Chapter 2
Iron in Plant–Pathogen Interactions

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Abstract Infectious diseases are the result of competitive relationships between a host organism and a pathogen. In host vertebrate–microbe interactions, the acquisition of iron for the essential metabolism of pathogenic organisms and the need of the host to bind and sequestrate the metal are central issues. Plants are also confronted with a wide variety of pathogenic microorganisms that can be highly devastating and compromise crop production. Investigated in a few cases in the past, the mechanisms involved in exchanging and withholding iron during plant–microbe interactions are becoming an emerging topic. This chapter surveys the wealth of information illustrating the role of iron acquisition, toxicity, and homeostasis in relevant pathosystem models of agricultural importance. There is now evidence that phytopathogenic bacteria and fungi can use siderophores and other iron uptake systems to multiply in the host and to promote infection. Moreover, plant can develop an iron-withholding response that changes iron distribution and trafficking during infection. Elucidating the mechanisms of competition for iron between plants and pathogens must help to develop innovative strategies for controlling diseases.

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Infectious diseases are the result of the interaction between a host organism and a pathogen. For the pathogen, entering the host and attaching to target tissues are issues that are tightly controlled by the production of virulence factors which promote the establishment of the microbe and the evasion of host defense. In the later stages of infection, pathogens may have the potential to proliferate beyond their infection sites, causing systemic diseases. In plants, the presence of rigid walls surrounding cells limits the possibilities for the microbe to thrive intracellularly. Plant pathogens produce cell wall degrading enzymes and/or type III secretion systems leading to cell injury and access to nutrients. They encounter diverse environmental conditions in the plant that depend on their colonization sites and their mode of attack. Concerning iron, the availability of this metal for the pathogen may be quite low (see Fig. 2.2). Indeed, plants acquire iron from the environment by the roots using mechanisms based on reduction or chelation (Morrissey and Guerinot 2009). Iron is then mobilized from the root tissues in xylem vessels by citrate which is involved in long distance transport from roots to shoots. Another molecule participating in distribution of iron throughout the plant via the phloem is the nonproteogenic amino acid nicotianamine that is able to chelate iron and other metals (Curie et al. 2009). Iron storage *in planta* occurs at the subcellular level in chloroplasts, where the photosynthetic process takes place (Briat et al. 2010). Plastids contain ferritin, an important iron reservoir protein (Nouet et al. 2011). Vacuoles are other crucial compartments for iron storage and sequestration in plant cells (Lanquar et al. 2005). At the tissue level, the apoplast which includes cell walls and extracellular spaces represent a major site of infection, but the status of iron is not well characterized.

In this context, how do microbes and plants control their iron homeostasis when they are mutually challenging? This chapter surveys the investigations that, in various plant microbial pathosystems, contribute to shed light on this question.

2.1 Iron and Microbial Virulence

2.1.1 Agrobacterium tumefaciens *and Crown Gall Disease*

In early 1980s, the role of iron well established in mammalian infections was still misunderstood in microbial pathogenesis to plants. Interestingly in 1979, J Neilands and collaborators reported the structure of agrobactin, a new catechol-type siderophore produced by the plant pathogenic bacterium *A. tumefaciens* (Ong et al. 1979), and a number of investigations addressing the role of siderophores in the virulence of plant pathogens came to light. *A. tumefaciens* is a soilborne Gram-negative bacterium which produces tumors in a broad range of plants (for a review,

Pitzchke and Hirt 2010). Galls appear at wound sites on the crowns of plants including tobacco, tomato, and potato. Tumor formation results from the transfer of a DNA region (T-DNA) located on a large plasmid present in virulent strains into plant cells, that integrates into the host chromosomal DNA. Expression of bacterial genes in the host leads to the production of enzymes catalyzing the synthesis of plant hormones responsible for cell proliferation, and enzymes generating new metabolites used by bacteria as carbon and nitrogen sources. This association between the pathogen and host cell requires plant signals from wound sites to activate the bacterial *vir* genes involved in T-DNA transfer and a suppressive mechanism of plant defense. To proliferate, tumors do not require the continuous presence of the bacteria.

Agrobactin was characterized as a threonyl peptide of spermidine acylated with three residues of 2, 3-dihydroxybenzoic acid. The production of agrobactin in iron deplete cultures of *A. tumefaciens* strain B6 was associated with the induction of several outer membrane proteins predicted to be involved in ferric-siderophore transport (Leong and Neilands 1981). Mutants unable to produce or utilize agrobactin as iron source were isolated after chemical mutagenesis; they were still capable of initiating tumors in sunflower plants or on carrot root disks. Interestingly, ferric citrate was shown to enhance the growth of the agrobactin deficient mutants exposed to iron deficiency. The authors concluded that the production of agrobactin *in planta* is not required for infection, but citrate could serve as an alternative iron transporter for *A. tumefaciens* within the host. More recently, a gene cluster called *agbCEBA* identified on the linear chromosome of *A. tumefaciens* strain MAFF301001 (Sonoda et al. 2002) was predicted to encode products showing similarity with enzymes involved in biosynthesis of catechol-type siderophores. This cluster revealed to be essential for bacterial growth and production of catechol compounds in iron-limited conditions. In addition, knowledge of the complete genome sequence from *A. tumefaciens* strain C58 allowed the identification of a new gene cluster encoding the biosynthesis of a metabolite with siderophore activity (Rondon et al. 2004). Designated as ATS, this cluster specifies proteins displaying no similarity with other known siderophore biosynthetic enzymes, suggesting that strain C58 produces a novel siderophore. Mutants disrupted in the ATS gene cluster have lost the capacity to produce this molecule and show impaired growth under iron limitation. However, they were able to induce tumors on carrot slices or on tomato seedlings. This result confirms the earlier observation made with mutants of strain B6, and further supports the idea that the production of siderophores is not essential for *A. tumefaciens* to acquire iron *in planta*. On the hand, a high-affinity uptake pathway dependent on a siderophore could be crucial for this bacterium to acquire iron in the soil, during its saprophytic life. As wounding is a prerequisite for successful infection by *A. tumefaciens,* it may be possible that the bacteria at the colonization sites use some iron substrates released in damaged tissues. Indeed, we noted that the C58 genome sequence can encode a number of iron-related transport routes, including two heme utilization pathways and a homologue of the Yfe system. The latter is an ABC permease predicted to transport diverse ferrous and ferric iron ligands (see Table 2.1 and Fig. 2.1).

Table 2.1 Siderophore independent iron routes in some phytopathogenic bacteria

Feo system:

Dickeya dadantii 3937; *D. chrysanthemi* 1591; *D. zeae* 586; *D. paradisiaca* 703

Ralstonia solanacearum GMI 1000; *R. solanacearum* CFBP2957;

Xanthomonas campestris pv. *campestris* B100; *X. oryzae* pv. *oryzae*;

X. oryzae pv. oryzicola; *Xylella fastidiosa*

EfeUOB system:

Dickeya dadantii 3937; *D. chrysanthemi* 1591; *D. zeae* 586; *D. paradisiaca* 703

Erwinia amylovora 1430; *Pseudomonas syringae* pv. *tomato* DC3000; *P. syringae* pv. *syringae* B728a; *P. syringae* pv. *tabaci* ATCC 11528, *P. syringae* pv. *phaseolicola*

FecABCDE system:

Pectobacterium atrosepticum SCRI 1043; *P. brasiliensis* 1692; *P. carotovorum* PC1;

Pseudomonas syringae pv. *syringae* B728a; *P. syringae* pv. *tomato* DC3000

P. syringae pv. *tabaci* ATCC 11528; *P. syringae* pv. *phaseolicola*

Hemophore system:

Pectobacterium atrosepticum SCRI 1043; *P. brasiliensis* 1692; *P. carotovorum* PC1 and WPP14; *P. wasabiae* WPP163; *Agrobacterium tumefaciens* C58

Hmu system:

Dickeya dadantii 3937; *D. chrysanthemi* 1591; *D. paradisiaca* 703; *Pectobacterium atrosepticum* SCRI 1043; *P. carotovorum* PC1 and WPP14; *P. brasiliensis* 1692; *P. wasabiae* WPP163; *Agrobacterium tumefaciens* C58

Yfe system:

Dickeya dadantii 3937, *D. chrysanthemi* 1591; *Pectobacterium atrosepticum* SCRI 1043; *P. carotovorum* PC1 and WPP14; *P. brasiliensis* 1692; *P. wasabiae* WPP163; *Erwinia amylovora* 1430; *Agrobacterium tumefaciens* C58

Analysis of the *A. tumefaciens* genome revealed the existence of three regulatory genes encoding a homolog of Fur repressor and two proteins, RirA and Irr, originally discovered in rhizobial species (see Chap.3, O'Brian and Fabiano 2010). Kitphati et al. (2007) constructed a *fur* null mutant. These authors showed that in *A. tumefaciens* strain NTL4, the *fur* gene is not involved in iron regulation of siderophore production and transport, but is important for the bacterial survival under iron limiting conditions. Like in *Sinorhizobium meliloti*, *A. tumefaciens fur* gene appeared to negatively regulate the expression of the *sit* operon encoding a manganese uptake system in response to manganese and iron. The *fur* mutant displays hypersensitivity to hydrogen peroxide, low catalase activity, and reduced ability to cause tumors on tobacco leaves compared to wild-type, indicating that the bacteria must accurately control their metal homeostasis and oxidative stress response in early steps of pathogenesis. The Fe–S protein RirA is the rhizobial iron regulator repressing many iron-responsive genes under iron repleted conditions. RirA homologues were found exclusively in members of α-Proteobacteria. A *rirA* null mutation in *A. tumefaciens* led to overexpression of siderophore biosynthesis and transport genes, iron overload, and increased sensitivity to oxidants (Ngok-Ngam et al. 2009). In addition, a functional *rirA* gene appeared to be required for full expression of several *vir* genes induced by the plant compound
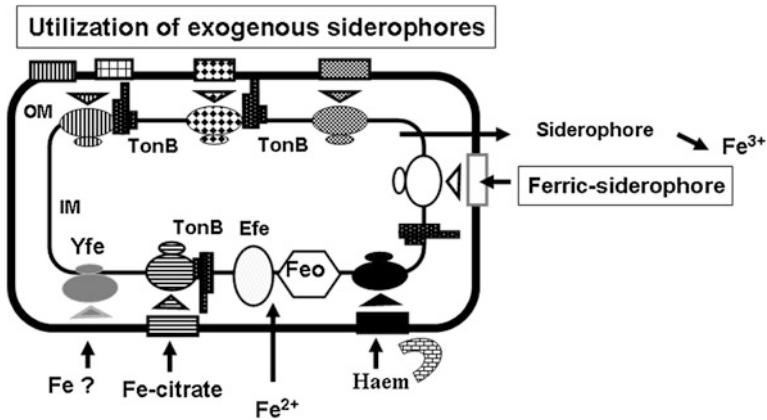
Fig. 2.1 Schematic representation of the various iron uptake routes in phytopathogenic Gram-negative bacteria (for a review see Crosa et al. 2004). Bacterial cells can synthesize and excrete siderophores that form with Fe^{3+} a siderophore ferric complex, designated as ferric-siderophore. Metal chelating power of siderophores are compared by calculating pFe values (pFe = −log $[Fe^{3+}]$ at a defined concentration and pH). A ferric-siderophore is specifically recognized by an outer membrane TonB-dependent transporter (*rectangles*), which is a gated-channel energized by the cytoplasmic membrane-generated proton motive force transduced by the TonB protein and its auxiliary proteins ExbB and ExbD. Transport of a ferric-siderophore across the inner membrane involves a less specific ABC permease (*triangle and circles*). Ferric complexes of citrate or of exogenous siderophores, i.e., produced by other microbes are imported in a similar way. Heme can be transported in two ways: either directly or bound to a secreted hemophore protein which delivers it in the cytoplasm, via specific TonB-dependent transporters and specific ABC permeases. Ferrous iron can be transported through the FeoAB and/or EfeUOB systems. FeoB, the main component of the Feo system, is an integral membrane protein with an N-terminal domain having GTPase activity essential for the transport function. Function of the FeoA peptide is unknown. EfeU is a potential integral inner protein acting as a permease for ferrous or ferric forms of iron. Functions of EfeO and EfeB proteins are unknown. The Yfe ABC permease can import an uncharacterized form of iron. The nature of the Fe-nicotianamine uptake system is unknown. OM: outer membrane, IM: inner membrane

acetosyringone, and for tumor formation on tobacco leaves. Thus, the RirA protein seems to play an important role in the control of *A. tumefaciens* pathogenicity. In *Bradyrhizobium japonicum*, the Irr protein controls iron homeostasis through heme biosynthesis. Heme biosynthesis is an iron-dependent process, the final step residing in insertion of iron into protoporphyrin, a reaction catalyzed by ferro-chelatase. In the absence of iron, Irr represses heme biosynthetic genes and acti-vates iron transport genes. In the presence of iron, Irr interacts with ferrochelatase, leading to Irr degradation via heme binding and derepression of the heme biosynthetic pathway (see Chap.3). The role of *A. tumefaciens irr* gene in iron metabolism was investigated. By constructing deletion mutations in *irr* and *rirA*, alone and in combination, Hibbing and Fuqua (2011) showed that these two genes work in a complementary way to maintain a tight control of iron homeostasis. Under conditions of iron limitation, the *irr* gene negatively controls the

transcription of genes involved in iron consuming processes such as heme and Fe–S cluster biosynthesis. It also negatively controls *rirA* expression leading to derepression of *sigI*, a gene encoding an ECF sigma factor (Extra Cytoplasmic Function) necessary for the expression of the ATS gene cluster described in strain C58. The authors found that *irr* and *rirA* mutants were not affected in the ability to induce tumors in a potato disk assay, a result which is quite different from that observed on tobacco leaves. As the growth of the *irr* mutant is strongly affected under conditions of iron limitation, this finding indicates that the potato disks might supply sufficient iron to compensate for the deficiency of this mutant.

2.1.2 Pseudomonas syringae, *a Plant Pathogen Adapted to Different Hosts*

Pseudomonads are widespread Gram-negative bacteria displaying various lifestyles and habitats. *P. aeruginosa*, an opportunistic pathogen infecting humans and animals is the most investigated species; *P. fluorescens/putida* and *P. syringae* are well known for their ecological importance in soils and implication in plant disease, respectively (for a review Silby et al. 2011). *P. syringae* comprises numerous pathovars causing symptoms that may vary according to the host species and the site of infection. A typical phenotypic trait of Pseudomonads is the production in iron deficient environments of green-yellow fluorescent compounds emblematic of a class of siderophores called pyoverdines. Pyoverdines contain a conserved dihydroxyquinoline chromophore linked to a variable peptide chain considered as a taxonomic hallmark (Meyer et al. 2008). Their high affinity for the ferric ion is conferred by the presence of catecholate and hydroxamate groups. Pyoverdine contributes to iron acquisition by *P. aeruginosa* in vivo. This class of siderophores also plays an important role in the biological control of phytopathogenic microrganisms of the rhizosphere and on the iron availability to plants in soils (Visca et al. 2002).

Interestingly, by investigating various pathovars of *P. syringae* for the production of phytotoxins, D Gross and collaborators found that a maximum yield of the necrosis inducing toxin syringomycin was obtained, if micromolar ferric iron concentrations were supplied to the growth medium (Gross 1985). This fact suggested that the pathogen could necessitate relatively high concentrations of iron during infection, and characterization of the fluorescent pigment of *P. syringae* pv. *syringae* was undertaken (Cody and Gross 1987). A pyoverdine-like molecule that confers on the bacterium the ability to grow under iron limitation was identified, and pyoverdine biosynthetic and transport mutants were isolated; these mutants were able to cause necrotic lesions on sweet cherry fruit equivalent to those observed with the wild-type strain indicating the dispensability of pyoverdine *in planta*. However, with emergence of complete genomic sequences of various *P. syringae* strains, it became apparent that most of pathovars not only have the capacity to produce

pyoverdines, but they also could synthesize pyoverdine unrelated siderophores. The genome of *P. syringae* pv. *tomato* DC3000 revealed to contain a gene showing similarity with the polyketide synthase gene *irp1* present in the YBT yersiniabactin gene cluster of *Yersinia pestis.* Yersiniabactin is a salicylate derived siderophore widespread among human and animal pathogenic bacteria and contributes to their virulence (Perry and Fetherston 2011). In *Yersinia* spp, the YBT locus is located in a genomic high-pathogenicity island. In *P. syringae,* Bultreys et al. (2006) reported the presence of an YBT locus in pathovars *tomato* and *phaseolicola*, the gene organization of which appeared to be well conserved through the different strains examined. In agreement with gene predictions, yersiniabactin was detected in the culture medium of the majority of strains tested. However, the occurrence of yersiniabactin nonproducers among natural isolates allowed the authors to consider that this siderophore is not essential for the pathogenicity in *P. syringae.*

The role of siderophores was further explored in the model strain *P. syringae* pv. *tomato* DC3000 that causes bacterial speck of tomato and is also virulent on *Arabidopsis*. Jones et al. (2007) found that this strain produces salicylic acid, yersiniabactin, and pyoverdine, in response to iron limitation. These authors showed that salicylic acid is a precursor for yersiniabactin synthesis and that formation of yersiniabactin is dependent on the isochorismate synthase gene *pchA*. Using a *pchA* insertion mutant, they investigated the physiological importance of yersiniabactin. Yersiniabactin appeared to be produced by the wild-type strain but not the *pchA* mutant during infection of *Arabidopsis* leaves. However, the *pchA* mutation had no effect on the ability of bacteria to colonize plant leaves, to multiply *in planta,* and to produce symptoms. The role of pyoverdine in pathogenesis was also studied (Jones and Wildermuth 2011). The loss of pyoverdine production in a *pvd* insertion mutant impaired in the pyoverdine biosynthetic pathway, had no impact on the virulence of DC3000 on tomato plants. Using a double mutant unable to synthesize yersiniabactin and pyoverdine, the authors showed that strain DC3000 produces a third siderophore-like molecule identified as citrate. A yersiniabactin and pyoverdine negative mutant missing the ferric citrate transporter FecA displayed a severe growth defect in iron-limited culture but was fully pathogenic, suggesting that DC3000 could obtain iron *in planta* by other means. Indeed, the genome of DC3000 reveals the presence of a homologue of the Yfe permease which might be effective during infection. An interesting hypothesis proposed by the authors is that during infection by DC3000 the leaf apoplast would not be limited in iron. In this regard, it is noteworthy that Kim et al. (2009, 2010) found that high concentrations of iron in the culture medium repressing expression of high-affinity iron transport systems can induce expression of the type III secretion system and virulence genes. Thus, there would be sufficient amounts of iron at the sites of infection by DC3000 to trigger the pathogenic process. A study of the transcriptional profile of *P. syringae* pv. *phaseolicola* NPS3121 in response to tissue extracts from a susceptible pea cultivar may also support this hypothesis, since most repressed genes were found to concern the uptake and metabolism of iron (Hernandez-Morales et al. 2009).

Contrastingly, Taguchi et al. (2010) reported the importance of pyoverdine as a virulence factor of *P. syringae* pv. *tabaci* 6605 in tobacco. The authors identified a pyoverdine gene cluster and constructed deletion mutants defective in biosynthesis of the pyoverdine peptide chain or the chromophore, as well as a mutant missing the ferripyoverdine receptor. On tobacco leaves, the pyoverdine-deficient mutants were less virulent than the wild-type and their growth *in planta* was reduced. Genetic complementation of the mutations restored pyoverdine production and virulence properties. However, the virulence of the receptor mutant was not affected. Further characterization of these mutants revealed that all have acquired lower abilities to produce tabtoxin, extracellular polysaccharide, and quorum-sensing molecules, as well as increased swarming ability and surfactant production. In light of the regulatory role conferred by pyoverdine and its receptor in *P. aeruginosa* PAO1 (Visca et al. 2002), it was proposed that in *P. syringae* pv. *tabaci* 6605, pyoverdine also acts as a signal triggering the production of several pathogenicity factors. Indeed, *P. aeruginosa* pyoverdine and its receptor mediate a transduction signal inducing the expression of ECF factors PvdS and FpvI that control a set of functions of which iron acquisition and pathogenicity. A *pvdS* gene orthologue was identified in the DC3000 genome. On the basis of a computational analysis, Swingle et al. (2008) characterized the DC3000 PvdS regulon and the PvdS-regulated promoter motif. They concluded that PvdS is a well conserved regulator of pyoverdine synthesis among fluorescent pseudomonads, although the promoters recognized by this factor are likely to differ from species to species. A homologue of the *E. coli* ECF sigma factor FecI involved in ferric citrate transport and signaling was also identified in DC3000. The DC3000 FecI protein was shown to regulate gene expression through a signal transduction cascade mediated by a TonB-dependent receptor in response to exogenous siderophores (Markel et al. 2011). In addition, different *P. syringae* pathovars appeared to contain a homologue of the *fur* gene (Butcher et al. 2011). By constructing a *fur* deletion mutant in *P. syringae* pv *tabaci* 11528, Cha et al. (2008) demonstrated that *fur* not only regulates siderophore production, but it also controls swarming motility as well as the production of tabtoxin and quorum-sensing molecules. The virulence of the *fur* mutant was compromised as revealed by the reduced size of necrotic lesions, the decline of bacterial populations and the absence of chlorosis around the lesions in infected tobacco leaves. These data indicate the importance of this iron sensory regulatory gene in the interaction between *P. syringae* pv *tabaci* and its host plant.

Later, it was found that many strains of the *P. syringae* group are able to secrete a second siderophore, achromobactin, structurally different from yersiniabactin. Achromobactin is a citrate/carboxylate siderophore originally described in the bacterial pathogen *Dickeya dadantii* (Münzinger et al. 2000, see Sect. 6.a). Among the six *P. syringae* pathovars the genome sequences of which could be consulted, only *P. syringae* pv. *tomato* DC3000 does not possess an achromobactin biosynthesis gene cluster. Mechanistically, achromobactin biosynthesis was reported as an excellent model for studying siderophore synthases having a mode of action different from that of nonribosomal peptide synthase (NRPS) (Schmelz et al. 2008, 2011). Indeed a major biosynthesis pathway for siderophores relies on the

extensively investigated NRPS multi-enzyme superfamily. Interestingly, an analysis of the synthetases AcsA, AcsC, and AcsD in *P. syringae* pv. *syringae* B728A allowed the in vitro reconstitution of achromobactin biosynthesis (Berti and Thomas 2009). Owen and Ackerley (2011) investigated the role of pyoverdine and achromobactin in the pathogenicity of *P. syringae* pv. *phaseolicola* 1448A. They tested the ability of siderophore negative mutants to form lesions on bean pods but neither pyoverdine nor achromobactin proved to be essential for this bacterium to cause halo blight. The authors suggested that the pathogenicity assay used in their study was unlikely the most appropriate to appreciate differences in disease progression, since lesion formation depends primarily on toxin production.

Interestingly, *P. syringae* comprises a number of strains exhibiting a pronounced epiphytic phase on plants during which they can establish high population levels on the leaf surface (Feil et al. 2005). Epiphytic populations of *P. syringae* pv. *syringae* B728A can subsequently invade the apoplastic spaces of plant tissue and initiate disease. On the other hand, there are natural isolates which are non-pathogenic and can behave as microbial antagonists able to suppress diseases induced by pathogenic strains. For example, *P. syringae* pv. *syringae* 22d/93 exerts a biocontrol activity against *P. syringae* pv. *glycinea*, the causal agent of bacterial blight of soybean (Wensing et al. 2010). Such epiphytes produce the siderophores, pyoverdine, and achromobactin. Disruption of either pyoverdine or achromobactin biosynthesis impairs the epiphytic fitness of strain 22d/93, as observed by a significant decrease in the population size of the mutants on soybean leaves compared to wild-type. The growth deficiency of these mutants was compensated for when wound inoculation was used, indicating the availability of iron in the presence of small lesions on the leaves. Thus, rather than serving as a virulence factor, production of achromobactin and pyoverdine by *P. syringae* pv. *syringae* 22d/93 contributes to the epiphytic fitness of this bacterium and improves its competitiveness as an antagonist of virulent strains. In *P. syringae* pv. *syringae* B728A, expression of achromobactin mediated iron transport was shown to depend on a gene cluster which also specifies the production of a new ECF sigma factor, AcsS, regulating the biosynthesis and secretion of achromobactin, as well as, other genes associated with epiphytic growth and survival (Greenwald et al. 2012). A complex iron regulatory network must exist in B728A, and further studies are needed to better define the role of siderophores on the leaf surface.

2.1.3 Diseases Caused by Xanthomonas Species

Plant pathogenic *Xanthomonas* spp. form a large group of Gram-negative bacteria which cause various diseases like vascular wilts, cankers, leaf spots, fruit spots, and blights on many plants and economically important crops (Ryan et al. 2011). These bacteria initially grow on leaf surfaces and enter into the host through hydathodes or wounds to spread systemically through the xylem elements of the vascular system or through stomata to colonize the mesophyll parenchyma.

X. campestris includes host-specific pathovars that infect different brassicaceous, solanaceous, and other plant species, whereas *X. oryzae* infects rice and some of its wild relatives.

Despite the availability of genome sequences of many *Xanthomonas* strains showing the existence of siderophore biosynthetic gene clusters in different pathovars, the structure of the siderophores produced is still unknown (Etchegaray 2004, Pandey and Sonti 2010). In this respect, the report published by Wiggerich and Puhler (2000) illustrating the difficulty to elucidate the role of a TonB-dependent ferric iron acquisition system in the pathogenicity of *X. campestris* pv. *campestris* B100 is interesting. These authors found that a TonB negative mutant, thus deficient in ferric iron ligand uptake did not induce the typical phenotype of black rot symptoms on leaf of cauliflower, but spread systemically in this plant, suggesting that this mutant could meet its iron needs *in planta* via a mechanism independent of the TonB machinery. However, this mutant neither grew and nor induced a hypersensitive response on pepper, a nonhost plant. These intriguing results remained unexplained. It may be assumed that the inability of the *tonB* mutant to cause black rot symptoms was due to a pleiotropic effect of the mutation thus altering a function unrelated to iron transport. In a mutant screening to find out new virulence functions of *X. oryzae* pv. *oryzae*, Chatterjee and Sonti (2002) isolated a strain deficient in the ability to produce lesions on a susceptible rice cultivar. Molecular characterization revealed that this mutant was impaired in a gene homologue to the *X. campestris* pv. *campestris rpfF* gene required for production of a diffusible extracellular factor that positively regulates virulence associated functions. In *X. oryzae* pv. *oryzae*, the virulence deficiency of the *rpfF* mutant was corrected by iron supplementation. This mutant overproduced siderophores and was sensitive to iron deficiency, a phenotype that could also be corrected by iron supplementation. Thus, iron metabolism plays a critical role in *X. oryzae* pv. *oryzae* virulence. Homologues of the *feoABC* genes that encode a ferrous iron uptake system called Feo and characterized in *E. coli* (Fig. 2.1) were identified in this pathovar (Pandey and Sonti 2010). A *feoB* mutation predicted to impair the production of the main component of ferrous iron transport caused severe defects in *X. oryzae* pv. *oryzae* growth under iron-limited conditions and virulence. In the other hand, mutations in a gene cluster encoding proteins involved in biosynthesis and utilization of the *X. oryzae* pv. *oryzae* siderophore, the *xss* operon, had no impact on the virulence. Using histochemical and fluoro-metric assays with *uidA* gene reporter fusions, the authors demonstrated that during infection of the susceptible rice cultivar, the bacteria express the *feoB* gene while the *xss* operon remains turned off. *X. oryzae* pv. *oryzae* grows in the rice xylem vessels and in this regard, it is worth noting that the iron content in the xylem sap of rice seedlings cultured under iron proficient conditions reaches a concentration range sufficient to satisfy the bacterial iron needs. In this fluid, the ferrous form of iron predominates while the ferric form is mainly present as ferric citrate (Yokosho et al. 2009). *X. oryzae* pv. *oryzae* genome analysis revealed the absence of a potential ferric citrate transport system, thus making particularly relevant the fact that the *feoB* mutant displays attenuated virulence. The role of the

Fur regulatory protein in pathogenicity of *Xanthomonas* spp was also investigated (Subramoni and Sonti 2005; Jittawuttipoka et al. 2010). First characterized in *X. campestris* pv. *phaseoli*, the *fur* gene was found to be well conserved among *Xanthomonas* spp, although it encodes a protein with unusual features (Loprasert 1999). The cysteine residues highly conserved at the Zn binding motifs are missing. In *X. oryzae* pv. *oryzae* as well as in *X. campestris* pv *campestris*, analysis of a *fur* mutant indicated that the Fur protein regulates the production of siderophores. In absence of a functional *fur* gene the bacterial cells are hypersensitive to oxidative stress, accumulate reactive iron species and have decreased catalase activity as well as aerobic growth defects. The *fur* mutants showed attenuated virulence properties on their respective hosts, rice, and chinese cabbage, as visualized by a decreased lesion size on the leaves. The growth deficiency in rice leaves of the *fur* mutant of *X. oryzae* pv. *oryzae* appeared to be rescued by application of an antioxidant such as ascorbic acid, indicating that this growth defect was likely due, in part to an impaired ability of the bacteria to cope with the oxidative stress conditions encountered during infection.

2.1.4 Diseases Caused by Xylella fastidiosa

X. fastidiosa is a Gram-negative bacterium associated with diseases of economical importance like the Pierce's disease of grape and the variegated chlorosis of citrus species (Chatterjee et al. 2008). This pathogen is vector-transmitted from one plant to another by various xylem sap-feeding insects. Symptoms are typically a leaf-scorch associated with the extensive colonization of xylem vessels. From infection sites, bacterial cells attach to the vessels walls and multiply forming biofilm-like colonies that can completely occlude xylem vessels, thereby blocking water transport. *X. fastidiosa* is closely related to various *Xanthomonas* species and the availability of complete genome sequences for both genera allowed the discovery that many genes implicated in the virulence of *Xanthomonas* species have homologues in *X. fastidiosa*. However, genes encoding type III secretion system machinery and associated effectors are missing in *X. fastidiosa*. Zaini et al. (2008) were interested in investigating the response to changes in iron availability of a strain pathogenic to citrus. By analyzing the transcriptional profile of *X. fastidiosa* strain 9a5c exposed to large variations of iron levels, these authors identified an iron stimulon encompassing the genes involved in uptake and storage of this metal as well as those responsible for type IV pilus and colicin V production. The type IV pilus contribute to twitching mediated motility and mutants deficient in type IV pili in the host are inhibited from colonizing upstream vascular regions. Thus, an attractive hypothesis is that iron could be sensed as an environmental signal susceptible to modulate the transcriptional control of this virulence factor. Investigations of the contribution of Fur protein in the regulation of this iron stimulon suggest that additional transcriptional regulators are involved in the response to iron. Of the genes participating in iron transport and up-regulated

under low iron conditions, those encoding the Feo system and several TonB-dependent receptors would be interesting to investigate as pathogenicity determinants.

2.1.5 Ralstonia solanacearum, *The Causal Agent of Bacterial Wilt on a Large set of Plants*

R. solanacearum is a soilborne Gram-negative bacterium that causes lethal wilt diseases of many plants including economically important crops around the world (Genin 2010). The bacterium enters plant roots through natural or mechanical wounds, multiplies in the root cortex and bacterial invasion of xylem vessels leads to systemic spreading. Wilting symptoms occur as a consequence of an intensive multiplication of the bacteria and are associated with the large production of exopolysaccharides blocking water traffic in the plant. Though a wide range of genes and functions playing a role in the colonization and multiplication of the bacterium within plant tissues are characterized, the way by which *R. solanacearum* meets its iron requirements is less well documented. It was interesting to discover that the *phcA* gene encoding a transcriptional regulator belonging to a cell density sensing system essential for production of *R. solanacearum* virulence factors is located immediately upstream of the *fur* gene on the genome of *R. solanacearum* strain AW1. Intrigued by the juxtaposition of these two sensory regulatory genes, Bhatt and Denny (2004) investigated the production of siderophores in this bacterium. They found that under iron limitation *R. solanacearum* AW1 releases a siderophore of the carboxylate family structurally determined as staphyloferrin B also produced by *R. metallidurans* and *Staphylococcus aureus*. The *phcA* gene was shown to negatively regulate the production of this siderophore and several pathogenicity factors. The authors isolated insertion mutants with reduced siderophore activity and identified a locus predicted to be involved in biosynthesis, export, and transport functions of staphyloferrin B. These mutants revealed to be fully pathogenic after inoculation on unwounded roots of young tomato plants. There was no production of siderophore in a culture of the wild-type strain grown in xylem sap from tomato plants. These data indicate that *R. solanacearum* AW1 acquires iron in the xylem vessels by a transport route different from that mediated by staphyloferrin B. The availability of the genome sequence of *R. solanacearum* GMI1000 led to the finding that this strain produces a metal-complexing antibiotic, micacocidin previously identified as a product structurally related to the siderophore yersiniabactin (Kreutzer et al. 2011). This molecule is synthesized by a number of staphyloferrin B producing strains, and it would be of interest to know whether it acts as a siderophore.

An in vivo expression technology (IVET)-like screen performed in *R. solanacearum* strain UW551 identified up-regulated genes in the presence of tomato root extracts (Colburn-Clifford et al. 2010). One of these genes, *dps*, encodes a

miniferritin initially defined in *E. coli* as a DNA-binding protein from starved cells and found to protect DNA from oxidative damages by interacting with DNA. However, Dps proteins possess a ferritin-like function that endows them with iron and hydrogen peroxide detoxification properties (for a review see Chiancone and Ceci 2010). Dps proteins are also called miniferritins, because they are assembled from only 12 identical subunits rather than the 'canonical' 24 subunits. It was demonstrated that expression of the *dps* gene of *R. solanacearum* contributes to oxidative stress tolerance and to colonization of tomato plants. However, the role that this protein may play in iron metabolism of this species has not been explored.

2.1.6 Enterobacterial Species and Plant Disease

In the past, the *Erwinia* genus was the only member of the *Enterobacteriaceae* family representative of the plant kingdom, including mostly phytopathogenic species. Exposed to many nomenclatural difficulties, the various pectinolytic species/subspecies classified in this genus were divided in three new genera, *Pectobacterium, Dickeya,* and *Brenneria.* These species are broad-host range pathogens causing soft rot diseases to economically important plants and crops, including vegetables and ornamentals (Czajkowski et al. 2011). Now, the *Erwinia* genus comprises non pectinolytic species displaying limited host range. The most important one, *E. am*ylovora, is responsible for fire blight to apple, pear, and other rosaceous plants (Eatsgate 2000).

2.1.6.1 Pectinolytic Species and Soft rot Disease

In the mid-1980s, a work aimed at investigating the pathogenicity determinants of *D. dadantii* (formerly *E. chrysanthemi*) revealed a possible role of iron assimilation in plant pathogenesis (Expert and Toussaint 1985). *D. dadantii* 3937 produces a systemic disease in African violets as well as in *Arabidopsis*. Symptoms consist of tissue disorganization due to the release of a set of bacterial pectinolytic enzymes that degrade plant cell walls. Cell wall deconstruction weakens plant cells, allows bacteria free access to cellular nutrients and facilitates their dissemination throughout the leaf and petiole. Colonization of leaf tissues begins with a symptom-less phase during which bacterial cells remain clustered in intercellular spaces and then migrate intercellularly without causing severe injury of cellular structures (Murdoch et al. 1999). Bacterial cells do not invade the vascular tissues. Searching for bacteriocin resistant mutants with impaired envelope structure, the authors isolated clones lacking low iron inducible proteins in their outer membrane and unable to cause systemic symptoms in African violets. A role in ferric-siderophore transport was hypothesized for these proteins and thus, the possibility that they contribute to iron nutrition of this pathogen *in planta* was considered. Induction of these proteins was correlated with production of a

monocatechol siderophore called chrysobactin (Persmark et al. 1989). Further investigations demonstrated that other strains of soft rot *Erwinia*, such as *E. carotovora* W3C105 (Barnes and Ishimaru 1999) and *D. chrysanthemi* EC16 (Sandy and Butler 2011) can produce chrysobactin when exposed to iron restriction. The genetics of chrysobactin mediated iron transport was explored in depth in *D. dadantii* 3937 (Franza and Expert 1991; Rauscher et al. 2002; Expert et al. 2004). Interestingly, chrysobactin biosynthesis mutants were found to respond positively to the chemical assay used for the detection of siderophores (Schwyn and Neilands 1987), and this led to the discovery of a second iron transport route mediated by achromobactin (Mahé et al. 1995; Münzinger et al. 2000). Chrysobactin-deficient mutants cannot compete with a strong ferric iron chelator but owing to achromobactin they can still thrive on a medium containing a ferrous iron chelator like 2,2' dipyridyl. A genetic cluster encoding all proteins necessary for achromobactin biosynthesis and transport was characterized in strain 3937 (Franza et al. 2005). The genome of related species, *D. chrysanthemi* 1591 and *D. zeae* 586, reveals the existence of a similar gene locus.

Several studies demonstrated that chrysobactin and achromobactin highly contribute to successful infection of the plant (Enard et al. 1988; Franza et al. 2005; Dellagi et al. 2005). Chrysobactin defective mutants produce only localized symptoms on African violets and compared to a chrysobactin proficient strain their growth *in planta* is reduced. This decline in population size appeared to coincide with the emergence of a necrotic border surrounding the lesion (Masclaux and Expert, 1995). Chrysobactin was detected in leaf intercellular fluids from plants inoculated with the wild-type strain, suggesting that this compound could sequester the iron present in colonized tissues and possibly induce a reaction from the plant able to deprive the bacterial cells of essential iron (Neema et al. 1993). By investigating the coordination properties of the different ferric complexes of this siderophore, the Albrecht-Gary's group (Tomisic et al. 2008) found that chrysobactin is a less effective ferric chelator than hexadentate siderophores, such as enterobactin or desferrioxamine B. However, due to a higher pFe value (as defined in Fig. 2.1) than citrate or malate which are major ferric ion carriers in plants (pFe of chrysobactin = 17.1 vs. pFe of citrate = 14.8), chrysobactin can effectively sequester the iron from these plant chelators. Achromobactin deficient mutants are also affected in their virulence but are more aggressive than the chrysobactin nonproducers and double mutants deficient in both achromobactin and chrysobactin production, are impaired in symptom initiation. The wild-type cells but not the double mutants can survive for several days in intercellular spaces of host tissues without multiplying substantially. During the symptomatic phase, only the wild-type cells can proliferate.

The low availability of iron for the bacteria in the apoplast acts as a signal that not only turns on the transcription of genes involved in iron assimilation, but also those encoding the major pectin-degrading enzymes encoded by the *pelD* and *pelE* genes (Franza et al. 1999, 2002). This regulation, mediated by the transcriptional repressor Fur, allows a fine control of the pathogenicity in response to intracellular iron levels. It implies two distinct mechanisms. DNase I footprinting experiments

demonstrated that the Fur binding sites covers the -35 and -10 promoter element of the ferric chrysobactin receptor encoding gene *fct* suggesting a direct competition between the RNA polymerase and Fur. On the other hand, for the *pelD* and *pelE* gene promoters, the sequence protected by Fur is located upstream from the -35 promoter element and includes a part of the binding site of the cAMP receptor protein CRP, required for activation of *pel* gene transcription. In this case, Fur would act as an antiactivator of transcription by blocking the action of CRP (Franza et al. 2002). Thus, the two pathogenicity determinants, iron acquisition and production of pectinases are regulated in a coordinated manner and this metabolic coupling can confer an important advantage on *D. dadantii* cells during pathogenesis. When inoculated on African violets, the *fur* mutant displayed an altered virulence in comparison to that of the wild-type strain. This reduced pathogenicity of the *fur* mutant on its host was explained by its altered growth capacity *in planta* (Franza et al. 1999) indicating that a tight control of the bacterial intracellular iron content is necessary for full virulence.

Indeed, during infection bacterial cells have to cope with the production of reactive oxygen species by plant cells (Santos et al. 2001; Fagard et al. 2007). Several studies demonstrated the importance of a regulatory link between iron metabolism and tolerance to oxidative stress. In particular, there was the discovery that the Suf machinery involved in the formation of Fe–S clusters under iron starvation and oxidative conditions is necessary for full virulence (Nachin et al. 2001). Microarray profiling of bacterial genes that are specifically up- or down-regulated in saintpaulia leaves, as well as in vivo expression analysis of promoter–GFP (Green fluorescent protein) reporter constructs in leaves of spinach allowed the identification of *E. chrysanthemi* genes that are regulated during plant infection (Okinaka et al. 2002; Yang et al. 2004). Upregulation of genes involved in iron uptake and in stress responses to reactive oxygen species was observed. *E. chrysanthemi* possesses three ferritins, of which the heme-free ferritin FtnA and the heme-containing bacterioferritin Bfr play differential roles in virulence, depending on the host (Boughammoura et al. 2008). FtnA constitutes the main iron storage protein. Indeed, an *ftnA* mutant that lacks a functional FtnA ferritin has increased sensitivity to iron deficiency compared to the wild-type. In addition, this mutant has increased sensitivity to oxidative and nitrosative stresses, as well as an increased content in ferrous iron. By limiting the concentration of reactive iron, FtnA reduces the cytotoxic effect of the Fenton reaction, and thus confers tolerance to oxidative stress. Bacterioferritin acts as a transient iron store which plays an important role in distribution of iron between essential metalloproteins, particularly under conditions of iron deficiency (Expert et al. 2008). In contrast, the *D. dadantii* miniferritin encoded by the *dps* gene has a minor role in iron homeostasis, but is important in conferring tolerance to hydrogen peroxide and for survival of cells that enter the stationary phase of growth (Boughammoura et al. 2012).

A comparative genomic study of *P. atrosepticum* and *D. dadantii* was carried out (Franza and Expert 2010). It appeared that besides the production and utilization of siderophores, *P. atrosepticum* and *D. dadantii* have the capacity to use

other iron sources (Table 2.1). Indeed, both species are able to use heme iron, whereas only *P. atrosepticum* can transport the ferric citrate complex and only *D. dadantii* can acquire ferrous iron. These different modes of iron capture indicate that these species have to cope with various environmental and ecological conditions during their pathogenic cycle. For instance, the *D. dadantii* FeoAB system is likely to be functional during plant infection. Construction of a *D. dadantii feoB* negative mutant allowed to show that when present in an achromobactin and chrysobactin negative background, the *feoB* mutation could confer a three-fold reduction in iron uptake under reducing conditions, compared to the *feoB* positive strain. With respect to the pathogenicity on Arabidopsis plants, there was no significant effect of the *feoB* mutation, when present in a wild-type background. On the other hand, in a siderophore negative background, the *feoB* mutation resulted in a reduced number of systemic infections, which was twofold lower than with the siderophore nonproducer and five times lower than with the wild-type strain (Franza and Expert 2010). Okinaka et al. (2002) also showed that *D. dadantii feoB* gene is up-regulated during infection of African violets.

2.1.6.2 Erwinia amylovora and Fire Blight Disease

Fire blight caused by *E. amylovora* is characterized by a progressive necrosis of tissues of infected aerial parts of the plant and often associated with ooze production. Natural infections mainly occur through wounds and natural openings, especially on flowers. On susceptible hosts, bacteria first move through the intercellular spaces of the parenchyma and at a later stage into the xylem vessels, provoking extensive lesions and sometimes complete dieback of the tree. The strategy of infection of *E. amylovora* differs from that of *D. dadantii* in that the two main pathogenicity factors for this bacterium are the exopolysaccharide amylovoran and secretion of effectors through the type III secretion system. These effectors are required during early infection steps that lead to local necrosis, whereas amylovoran is required during later stages which result in bacterial progression *in planta*. *E. amylovora* effector proteins contribute to pathogenesis by controlling plant defenses and triggering cell death (Degrave et al. 2008).

In iron-limited environments, *E. amylovora* produces cyclic trihydroxamate siderophores belonging to the desferrioxamine family, with a predominance of deferrioxamine E. First isolated from actinomycetes, desferrioxamines were also found in other bacteria such as *E. herbicola* (Feistner et al. 1993; Kachadourian et al. 1996). Unlike nonpathogenic mutants lacking a functional type three secretion system, mutants affected in ferrioxamine-mediated iron transport still cause fire blight (Dellagi et al. 1998, 1999). The lack of ferrioxamine-dependent iron uptake had no effect on pathogenicity when tested on apple seedlings. However, the mutants were less able to colonize floral tissues and to initiate necrosis on apple flowers, indicating that the production of desferrioxamine is critical at the onset of infection. This observation led the authors to speculate that desferrioxamine is involved not only in iron acquisition, but also in oxidative

stress responses of the pathogen. Indeed, *E. amylovora* wild-type strains induce electrolyte leakage from host plant cells as the result of cell death. The ability of desferrioxamine biosynthetic mutants to induce this reaction is severely reduced and this defect is rescued by the addition of this siderophore. As desferrioxamine alone does not induce electrolyte leakage, it was proposed that this compound, by inhibiting the generation of toxic radicals via the Fenton-type redox chemistry, protects the bacterial cells against the toxic effects of reactive oxygen species produced at the onset of infection. The reduced ability of the desferrioxamine biosynthetic mutants to cause electrolyte leakage would result from the transient decrease in bacterial population size following the initial oxidative burst. In support of this interpretation was the increased survival of *E. amylovora* cells treated with hydrogen peroxide in the presence of desferrioxamine B, which is known to protect animal tissues from damage by reactive oxygen species. By forming high-stability iron complexes, compounds of the desferrioxamine family inhibit the generation of hydroxyl radicals via the Fenton reaction. Interestingly, Zhao et al. (2005) found that the gene encoding the ferritin Ftn is induced during infection in pear tissue.

A search of the complete genome of *E. amylovora* 1430 for the presence of the *foxR* gene encoding the ferrioxamine receptor allowed Smits and Duffy (2011) to identify a genetic cluster involved in desferrioxamine biosynthesis and transport. These authors found the existence of a high conservation of the desferrioxamine biosynthetic proteins in genomes of members of the related genera *Erwinia* and *Pantoea* indicating the ancestral nature of this feature.

2.1.7 Pathogenicity of Ascomycete Fungi

The filamentous ascomycete *Cochliobolus heterostrophus* is representative of a genus that attacks monocots, including all major cereal crops, worldwide. This fungus was known as a mild pathogen of corn until 1970, when a highly virulent race caused the Southern Corn Leaf Blight (SCLB), which devastated the US corn crop along the eastern seaboard. Thus, *C. heterostrophus* emerged as a model necrotrophic fungus for the study of plant pathogenesis (Turgeon and Baker 2007). The fungus overwinters as conidia and mycelia on debris of dead corn plants. Conidia infect corn leaves by direct penetration and cause small lesions, which, under epidemic conditions, can cover the entire leaf, thereby killing the plant. *C. heterostrophus* and related taxa are known for their ability to produce host-specific toxins that serve as pathogenicity factors. Synthesized by NRPS, each toxin appears to be necessary for development of a particular disease. However, in a number of physiological and genetic studies, they were found to be insufficient to explain pathogenicity entirely. Looking for general pathogenicity factors, Lee et al. (2005) undertook a genome-wide search for NPRS-encoding genes in *C. heterostrophus*. Twelve loci predicted to encode NPRS were deleted singly, and only one, *NPS6*, appeared to be involved in virulence to maize (Oide et al. 2006). Interestingly, *NPS6* is the only gene

that has an ortholog in all other ascomycetes examined. To examine the functional conservation of *NPS6*, *NPS6* orthologs were deleted in the rice pathogen *Cochliobolus miyabeanus*, the *Arabidopsis* pathogen *Alternaria brassicicola*, and the wheat/maize/barley pathogen *Fusarium graminearum*.

Lesions caused by *C. heterostrophus nps6* mutants are smaller compared to those of the wild-type. Conidia of the *nps6* mutant germinate normally, form appressoria and penetrate successfully into the host. However, the extent of colonization by this mutant is less than that of the wild-type strain, indicating that deletion of *NPS6* does not cause a defect in pre-penetration growth or penetration efficiency. Lesion sizes upon infection with the *nps6* mutants of *C. miyabeanus* and *A. brassicicola* are also reduced. Similarly, symptom development on wheat spikes inoculated with the *nps6* mutants of *F. graminearum* is much delayed compared with the wild-type strain. Besides their reduced virulence, *nps6* mutants have increased sensitivity to iron depletion and increased sensitivity to oxidative stress. Indeed, *C. heterostrophus*, *Al. brassicicola*, and *F. graminearum NPS6* genes are upregulated under iron-depleted conditions. These observations suggested that the NPRS encoded by *NPS6* are involved in siderophore biosynthesis. HPLC analyses of the culture filtrate and mycelial fractions of *C. heterostrophus* wild-type and *nps6* mutants confirmed this assumption. Further analyses demonstrated that the siderophores produced are coprogens, which are hydroxamates widely distributed among fungal species, including the saprophyte *Neurospora crassa* (Winkelmann 2007). The role of these siderophores in iron nutrition of these fungi *in planta* was supported by the observation that exogenous application of iron enhances the virulence of *nps6* mutants. The application of the extracellular siderophore of *A. brassicicola* restores wild-type virulence to the *nps6* mutant on *Arabidopsis*. Whether these siderophores play a role in virulence through the protection of fungal pathogens against reactive oxygen species generated *in planta* requires further investigation.

The filamentous ascomycete *Magnaporthe grisea* causes rice blast disease, which is very devastating in cultivated rice (Caracuel-Rios and Talbot 2007). Infection occurs through the formation of specialized structures called appressoria that differentiate from the end of fungal germ tubes and generate mechanical force necessary to romper the leaf cuticle and entry to internal tissues. *M. grisea* can also infect roots by development of distinct infection structures. Once inside roots, the fungus can invade the plant vascular system and spread to aerial parts of the plant where it produces disease lesions. *M. grisea* produces extracellular siderophores of the coprogen family to acquire iron from the environment. It also synthesizes the siderophore ferricrocin, a cyclic hexapeptide located intracellularly and considered as an iron storage molecule under iron-replete conditions. In the *M. grisea* genome, Hof et al. (2007) identified a NRPS gene, *SSM1*, clustered with other genes potentially involved in siderophore biosynthesis. Disruption of *SSM1* confirmed that the gene encodes ferricrocin synthase. The ability of the ferricrocin deficient mutants to infect rice leaves was examined in detached leaf assays. On intact leaves, the mutants caused almost no disease symptoms, while on wounded leaves they induced lesions similar to those of the wild-type strain. Using a whole plant

infection assay with a susceptible rice cultivar, the authors confirmed that the mutants are impaired in their ability to develop the typical symptom of gray-centered lesion. The mutants showed no defects in term of sporulation, germination of conidia or appressorium formation however, a cell penetration assay revealed that they have reduced ability to penetrate the plant surface. Therefore, ferricrocin appears to play a role in an early plant infection step which is critical for making the appressorium functional.

2.1.8 Ustilago maydis, a Basidiomycete Model

The plant-pathogenic basidiomycete *U. maydis* causes smut disease of maize. The main symptoms are tumors which can develop on all green parts of the plant (Bölker 2001). The fungal hyphae proliferate in these tumors and differentiate into diploid spores. Spores are distributed by air and can germinate after landing on plant surfaces. During germination, meiosis occurs and results in the production of nonpathogenic haploid cells. Fusion of compatible haploid cells is required to generate a dikaryotic mycelium which is infectious. The fusion of cells and the development of the pathogenic dikaryon are governed by the a and b mating-type loci. During mating, cells secrete specific pheromones and respond to the presence of cells of the opposite mating type. This process involves coordinated cAMP-dependent kinase A (PKA), as well as mitogen-activated protein kinase (MAPK) signaling. Transcriptome analysis using whole genome microarrays to identify putative targets of the PKA catalytic subunit Adr1 revealed nine genes with putative functions in two high-affinity uptake systems, located in three gene clusters on chromosomes 1, 2, and 4 of the *U. maydis* genome (Eichhorn et al. 2006).

   The cluster on chromosome 1 contains the *sid1* and *sid2* genes previously shown to be involved in ferrichrome biosynthesis (Mei et al. 1993). Chromosome 2 harbors a cluster of genes, *fer3* to *fer10* (for Fe-regulated), several of which were predicted to encode the enzymes catalyzing the biosynthesis of ferrichrome A, the second siderophore released by *U. maydis*, as well as the corresponding transporters. The two genes found on chromosome 4, *fer1* and *fer2*, encode a putative ferroxidase and a high-affinity ferric permease, respectively. The *fer2* gene is able to complement the growth defect of an *FTR1* mutant of *Saccharomyces cerevisiae* affected in high-affinity iron uptake due to a lack of functional ferric iron permease. It is plausible that, like in *S. cerevisiae*, the iron permease and oxidase in *U. maydis* are part of a ferrous uptake system in which a plasma membrane reductase reduces ferric ions present in the medium. The resulting ferrous ions are then reoxidized by the multicopper oxidase before transport by the permease. Like the *sid1* and *sid2* genes, the *fer* genes were found to be negatively controlled by the GATA transcription factor Urbs1 in the presence of iron. As these genes are also regulated through a cAMP-dependent pathway, it was postulated that the

regulatory activity of Urbs1, which has eight putative PKA phosphorylation sites, could depend on phosphorylation.

When maize plants are infected with a compatible combination of wild-type strains, tumors develop in most infected plants and a high proportion of these plants die. Upon infection with either *fer2* or *fer1* mutants fewer plants develop tumors and these tumors remain small. As a consequence, plant death is only rarely observed. These mutants are not affected in early development on the leaf surface: they form appressoria and penetrate normally. However, proliferation of the fungal cells in the plant is not observed. Interestingly, the *fer2* mutant is more virulent on a wild-type host than on the maize *ys1* mutant, which has a defect in ferric phytosiderophore uptake. Thus, the ferroxidation/permeation system is decisive for iron acquisition during plant colonization by *U. maydis*. As ferrichrome biosynthetic mutants are not affected in virulence, an intriguing question is why phytopathogenic fungi use different strategies for iron acquisition during plant colonization. It is possible that the lifestyle of the fungus during infection, i.e., growing as a biotroph or a necrotroph may determine in which form the iron present in the host plant can be acquired. At which stages of its life cycle *U. maydis* makes use of the ferrichrome-mediated iron uptake system is another remaining question.

The role of the siderophore rhodotulic acid in the phytopathogenesis of *Microbotryum violaceum*, an obligate anther smut fungus infecting species of the Caryophyllacea family, was also investigated by characterizing a mutant deficient in extracellular production of this molecule. Although the growth of this mutant compared to wild-type is altered when starved for iron, the production of rhodotulic acid proved to be unnecessary for fungal growth *in planta* (Birch and Ruddat 2005).

2.2 Iron and Plant Defense

2.2.1 Iron Homeostasis in Wheat Upon Infection by Blumeria graminis

The powdery mildew fungus *Blumeria graminis* is a biotrophic pathogen that requires successful host cell wall penetration, development of a functional haustorium and maintenance of host cell integrity to establish a compatible interaction with its host. Resistance to this pathogen is expressed by prevention of penetration through localized cell wall strengthening by the apposition of new wall material. During *B. graminis* attack, there is production of reactive oxygen species in epidermal and mesophyll cells close to the infection sites. In an expressed sequence tag (EST) library developed from wheat epidermis challenged with the wheat powdery mildew fungus *B. graminis* sp. *tritici*, Liu et al. (2007) noted a high

occurrence of Fe-related transcripts, which prompted these authors to investigate changes in iron homeostasis in wheat leaves during the fungal attack.

Using ICPMS (inductively coupled plasma mass spectrometry) to track changes in metal concentrations in the epidermis and mesophyll of wheat leaves infected with *B. graminis*, Liu et al. (2007) found that ferric ions accumulate in the infected epidermis but not in the mesophyll. This accumulation takes place when cell wall appositions are mature. Further analysis indicated that the accumulated iron enhances the production of reactive oxygen species triggered during pathogen attack. Indeed, a pretreatment of the inoculated leaves with the ferric iron chelator desferrioxamine decreased the oxidative burst detected by diaminobenzidine (DAB) staining. The iron-regulated genes *TmNAS1* and *TmFER1*, encoding a nicotianamine synthase and a ferritin isoform, respectively, appeared to be down-regulated during pathogen attack, suggesting cytosolic iron depletion in the infected tissues. In addition, a correlation was established between cytosolic iron depletion and activation of the pathogenesis-related gene *TmPR1b*. Otherwise, using the membrane-permeable fluorescent chelator calcein, the authors showed that a treatment of wheat suspension cells with H_2O_2 promoted active cytosolic Fe efflux. Collectively, these findings led to the proposal of a model of the involvement of iron in wheat leaves during *B. graminis*, where the redistribution of this metal and the oxidative burst triggered after infection contribute to the amplification of a redox signal involved in the defense response.

2.2.2 Involvement of Ferritin in the Response of Potato to Phytophthora infestans

Oomycetes in the genus *Phytophthora* are fungus-like plant pathogens that are devastating for virtually all dicots (Attard et al. 2008). The species *Phytophthora infestans* causes potato late blight and this disease is initiated when wind-blown sporangia release zoospores onto the plant surface. The zoospores encyst to form appressoria, after which hyphae develop and can spread throughout the plant. When these structures penetrate plant cells, they remain enveloped by a lipid membrane derived from the plant plasma membrane. Treatment of potato leaves with desferrioxamine before spraying a suspension of sporangia resulted in a reduction of lesion development and a decrease in the production of reactive oxygen species (Mata et al. 2001). Upon infection with *P. infestans* expression of the *StF1* potato ferritin gene was increased, suggesting that ferritin may be a protective molecule for plant cells. By scavenging the intracellular iron, ferritin can limit the generation of reactive oxygen species. Although increased ferritin gene expression does not suffice to confer resistance to *P. infestans*, a previous study indicated that transgenic tobacco plants ectopically expressing an alfalfa ferritin gene has increased tolerance to viral (tobacco necrosis virus) and fungal (*Alternaria alternata*, *Botrytis cinerea*) infections (Deak et al. 1999). These reports support the idea that ferritin can be part of host defense responses triggered during infection.

2.2.3 Iron Homeostasis and Resistance of Arabidopsis to D. dadantii

As efficient iron uptake/storage mechanisms are of critical importance to the survival of *D. dadantii* during infection, the question of whether this pathogen could induce iron-withholding reactions in the plant has been addressed. A first observation made by Neema et al. (1993) indicated that iron incorporated into plant ferritins drastically decreased in soybean suspension cells challenged with *D. dadantii.* This effect was also observed during treatment of the cells with chrysobactin. The possibility of a competition for iron between the pathogen and the host was also illustrated by the observation that accumulation of polyphenols in plant tissues inhibits growth of mutants of *D. dadantii* affected in their sidero-phore-mediated iron transport pathway (Mila et al. 1996). Polyphenols have iron-chelating properties and could play the role fulfilled by iron-binding proteins, such as transferrin in animal immunity.

In order to identify plant genes that are regulated in response to infection by *D. dadantii*, Dellagi et al. (2005) differentially screened cDNA libraries from *Arabidopsis* and found that the gene encoding the ferritin AtFer1 is upregulated in infected plants. These authors established that accumulation of *AtFer1* transcripts and production of ferritins during infection is a defense reaction against proliferation of the pathogen. The siderophore chrysobactin, as well as desferrioxamine are elicitors of this response. As only the iron-free siderophores induce this reaction, it was suggested that these iron sequestering molecules could cause severe iron depletion in *Arabidopsis* leaf tissues, resulting in the redistribution of intracellular iron stores and/or the activation of iron acquisition systems of the cell. Intracellular redistribution could involve remobilization of vacuolar iron by the specific metal transporters Nramp3 and Nramp4 (Fig. 2.2). Indeed, among the six *NRAMP* genes present in *Arabidopsis*, *AtNramp3* was found to be strongly upregulated in response to several biotic stresses.

Because there is a functional redundancy between AtNRAMP3 and AtNRAMP4 in seed germination and the encoded proteins share 50 % sequence identity with the mouse NRAMP1 metal ion transporter involved in innate immunity, the role of these genes in resistance to *D. dadantii* was investigated further (Segond et al. 2009). *AtNRAMP3* is upregulated in leaves challenged with *D. dadantii*, while *AtNRAMP4* expression does not change. Using simple and double *nramp3* and *nramp4* mutants, as well as lines ectopically expressing either of these genes, Segond et al. (2009) showed that *AtNRAMP3*, and to a lesser extent *AtNRAMP4*, are involved in the resistance of *Arabidopsis* against this bacterium. The susceptibility of the *nramp3 nramp4* double mutant was associated with a reduced accumulation of reactive oxygen species and AtFER1, which are effective defense components against *D. dadantii.* By promoting an efflux of iron and possibly other metals from the vacuole to the cytosol, the activity of Nramp3 and Nramp4 proteins may contribute to exacerbation of the oxidative stress generated during infection and be responsible for basal resistance to this pathogen. Both increased oxidative stress and efflux of
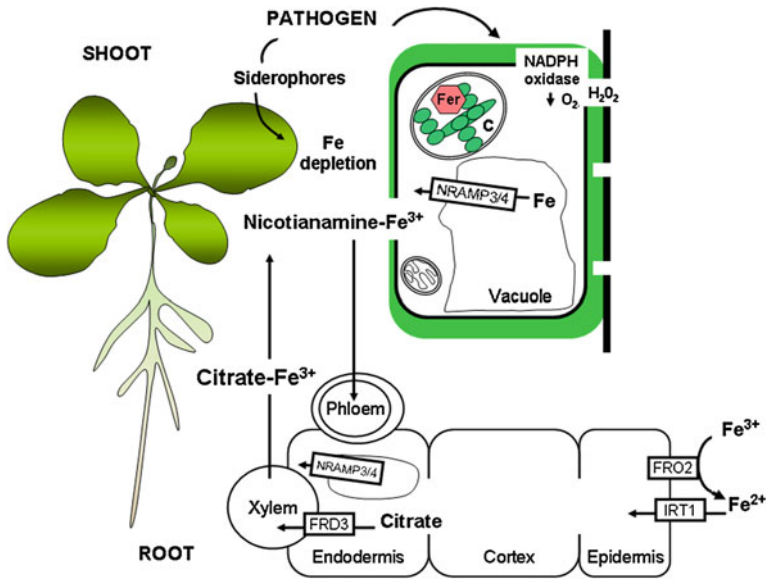
Fig. 2.2 Schematic representation of plant iron acquisition and changes in iron trafficking during pathogenesis. This model is based on the reactions triggered by *D. dadantii* during infection of *Arabidopsis*. Plants acquire iron from the soil. In dicots, Fe^{3+} is reduced to Fe^{2+} by the FRO2 ferric chelate reductase, and then transported through the plasma membrane by the iron-regulated transporter IRT1. Inside the plant, iron is transported essentially as ferric complexes of citrate in the xylem and of nicotianamine in the phloem. Storage and buffering occur in the apoplast and the organelles including vacuoles and plastids (C) that contain ferritins (Fer). Bacterial invasion triggers iron depletion in leaves, leading to a mobilization of vacuolar iron mediated by transporters AtNramp3 and AtNramp4. The reactive iron released in the cytosol contributes to amplify the production of reactive oxygen species and to induce ferritin synthesis in the chloroplast depriving the bacteria of iron. Infection also results in iron mobilization in the roots, from both the vacuole and the soil

iron could be at the origin of ferritin up-regulation in infected leaves. In addition, roots from *D. dadantii*-challenged plants accumulate transcripts of *AtNRAMP3* as well as the root iron deficiency markers *IRT1* and *FRO2*. This finding suggests the existence of a shoot to root signal activated by pathogen infection. Whether the redistribution of iron in the infected leaf and uptake of iron by the roots are physiologically linked is an appealing question. Collectively, these data indicate that the functions of NRAMP proteins in innate immunity (Neves et al. 2011) have been conserved between animals and plants.

Are the siderophores produced by the bacterium the main elicitors of these plant reactions? To approach this question, Dellagi et al. (2009) analyzed the effect of diverse types of siderophores including chrysobactin and desferrioxamine in Arabidopsis plants following leaf infiltration. It was found that these siderophores, only when iron-free, could activate the salicylic acid-dependent reactions

belonging to a major signaling pathway involved in the plant's immune network. They also could activate the iron deficiency response from the root, showing the existence of a leaf to root deficiency signal mediated by these microbial molecules, and revealing a new link between two processes controlled by salicylic acid and iron. Thus, expression of the plant immune response and activity of the plant iron acquisition system could be modulated during infection via the fluctuations of siderophore production by the pathogen. This effect may be to the advantage of the pathogen or may help the plant to resist the infection. A future challenge is to better understand the molecular mechanisms by which siderophores can activate this dual response.

2.2.4 Effect of the Plant Iron Status on Susceptibility/Resistance to Pathogens

As mineral nutrition may affect the interaction of plants with microorganisms because of changes in metabolism, several investigators wondered whether the iron status of the host could exert a significant effect on the disease evolution. Indeed, iron deficiency can compromise the activity of metalloenzymes important for the host immune response. This question was addressed in several studies in which plants grown under iron deficiency conditions were challenged with fungal pathogens. In particular, solanaceous crop plants were infected by species of *Fusarium* and *Verticillium* which are ascomycetes responsible for wilt diseases. These fungi usually enter the plant through young roots and then grow into the water conducting vessels of the roots and stem. As the vessels are plugged and collapse, the water supply to the leaves is blocked. Anderson and Guerra (1985) observed increased lesion size in beans infected by *F. solani* by reducing iron in the nutrient solution of plants grown under hydroponic conditions. In infection by *V. dalhliae* of several crops, including peanuts, egg-plants, tomato, and potato, iron deficiency resulted in increased sensitivity to symptom development. Supplementation of plants with iron significantly alleviated disease symptoms (Barash 1988), however in peanuts, this effect was not correlated with production of phytoalexins, a typical antimicrobial plant reaction. Studies recently conducted on *Arabidopsis* with two aerial pathogens *D. dadantii* and the ascomycete *Botrytis cinerea*, showed a different result (Kieu et al. 2012). Iron-starved plants displayed reduced susceptibility to infection and iron supplementation restored the symptom severity. In the case of *D. dadantii*, further examination revealed that iron deficiency causes a reduction in bacterial fitness and expression of virulence genes as well as an exacerbation of the salicylic acid-mediated defense pathway. But disease reduction did not correlate with the involvement of defenses known to be effective against the bacterium. Thus, the plant iron status can influence host-pathogen relationships in different ways by affecting the pathogen's virulence as well as the host's defense.

2.3   Conclusion

Like other members of the microbial world interacting with animals and humans, plant pathogenic microorganisms have evolved a diversity of systems allowing them to capture iron from various environments in response to their metabolic needs. Indeed, experimental investigations have permitted to underscore the importance of siderophores and corresponding transport machineries in iron nutrition of phytopathogenic species. Moreover, knowledge of complete sequences of bacterial and fungal genomes has greatly contributed to enlarge our vision of how plant pathogens can manage their iron homeostasis. These microbes produce siderophores belonging to the three broad classes, hydroxamate, catecholate, and α-hydroxycarboxylate as chelating groups. Bacterial phytopathogens are also gifted with specific transport systems for ferrous iron, ferric citrate, and other sources of ferric iron, heme and exogenous microbial siderophores (Table 2.1). Reductive iron uptake mechanisms are likely to exist in plant pathogenic fungi, but the possibilities of transporting heme and ferric ligands other than siderophores have not been reported. The plurality of these systems illustrates the importance of iron acquisition to the fitness of the bacteria associated to plants. Outside the plant, the natural habitats of pathogens are the rhizosphere and soils where many microbial populations interact with each other or with the plant. Very likely, the possibility to express well-suited mechanisms for iron acquisition with the capacity to utilize exogenous siderophores is essential for their survival.
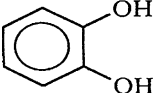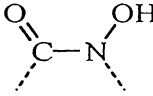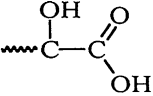
Although the potentiality to produce siderophores seems to be a common trait to most phytopathogens, the requirement of a functional siderophore-mediated iron transport route for pathogenicity is not absolute. As siderophores are powerful iron-chelating compounds and can compete out with known plant iron transporters including organic acids and nicotianamine, or with iron containing proteins, this indicates that in certain pathological situations, the availability of this metal is sufficient to enable the pathogen to thrive *in planta*. Indeed, depending on the infected organ or tissue and the strategy of microbial attack, it might be beneficial for the pathogen to use alternative iron transport routes or a heme acquisition system. A preference for the Feo ferrous iron acquisition system was demonstrated in the case of *X. oryzae* infection and a ferric iron permease is required for the virulence of *U. maydis*. Production of a siderophore dependent iron transport system requires the involvement of NRPS and a specific TonB-dependent transporter and the whole process is metabolically energy costing. Secretion of virulence factors is likely to be expensive in energy as well and co-expression of both functions could be metabolically incompatible. The fact that expression of the *P. syringae* DC3000 type III secretion system in culture requires sufficient iron levels supports this assumption. In the same way, several genes encoding the *D. dadantii* pectate-lyase involved in the virulence are repressed when the host is iron-depleted. The iron sensory protein Fur was shown to act as a global regulator of phytopathogenicity in several pathosystems and the discovery of new virulence regulatory networks responding to iron levels highlights the importance of a tight control of the bacterial intracellular iron level in

relation to metabolic activities during infection. It is well known that during the infectious process, phytopathogenic bacteria encounter an oxidative environment. Reactive oxygen species are generated by host plants as a defense mechanism against microbial invasion. This plant defense response consists of the production of superoxide, hydrogen peroxide, and nitric oxide, which function either directly in the establishment of defense mechanisms or indirectly via synergistic interactions with other signaling molecules, such as salicylic acid (reviewed in Bolwell and Daudi 2009). Under these conditions, a tight control of the iron concentration is essential for the invading bacteria to avoid exacerbation of this oxidative stress through Fenton's reaction, which generates the highly toxic and reactive hydroxyl radical OH·. Interestingly, siderophores can interfere with this reaction by sequestering ferric ions and thus influencing the ferric/ferrous iron ratio; for instance, desferrioxamine produced by *E. amylovora* was proposed to function as such.

An obstacle in the comprehension of iron competition between bacteria and plants is the lack of information on the siderophores produced by phytopathogenic bacteria. Very few siderophores have been chemically and structurally characterized in plant pathogenic species (Table 2.2). Depending on their iron-chelating functional groups, siderophore molecules possess different affinity for the ferric ion and exhibit different chelating capacity and stability under pH variations. These parameters must be evaluated in order to determine the functional role that a given siderophore can play during the infectious cycle. Several phytopathogenic bacteria encode more than one siderophore iron uptake system that can be distinct in very close strains or pathovars. An illustration of this complexity is illustrated by the strain EC16 of *D. dadantii* which produces dichrysobactin and linear/cyclic trichrysobactin in addition to the monomeric siderophore chrysobactin (Sandy and Butler 2011). Synthesis of different siderophores may help bacteria to cope with fluctuations of the iron status encountered within plant tissues. Indeed, different siderophore dependent iron transport routes can be differentially expressed and play distinct roles according to environmental conditions or pathological situations. These aspects are important to further consider the role of siderophores in pathogenicity.

The role of iron in microbial plant pathogenesis is an emerging topic with exciting recent developments. There is now evidence that a competition for iron between the host and the microorganism can take place. In particular, it is noteworthy that during infection by *D. dadantii*, Arabidopsis plants develop an iron-withholding response that changes iron distribution and trafficking. This effect is likely to be caused by the *D. dadantii* siderophores that can interfere with plant defense responses and exacerbate iron mobilization from the root. The fact that the plant nutritional iron status also affects the development of diseases, by acting on both the pathogen's virulence and the host's defense is demonstrative. Elucidating mechanisms involved in exchanging and withholding iron during plant–microbe interactions should help to develop integrative strategies for controlling plant diseases. Such investigations are of primary importance in an agronomical context, where reductions in mineral fertilization and pesticide crop treatment are becoming necessary. They should help to the development of crop genotypes with

Table 2.2 Siderophores produced by phytopathogenic bacteria, with their role in virulence

Common bidentate coordination sites in microbial siderophores

Catechol	Hydroxamic acid	Hydroxy carboxylic acid

Species/Strain	Typical disease	Characterized siderophore	Role in pathogenicity (reference)
P. syringae pv. *syringae* strain B301D	Fruit necrotic spots	Pyoverdin	Not required for cherry fruit disease (Cody and Gross 1987)
P. syringae pv. *tomato* strain DC3000	Bacterial speck of tomato	Yersiniabactin Pyoverdin	Not required for tomato and Arabidopsis diseases (Jones et al. 2007) (Jones and Wildermuth 2011)
P. syringae pv. *tabaci* strain 6605	Wildfire disease on host tobacco plants	Pyoverdin	Necessary for tobacco leaves infection (Taguchi et al. 2010)
P. syringae pv. *phaseolicola* strain 1448a	Bean halo blight	Pyoverdin and Achromobactin	Unnecessary for bean pods infection (Owen and Ackerley 2011)
A. tumefaciens strain B6	Crown gall tumors on dicot plants	Agrobactin	Not required for carrot or sunflower disease (Leong and Neilands 1981)
R. solanacearum strain AW1	Bacterial wilt on many plants	Staphyloferrin B	Unnecessary for tomato plants infection (Bhatt and Denny 2004)
E. chrysanthemi (*D. dadantii*) strain 3937	Soft rotting on many plants	Chysobactin and achromobactin	Required for virulence on host plants (Enard et al. 1988; Neema et al. 1993; Franza et al. 2005)
E. carotovora strain W3C105	Potato stem rot	Chrysobactin and aerobactin	No role in aerial stem rot (Ishimaru and Loper 1992)
E. amylovora strain 1430	Fire bight on *Pomoideae*	Deferrioxamines	Necessary for virulence apple flowers (Dellagi et al. 1998)

efficient mineral use and uptake properties without an increase in susceptibility to plant pathogens and/or decrease in yield or crop quality. In addition, the use of naturally occurring microbial antagonists to suppress plant diseases offers an alternative to classical methods of plant protection and in this regard, it is worth considering that production of high-affinity iron transport systems by such antagonists can be a determining factor in their competitiveness.

Furthermore, iron deficiency represents a major nutritional disorder and is estimated to affect a great part of the world's population. A commonly used

strategy to increase dietary intake of iron is to develop iron biofortified plants (Murgia et al. 2012). Potential changes in susceptibility of plants to microbial diseases must be considered, when biotechnological approaches aimed at enhancing the bioavailability of iron in the tissues are developed.

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