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Iron competition in fungus-plant interactions

The battle takes place in the rhizosphere

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Abbreviations: TCA, tricarboxylic acid; pvd, pyoverdine; DEGs, differentially expressed genes; GO, gene ontology

Soilborne fungal pathogens are highly persistent and provoke important crop losses. During saprophytic and infectious stages in the soil, these organisms face situations of nutrient limitation and lack of essential elements, such as iron. We investigated the role of the bZIP transcription factor HapX as a central regulator of iron homeostasis and virulence in the vascular wilt fungus *Fusarium oxysporum*. This root-infecting plant pathogen attacks more than hundred different crops and is an emerging human opportunistic invader. Although iron uptake remains unaffected in a strain lacking HapX, de-repression of genes implicated in iron-consuming processes such as respiration, amino acid metabolism, TCA cycle and heme biosynthesis lead to severely impaired growth under iron-limiting conditions. HapX is required for full virulence of *F. oxysporum* in tomato plants and essential for infection in immunodepressed mice. Virulence attenuation of the Δ*hapX* strain on tomato plants is more pronounced by co-inoculation of roots with the biocontrol strain *Pseudomonas putida* KT2440, but not with a mutant deficient in siderophores production. These results demonstrate that HapX is required for iron competition of *F. oxysporum* in the tomato rhizosphere and establish a conserved role for HapX-mediated iron homeostasis in fungal infection of plants and mammals.

Iron (Fe) is a key element for virtually every organism and functions as an essential cofactor of a wide range of cellular processes. However, the excess of this metal can be highly toxic promoting the production of reactive oxygen species.[1] Because of this duality and the limited bioavailability of iron given its conversion into insoluble forms, organisms have developed tightly controlled mechanisms to maintain iron homeostasis, i.e., the balance between uptake, storage and utilization of this element.

Previous studies suggested that human pathogens must cope with the extreme iron-limiting conditions originated by the mammalian immune system to keep invading microorganisms at bay.[2-5] Here we investigated the role of iron homeostasis in the soilborne fungal plant pathogen *Fusarium oxysporum*. Since soluble Fe^{3+} in natural soils represents only ~10^{-10} M at equilibrium with soil iron[6] and plant roots have efficient iron-sequestering mechanisms,[7] we hypothesized that iron homeostasis should play an important role during root infection. *F. oxysporum* infects and kills both tomato plants and immunodepressed mice, and thus provides an excellent model to study the role of iron homeostasis during fungal infection of plant and mammalian hosts.[8]

The bZIP transcription factor HapX, which was was originally identified in *Schizosaccharomyces pombe*[9] and *Apergillus nidulans*,[10] has been reported to regulate iron-dependent pathways in several fungal species. *HapX* transcript levels are upregulated under iron-depleted conditions.[2,4,5,11] We found that deletion of *hapX* in *F. oxysporum* had no effect on mycelial growth on iron-sufficient media, but dramatically reduced growth under iron-depleted conditions.[12] Interestingly, as reported in *Candida albicans*,[4] the *F. oxysporum* Δ*hapX* mutant was fully competent in iron uptake. In agreement with this result, transcription of siderophore genes was strongly induced during iron-depleted conditions both in the wild type strain and the Δ*hapX* mutant.[12] Moreover, significant levels of extracellular siderophores were detected in culture supernatants of the fungal strains grown under iron-depleted conditions, but not during iron sufficiency.[12] Intracellular siderophore

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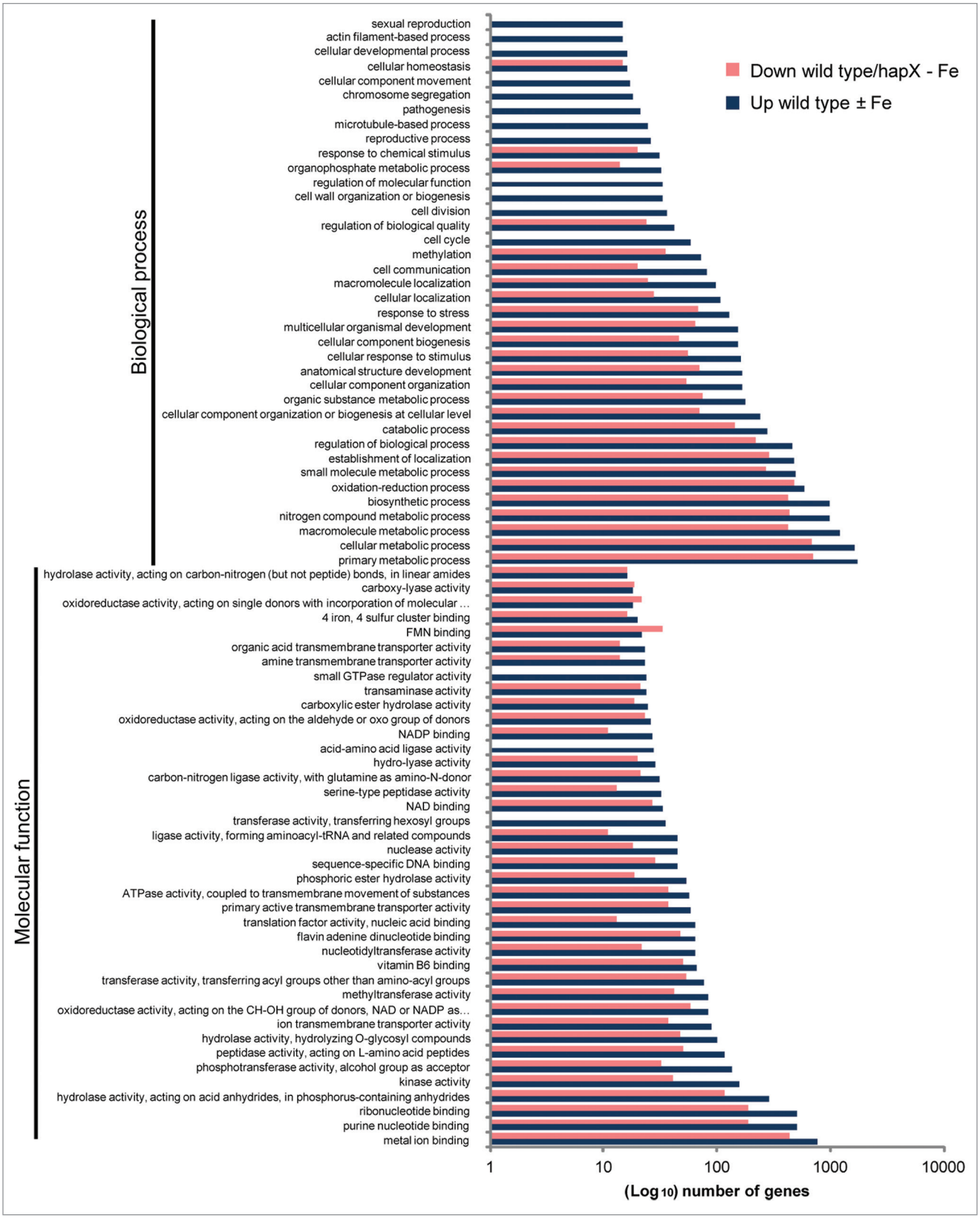

Figure 1. For figure legend, see following page.

Figure 1. (Previous page.) Functional categories of differentially expressed genes (DEGs) repressed by HapX under steady-state iron starvation. GO functional enrichment analysis of *F. oxysporum* DEGs that fulfill the following criteria in microarray-based transcriptional profiling: (1) Downregulation in the wild type under steady-state iron starvation vs. iron sufficiency (with or without 50 μM $Fe_2(SO_4)_3$; wild type ± Fe); (2) Upregulation during steady-state iron-starved growth in Δ*hapX* compared with the wild type (wild type/hapX - Fe). DEGs were assigned to different functional categories using Blast2GO (version 2.3.5; www.blast2go.org) with the default parameters. The Blast2GO program extracts the GO terms associated with homologies identified with NCBI's QBLAST and returns a list of GO annotations represented as hierarchical categories of increasing specificity. The level presented in each principal GO category corresponds to 5 and 3 for the molecular function and biological process categories, respectively, with 40 and 38 categories shown, respectively.

content in the Δ*hapX* mutant was even higher than that of wild type or the Δ*hapX*+*hapX* complemented strain. Collectively, these results suggest that impaired growth of the Δ*hapX* mutant under iron starvation is unlikely to be caused by a defect in iron acquisition.[12]

An alternative explanation would be that the Δ*hapX* mutant wastes iron because it is unable to reduce iron consumption under limiting conditions. We used microarrays to compare genome-wide transcription in the *F. oxysporum* wild type strain and Δ*hapX* mutant under steady-state iron starvation vs. iron sufficiency (with or without 50 μM $Fe_2(SO_4)_3$), with the aim to search for differentially expressed genes (DEGs) downregulated in the wild type but not in the Δ*hapX* mutant.[12] Functional classification of DEGs repressed by HapX under steady-state iron starvation evidenced specific subcategories of molecular function and biological process (wild type ± Fe in **Fig. 1**), including hexosyl group transferase activity, acid-amino acid ligase activity, small GTPase regulator activity and cell cycle, cell division, cell wall organization and biogenesis, regulation of molecular function, reproductive processes, microtubule-based processes, pathogenesis, chromosome segregation, cellular component movement, cellular developmental process, actin filament-based process, sexual reproduction, respectively.

Additional molecular function subcategories over-represented in the wild type ± Fe comparison include metal ion binding, purine nucleotide binding, ribonucleotide binding and hydrolase activity acting on acid anhydrides in phosphorus-containing anhydrides with 341, 318, 318 and 173 genes, respectively, more in wild type ± Fe than in the wild type/hapX-Fe comparison. Biological process subcategories such as primary metabolic process, cellular metabolic process, macromolecule metabolic process, nitrogen compound metabolic process and biosynthetic process had 1027, 950, 807, 546, 546 more genes, respectively, in the wild type ± Fe than in the wild type/hapX-Fe comparison (**Fig. 1**).

In line with the starting hypothesis, this group of DEGs includes a significant number of genes from iron-consuming pathways such as respiration, TCA cycle, Lys biosynthesis or heme biosynthesis.[12] Real-time qRT-PCR of representative genes from these categories confirmed that they were strongly repressed during iron starvation in a HapX dependent manner.[12] Taken together, these results suggest that growth deficiencies of the Δ*hapX* mutant under iron starvation are due to de-repression of iron-consuming pathways leading to iron misuse.[12]

Role of HapX-mediated iron homeostasis in fungal virulence on plants and mammals. When roots of tomato seedlings were inoculated with the different fungal strains, mortality rates of plants infected with the Δ*hapX* mutant were significantly lower than those of plants infected with the wild type or with the Δ*hapX*+*hapX* complemented strain.[12] Furthermore, fungal biomass inside the plant roots 7 d after fungal inoculation was decreased in plants inoculated with the Δ*hapX* mutant relative to those inoculated with the wild type strain. Moreover, mortality rates, as well as the concentration of fungal propagules in kidneys and lungs of immunodepressed mice infected with the Δ*hapX* mutant were significantly reduced compared those of mice infected with the wild type or with the Δ*hapX*+*hapX* complemented strain.[12] This is in line with reports from other human pathogens such as *A. fumigatus, C. albicans* or *Cryptococcus neoformans*.[2-5]

Transcriptional upregulation of genes during iron starvation, including *hapX*, *sidA* (siderophore biosynthesis precursor) or *srbA* (iron acquisition in response to hypoxia), was observed during early stages of plant infection and when *F. oxysporum* was shifted from minimal medium to human blood.[12] Genome-wide transcript profiling revealed a large number of genes whose expression is activated under iron starvation conditions in a HapX-dependent manner. Functional analysis of DEGs identified four molecular functions that were specifically upregulated in the wild type under steady-state iron starvation vs. iron sufficiency (wild type ± Fe): FMN binding, secondary active transmembrane transporter activity, carboxy-lyase activity and carbon-nitrogen ligase activity with glutamine as amido-N-donor. We also detected one molecular function (small GTPase regulator activity) downregulated during steady-state iron-starved growth in the Δ*hapX* mutant compared with the wild type (wild type/hapX-Fe) (**Fig. 2**). Among the 30 subcategories under biological processes, transcripts related to cell division, pathogenesis, and microtubule-based process were specifically downregulated genes in the *hapX* mutant compared with the wild type under iron-limiting conditions (**Fig. 2**). Other subcategories overrepresented among the genes upregulated in the wild type ± Fe comparison include the molecular function subcategory metal ion binding and the biological process subcategories establishment of localization and oxidation-reduction process. Interestingly, the function of some of these genes is linked to virulence (**Fig. 2**).

Collectively, these results establish a key role of HapX in reprogramming iron-regulated gene expression during infectious growth of *F. oxysporum* on plant and mammalian hosts (see model in **Fig. 3**).

Iron homeostasis is required for rhizosphere competence of *F. oxysporum*. The rhizosphere is defined as the area influenced by the root plant system where different types of microorganisms, pathogens or not, may coexist. *F. oxysporum* must compete for the limited iron with different rhizosphere-inhabiting microorganisms such as siderophore producing bacteria of

the genus *Pseudomonas*.[13,14] Two different *Pseudomonas* strains exhibited an in vitro antagonistic effect against *F. oxysporum*, which was exacerbated in the Δ*hapX* mutant, specifically under iron-limiting conditions and dependent on siderophore production.[12] Even more striking, coinoculation of tomato roots with *F. oxysporum* and the pyoverdine (pvd) producing bacterium *P. putida* KT2440 resulted in a significant delay in plant mortality, confirming the previously reported biocontrol activity of this bacterial isolate. Virulence attenuation by *P. putida* KT2440 was much more pronounced in plants infected with the Δ*hapX* mutant than in those infected with the wild type strain. Importantly, the decrease in biomass of the Δ*hapX* mutant was 2.5× stronger in plants coinoculated with the *P. putida* wild type strain in comparison with the *pvd*⁻ mutant.[12] Taken together, these results demonstrate that HapX plays a key role during iron competition of *F. oxysporum* against siderophore producing pseudomonads, and directly affects the ability of the fungus to proliferate in the rhizosphere and cause disease on tomato plants (**Fig. 3**).

Biological control of plant disease implies any means of controlling disease or reducing the amount or effect of pathogens that relies on biological mechanisms or organisms other than man. Among different reported mechanisms of biocontrol, iron competition mediated by siderophores ranks among the most important ones.[15] Our results show that bacterial siderophore production is required for efficient in vitro antagonism of pseudomonads against *F. oxysporum* under iron-depleted conditions, and that co-inoculation with a siderophore producing *P. putida* strain causes a clear attenuation of vascular wilt disease caused by *F. oxysporum* on tomato plants. Interestingly, part of the protecting effect of *P. putida* was independent of siderophore-mediated iron uptake, since it was detectable both in the bacterial wild type strain and the *pvd*⁻ mutant. However, plants inoculated with the *F. oxysporum* Δ*hapX* mutant displayed an additional decrease in vascular wilt symptoms and fungal biomass, which was specifically observed in combination with the *P. putida* wild type strain.[12] While confirming the presence of additional biocontrol mechanisms other than siderophore-mediated iron competition, these results establish a key role of HapX during iron competition of *F. oxysporum* with fluorescent pseudomonads and highlight its importance for iron competence in the rhizosphere (**Fig. 3**).

Clearly, more research is required to improve biocontrol applicability and success rate. The availability of complete genomes from both pathogens and biocontrol organisms should advance our understanding of the different modes of action, leading to an improved production, formulation and application of biocontrol agents.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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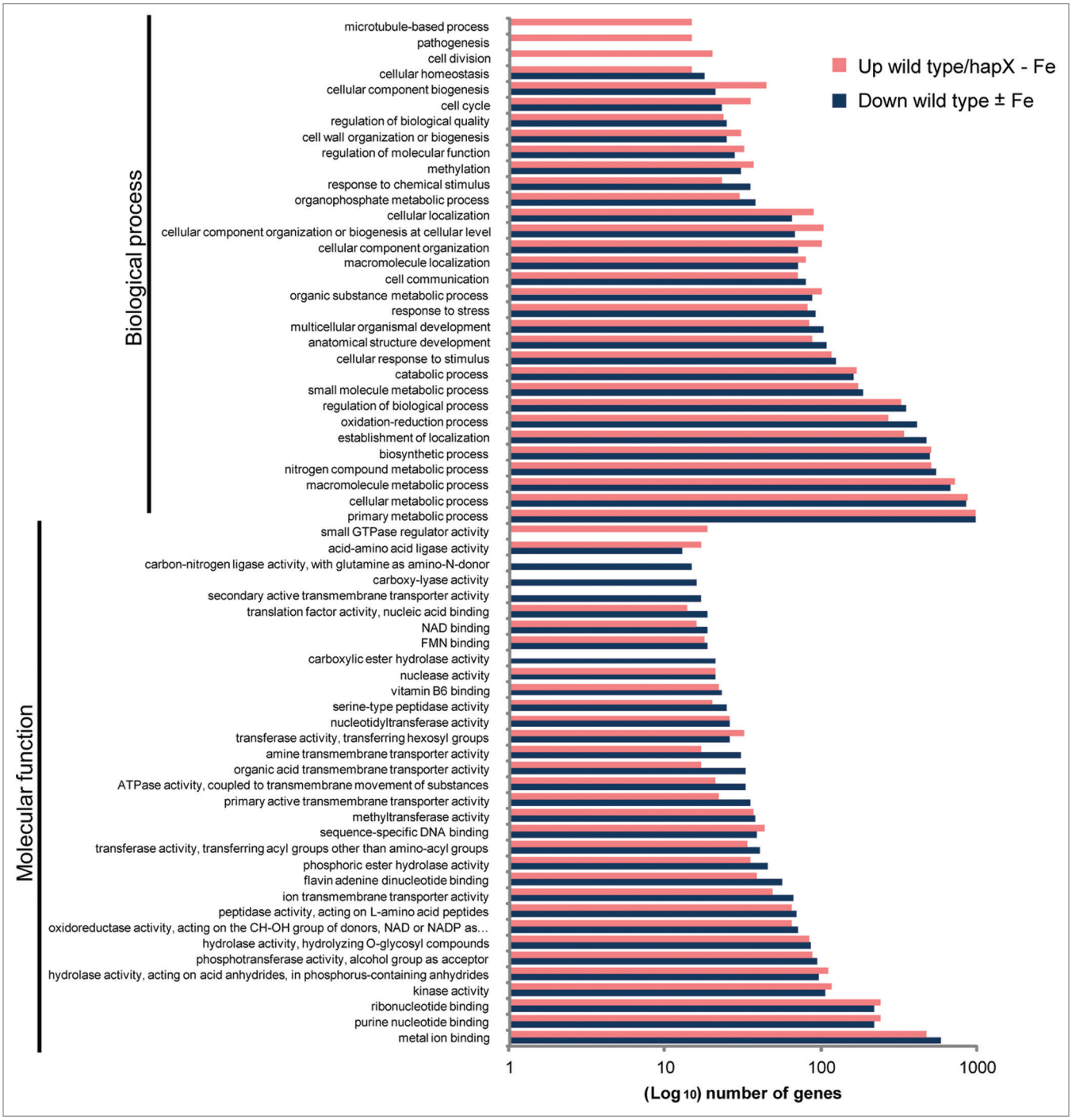

Figure 2. Functional categories of DEGs induced by iron starvation in a HapX-dependent manner. GO functional enrichment analysis of *F. oxysporum* DEGs that fulfill the following criteria in microarray-based transcriptional profiling: (1) Upregulation in the wild type under steady-state iron starvation vs. iron sufficiency (wild type ± Fe); (2) Downregulation during steady-state iron-starved growth in Δ*hapX* compared with the wild type (wild type/hapX - Fe). DEGs were assigned to different functional categories using Blast2GO (see **Fig. 1**) according to molecular function or biological process, with 33 and 30 categories, respectively.

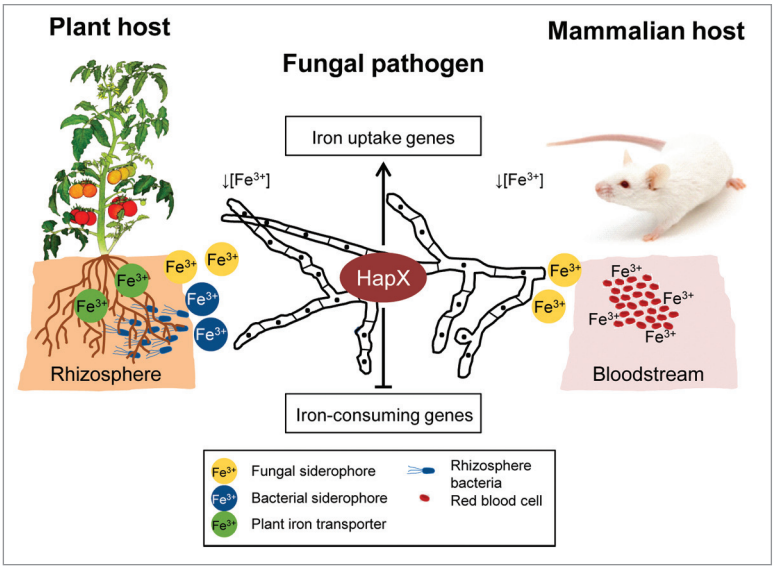

Figure 3. HapX-mediated iron homeostasis is crucial for fungal rhizosphere competence and for virulence on plant and mammalian hosts. During plant infection, *F. oxysporum* must compete for limited iron resources with rhizosphere colonizing bacteria and with plant roots. During infection of mammalian hosts, free iron levels are very low in serum and tissues due to iron sequestration by iron-sequestering host proteins. Under conditions of iron starvation, HapX mediates upregulation of iron-acquisition genes (e.g., biosynthesis of siderophores) and downregulation of genes functioning in iron-consuming processes (e.g. respiration, TCA cycle, heme biosynthesis).