpectedly, we found that genes associated with mobile genetic elements, such as hAT family transposases and reverse transcriptase, are also induced by iron starvation via HapX. Collectively, these results suggest a role for HapX in the activation of iron starvation–induced virulence factors during fungal growth in the host. Further characterization of these potential virulence targets will provide new insights into the conserved role of the HapX-mediated iron response in fungal pathogenicity on plants and mammals.

**METHODS**

**Fungal Strains and Culture Conditions**

*Fusarium oxysporum* f.sp *lycopersici* race 2 wild-type isolate 4287 (FGSC 9935) was used in all experiments. All fungal strains were stored as microconidial suspensions at −80°C with 30% glycerol. For extraction of genomic DNA and microconidial production, cultures were grown in potato dextrose broth at 28°C with shaking at 170 rpm (Di Pietro and Roncero, 1998). For analysis of gene expression, freshly obtained microconidia were germinated for 24 h in iron-depleted MM (Puhalla, 1968); mycelia were harvested by filtration, washed three times in sterile double-distilled water (ddH$_2$O), and transferred for 10 h to fresh MM with or without 50 μM of Fe$_2$(SO$_4$)$_3$. For analysis of gene expression in human blood, wild-type microconidia were germinated for 16 h in iron-depleted MM (Puhalla, 1968); mycelia were harvested by filtration, washed three times in sterile double-distilled water (ddH$_2$O), and transferred for 10 h to fresh MM with or without 50 μM of Fe$_2$(SO$_4$)$_3$. For analysis of gene expression in human blood, wild-type microconidia were germinated for 16 h at 28°C in iron-depleted MM containing 25 mM of sodium glutamate and 20 mM of HEPS, pH 7.4, transferred for 4 h to 37°C, and then transferred for different time periods to fresh MM or to heparinized human whole blood (Dunn Labortechnik GmbH) at 37°C. For analysis of in planta gene expression, wild-type microconidia were inoculated in iron-depleted MM in the presence of tomato (*Solanum lycopersicum*) roots as previously described (López-Berges et al., 2010), and the roots with the adhered mycelium were frozen after 2 d for RNA extraction. For later stages of infection, tomato plants inoculated with wild-type conidia were planted in vermiculite (see below), and roots were obtained 5 or 10 DAI and washed carefully before RNA extraction.

For intracellular iron determination, freshly obtained microconidia were germinated for 24 h in iron-depleted MM. Mycelia were harvested by filtration, washed three times in sterile ddH$_2$O, and transferred for 1 h to fresh MM with or without 50 μM of Fe$_2$(SO$_4$)$_3$. For analysis of colony growth, 2 × 10$^3$ fresh microconidia were spotted onto potato dextrose agar (PDA) (Scharlau) or MM with or without 50 μM of Fe$_2$(SO$_4$)$_3$ and/or 0.2 mM of BPS. 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. For quantification of siderophores and PpxX, strains were grown at 28°C in *Aspergillus* MM (according to Pontecorvo et al. [1953]) containing 1% Glic as the carbon source and 20 mM of Gln as the nitrogen source for 5 d. Iron-replete media contained 30 μM of FeSO$_4$. For iron-depleted conditions, iron was omitted.

**Bacterial Strains**

Two *Pseudomonas* isolates were used: *Pseudomonas putida* KT2440 producing the siderophore Pvd and its pvd” mutant (SA12) (Matthijs et al., 2008), and *Pseudomonas fluorescens* SBW25 producing both Pvd and Ocg and its pvd” mutant (SBW25) (Moon et al., 2008). For construction of the pvd” ocg” double mutant (15F3), the suicide plasmid pUT harboring the transposon mini-TnSphoA3 (gentamycin-resistant) (de Chial et al., 2003) was used to generate transposon insertions in the chromosome of *P. fluorescens* SBW25::pvdL. Mid-log phase cultures of *Escherichia coli* SM10 (λpir), the host of pUT-mini-TnSphoA3, were mixed with strain SBW25::pvdL in a 1:1 ratio. *P. fluorescens* SBW25::pvdL was kept at 45°C for 20 min just before mixing both strains to inactivate its restriction system. After overnight incubation on Luria-Bertani medium at 26°C, transposon insertions were selected on CAA supplemented with 100 μg mL$^{-1}$ of gentamycin and 25 μg mL$^{-1}$ of chloramphenicol. To avoid counterselection of mutants affected in iron uptake and metabolism, transposon-mutagenized pvdL recipients were selected on CAA amended with 50 μg of FeCl$_3$. A bank of 2000 transconjugants was screened for mutants with loss of siderophore production as detected by the CAS assay (Schwyn and Neilands, 1987). Chromosomal DNA of 15F3 was isolated using the Genta Puregene Genomic DNA Purification Kit (Qiagen), digested with Sall (Fermentas) and self-ligated, and the DNA flanking the mini-TnSphoA3 was isolated and sequenced.

**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). The quantity and quality 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).

**Targeted Gene Knockout**

PCR reactions were routinely performed with the High Fidelity Template PCR system (Roche Diagnostics) using a MJ Mini Bio-Rad personal thermal cycler (see Supplemental Table 3 online for a complete list of primer sequences used in the study). All fungal transformations and purification of the transformants by monoclonal isolation were performed as described (Di Pietro and Roncero, 1998). Targeted replacement of the entire coding region of the *F. oxysporum* hapX gene with the hygromycin-resistance cassette (Punt et al., 1987) was performed using the double-joint PCR method (Yu et al., 2004) (see Supplemental Figure 2A online). DNA fragments flanking the hapX coding region were amplified from genomic DNA of *F. oxysporum* with primer pairs hapX-1 + hapX-1n of gentamycin and/or 0.2 mM of BPS. 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. For quantification of siderophores and PpxX, strains were grown at 28°C in *Aspergillus* MM (according to Pontecorvo et al. [1953]) containing 1% Glic as the carbon source and 20 mM of Gln as the nitrogen source for 5 d. Iron-replete media contained 30 μM of FeSO$_4$. For iron-depleted conditions, iron was omitted.
B–resistance gene under control of the *Aspergillus nidulans* gpdA promoter and trpC terminator, amplified with primers gpdA-15b + trpC-8b from the plasmid pAN8-1 (Matern et al., 1988). Three out of eight pleomycin-resistant cotransformants were selected for their wild-type growth phenotype on solid MM without iron and were analyzed for the presence of a functional hapX allele by PCR with gene-specific primer pair hapX-4 + hapX-5 (see Supplemental Figure 2D online). We concluded that these transformants, designated ΔhapX+hapX, had integrated an intact copy of the *F. oxysporum* hapX gene into the genome.

**Sequence Alignments, Phylogenetic Analysis, and Accession Numbers**

HapX orthologs were identified by BLASTP searches in the Fusarium Comparative Genome database of the Broad Institute (http://www.broadinstitute.org/annotation/genome/fusarium_graminearum/Multi-Home.html) or the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST.cgi) websites, using the *Aspergillus fumigatus* HapX protein as bait. Full-length sequences were aligned with ClustalW (Thompson et al., 1994) and manually inspected. The SidC phylogenetic tree was built by maximum likelihood from a ClustalW alignment with PhyML version 4.0 using both parsimony and distance analysis (neighbor joining) with 1000 bootstrap replicates and represented as a phylogram with Dendroscope v1.2.3 (Guindon and Gascuel, 2003). Putative SidC orthologs in each fungal genome were identified by BLASTP searches on the Broad Institute or the National Center for Biotechnology Information websites, using the *A. fumigatus* SidC protein as bait.

**Intracellular Iron Quantification**

Intracellular iron concentration was measured using the BPS-based colorimetric assay (Tamarit et al., 2006; Hsu et al., 2011), with modifications. Briefly, mycelia were harvested by filtration, washed three times with ddH2O, and resuspended in 500 μL of 3% nitric acid. Suspensions were boiled for 2 h and centrifuged to discard cell debris. A total of 400 μL of supernatant was mixed with 160 μL of sodium ascorbate, 320 μL of BPS, and 126 μL of ammonium acetate, and reactions were incubated at room temperature for 5 min. OD535 of the BPS–Fe complex was measured with a SmartSpec Plus spectrophotometer (Bio-Rad). To eliminate the nonspecific absorbance, OD600 was subtracted from OD535. Intracellular iron concentration was expressed relative to that of the wild-type strain grown under iron-depleted conditions.

**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 3 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 Δcycle threshold (Livak and Schmittgen, 2001; Pfaffl, 2001) and normalized to act1. Expression values are presented as values relative to the expression in the wild-type strain under iron-replete conditions.

**Transcriptional Profiling**

A custom-made *F. oxysporum* microarray chip in the 4 × 44 K format (Agilent Technologies) containing 45,220 distinct 60-mer probes representing a total of 17,781 genes was used in this study. Three different probes were used for genes larger than 885 bp, and two were used for those smaller than 885 bp. RNA was prepared from the wild type or the ΔhapX strain grown in the presence or absence of Fe2(SO4)3 as described above. In total, three arrays were analyzed, representing three biological replicates. Microarray services were performed by the company Bioarray SL, an Agilent Certified Service Provider. Quality of RNA was analyzed using Nanodrop (Thermo Fisher Scientific) and Bioanalyzer 2100 (Agilent Technologies). RNA labeling with Cy3, array hybridization, and scanning were performed following Agilent’s One-Color Microarray-Based Gene Expression Analysis protocol (http://www.chem.agilent.com/Library/ usemanuals/Public/G4140-90040_GeneExpression_One-color_v6.5.pdf). Expression of a given gene was calculated based on the averaged processed data of the different probes corresponding to each gene.

Data background subtraction was performed using the “normexp” method with an offset value of 10. Interarray normalization was done using the “quantiles” method, which was implemented in R and included in the Bioconductor package (http://www.r-project.org/; Workman et al., 2002). Statistical analysis was conducted using the Bioconductor packages Limma, Maruy, affy, Pqacmethods, and EMA. The unequal f test assuming equal variances was used for statistical comparison between the different data sets. A false discovery rate of 0.05 was considered significant.

**Fluorescence Microscopy**

Red autofluorescence of PpIX was visualized as described (Hortschansky et al., 2007) using an Imager M2 Zeiss Axiosplan fluorescence microscope with a dsRed2 filter (excitation/emission at 546/590 nm) and a digital Photomicros Evolve camera for documentation.

**Analysis of Fungal Siderophores and PpIX**

Analysis of siderophores and PpIX was performed by CAS assay, reverse-phase HPLC, and high-resolution electrospray ionization MS as previously described (Oide et al., 2006). Siderophore analysis with reverse-phase HPLC was performed after saturation with FeSO4.

**In Vitro Antagonism Assays with Pseudomonas spp**

For the in vitro determination of *F. oxysporum*-Pseudomonas antagonism, 2.5 × 106 fresh fungal microconidia were spread onto Glic casamino acids solid medium (Cornelis et al., 1992) with or without 50 μM of Fe2(SO4)3 using a 1% agar-water top solution. A total of 5 μL of a bacterial overnight culture grown in Luria-Bertani medium and washed three times with ddH2O was spotted immediately on the surface of the plate, and plates were incubated for 4 d at 28°C. Alternatively, bacterial strains were point-inoculated with a toothpick on 25-mM-Gln MM plates with or without 50 μM of Fe2(SO4)3 and incubated at 28°C. After 24 h, 5 × 104 freshly obtained fungal microconidia were spotted at a distance of 20 mm, and cultures were incubated for an additional 5 d.

**Plant Infection Assays**

Tomato root inoculation assays were performed as described (Di Pietro and Roncero, 1998). Briefly, 2-week-old seedlings of tomato cv Monika were inoculated with *F. oxysporum* strains by immersing the roots in a microconidial suspension, planted in vermiculite, and maintained in a growth chamber. For bacterial/fungal coinoculation assays, seedlings were first dip-inoculated into a suspension of *P. putida* strains for 2 h and then infected with *F. oxysporum* as described above (Vitullo et al., 2011). The severity of disease symptoms and percentage survival were recorded each day for 30 to 45 d. Ten plants were used for each treatment. Virulence experiments were performed at least three times with similar results. Survival was estimated by the Kaplan-Meier method and compared
among groups using the log-rank test. Data were analyzed with the software GraphPad Prism 4.

In planta quantification of fungal biomass was performed as described (Pareja-Jaime et al., 2010), with modifications. Briefly, total genomic DNA was extracted from infected tomato roots at 7 DAI. Quantitative real-time PCR was performed, and relative amounts of fungal genomic DNA were calculated by comparative cycle threshold of the fungus-specific six1 gene (Rep et al., 2004) normalized to the tomato gadph gene.

**Animal Infection Assays**

Mice were cared for in accordance with the principles outlined by the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (http://conventions.coe.int/Treaty/Conventions/Html/Treaty123.htm). Experimental conditions were approved by the Animal Welfare Committee of the Faculty of Medicine, Universitat Rovira i Virgili.

Experimental assays with immunodepressed mice were performed as described (Ortoneda et al., 2004). Briefly, groups of 10 Oncins France 1 male mice (Charles River, Criffa S.A.) were immunosuppressed with a single intraperitoneal 200 mg kg\(^{-1}\) dose of cyclophosphamide (Laboratorios Funk S.A.) and with a single intravenous 150 mg kg\(^{-1}\) dose of 5-fluorouracil (Fluoro-uracil, Roche S.A.) and infected by injecting 0.2 mL of an inoculum of 10\(^6\) conidia mL\(^{-1}\) of sterile saline into a lateral vein of the tail. Survival was recorded each day for 15 d. Infection experiments with each individual strain were performed at least three times. Survival was estimated by the Kaplan-Meier method and compared among groups using the log-rank test. To determine fungal tissue burden, randomly chosen surviving mice were sacrificed 7 DAI. Kidneys and lungs were aseptically removed, weighed, and homogenized in sterile saline, and 10-fold serial dilutions were spread onto PDA. Plates were incubated at 28°C, colonies were counted after 3 d, and the number of colony forming units per gram of organ was calculated. Fungal colony counts were converted to log\(_{10}\) and compared using the analysis of variance test. Data were analyzed with the software GraphPad Prism 4.

**Supplemental Data**

**Supplemental Figure 1.** Amino Acid Sequence Alignment of Fungal Orthologs of the bZIP Protein HapX.

**Supplemental Figure 2.** Targeted Disruption of the *F. oxysporum* hapX Gene.

**Supplemental Figure 3.** The Genus *Fusarium* Contains an Extra Ortholog of the NRPS SidC.

**Supplemental Figure 4.** CCAAT Sequences in the Promoter Regions of HapX-Regulated Genes.

**Supplemental Figure 5.** *P. putida–F. oxysporum* Antagonism Assay.

**Supplemental Figure 6.** *P. fluorescens–F. oxysporum* Antagonism Assay.

**Supplemental Table 1.** Predicted Orthologs of Siderophore Bio-synthetic and Regulatory Genes of *A. fumigatus* in *F. oxysporum*.

**Supplemental Table 2.** Statistical Significance (P Values) of Tomato Plant Survival Curves in Coinoculation Experiments of *F. oxysporum* and *P. putida*.

**Supplemental Table 3.** Primer Sequences Used in This Study.

**Supplemental Data Set 1.** Genes Derepressed in ΔhapX under Iron Starvation.

**Supplemental Data Set 2.** Genes Induced by Iron Starvation in a HapX-Dependent Manner.

**ACKNOWLEDGMENTS**

We thank Esther Martínez Aguilera for valuable technical assistance. This research was supported by the following grants: BIO2010-15505 from Ministerio de Ciencia e Innovación (MICINN), European Research Area (ERA)-NET(PathoGenoMics project TRANSPAT (BIO2008-04479-E from MICINN). EU2009-03942 from MICINN/Plant KBBE, BIO-3847 from Junta de Andalucia to A.D.P., Marie Curie Initial Training Network ARIADNE (FP7-PEOPLE-ITN-237936) to A.D.P., and ERA-Net/PathoGenoMics project TRANSPAT (FWF I282-B09 from Austrian Science Foundation) to H.H. M.S.L.-B. received a PhD fellowship from MICINN.

**AUTHOR CONTRIBUTIONS**


Received March 23, 2012; revised August 2, 2012; accepted August 27, 2012; published September 11, 2012.

**REFERENCES**


HapX-Mediated Iron Homeostasis Is Essential for Rhizosphere Competence and Virulence of the Soilborne Pathogen *Fusarium oxysporum*

Manuel S. López-Berges, Javier Capilla, David Turrà, Lukas Schafferer, Sandra Matthijs, Christoph Jöchl, Pierre Cornelis, Josep Guarro, Hubertus Haas and Antonio Di Pietro

*Plant Cell* 2012;24;3805-3822; originally published online September 11, 2012; DOI 10.1105/tpc.112.098624

This information is current as of June 26, 2017