

NPS6, Encoding a Nonribosomal Peptide Synthetase Involved in Siderophore-Mediated Iron Metabolism, Is a Conserved Virulence Determinant of Plant Pathogenic Ascomycetes^W

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NPS6, encoding a nonribosomal peptide synthetase, is a virulence determinant in the maize (*Zea mays*) pathogen *Cochliobolus heterostrophus* and is involved in tolerance to H₂O₂. Deletion of NPS6 orthologs in the rice (*Oryza sativa*) pathogen, *Cochliobolus miyabeanus*, the wheat (*Triticum aestivum*) pathogen, *Fusarium graminearum*, and the *Arabidopsis thaliana* pathogen, *Alternaria brassicicola*, resulted in reduced virulence and hypersensitivity to H₂O₂. Introduction of the NPS6 ortholog from the saprobe *Neurospora crassa* to the $\Delta nps6$ strain of *C. heterostrophus* restored wild-type virulence to maize and tolerance to H₂O₂, demonstrating functional conservation in filamentous ascomycete phytopathogens and saprobes. Increased sensitivity to iron depletion was identified as a conserved phenotype of $\Delta nps6$ strains. Exogenous application of iron enhanced the virulence of $\Delta nps6$ strains of *C. heterostrophus*, *C. miyabeanus*, *F. graminearum*, and *A. brassicicola* to each host. NPS6 is responsible for the biosynthesis of extracellular siderophores by *C. heterostrophus*, *F. graminearum*, and *A. brassicicola*. Application of the extracellular siderophore of *A. brassicicola* restored wild-type virulence of the $\Delta Abnps6$ strain to *Arabidopsis*. It is proposed that the role of extracellular siderophores in fungal virulence to plants is to supply an essential nutrient, iron, to their producers in planta and not to act as phytotoxins, depriving their hosts of iron.

INTRODUCTION

Nonribosomal peptide synthetases (NRPSs) are multifunctional proteins that biosynthesize small peptides independently of the ribosomal protein synthesis machinery. To date, the NRPS method of peptide biosynthesis has been described mostly for filamentous ascomycete fungi and for bacteria and has not been shown to occur in plants. The products of certain fungal NRPSs play critical roles in plant–microbe interactions. For example, AM-toxin, produced by the apple (*Malus domestica*) pathotype of *Alternaria alternata*, and HC-toxin, produced by race 1 of the maize (*Zea mays*) pathogen, *Cochliobolus carbonum*, are determinants of pathogenicity; if not producing toxin, these fungi are not effective as pathogens (Panaccione et al., 1992; Johnson et al., 2000). Another example is the metabolite produced by the *Magnaporthe grisea* Avirulence *Conferring Enzyme1*-encoded NRPS, which acts, directly or indirectly, as an avirulence determinant (Bohnert et al., 2004).

In a previous genome-wide search for NRPS-encoding genes (*NPSs*) in the maize pathogen *Cochliobolus heterostrophus*, we determined that the genome encodes 12 *NPSs* and that, when deleted singly, only one, *NPS6*, is involved in virulence to maize (Lee et al., 2005). Deletion of *NPS6* causes, concomitantly, a reduction in virulence and increased sensitivity to H₂O₂. Phylogenetic and structural analyses suggest that *NPS6* is conserved among diverse species of filamentous ascomycetes, in contrast with the previously identified fungal *NPS* genes involved in virulence, which appear to be restricted to one or a few species or races (Lee et al., 2005).

The dual phenotype of reduced virulence and sensitivity to oxidative stress and phylogenetic conservation of *NPS6* led us to the hypothesis that the product of the *NPS6* protein might be a siderophore, as most fungal siderophores are products of NRPSs. These Fe³⁺-specific iron-chelating ligands are produced by diverse species of bacteria and fungi and also are reported in marine organisms such as phytoplankton and cyanobacteria (Armstrong and Baalen, 1979; Trick et al., 1983). Iron, one of the most abundant elements on earth, is an essential nutrient for virtually all organisms (Winkelmann, 1991); however, bioavailable forms are very limited in aerobic environments, as a result of oxidation and the formation of insoluble iron hydroxides and oxyhydroxides (Neilands and Leong, 1986; Lesuisse and Labbe, 1994; Haas, 2003). Hence, efficient iron uptake/storage mechanisms are of critical importance to the survival of all organisms in nature. With their strong iron binding activity, siderophores

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function in both the acquisition and storage/sequestration of iron (Neubauer et al., 2000). In fungi, intracellular siderophores play a cytoprotective, antioxidant role, in addition to their role in iron storage, by preventing the Fenton reaction ($\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^\cdot + \text{OH}^-$), which generates highly cytotoxic reactive oxygen species (i.e., hydroxyl radicals) from less toxic H_2O_2 .

In terms of virulence, the crucial role of siderophores is well established for animal pathogenic bacteria (Bearden and Perry, 1999; Takase et al., 2000; Weinberg, 2000), which use siderophores to acquire iron from their animal hosts, in which iron is usually sequestered by iron binding proteins such as transferrin and lactoferrin and, hence, limited. Recent studies on the opportunistic fungal human pathogen *Aspergillus fumigatus* showed that siderophore-mediated iron metabolism plays a role in fungal infection of animals (Schrettl et al., 2004; Hissen et al., 2005).

A role for siderophores in plant-pathogen interactions has not been established as clearly as in animal-pathogen interactions, especially for fungus-plant interactions. Pioneering work with the bacterial plant pathogen *Erwinia chrysanthemi* revealed the role of the siderophore chrysobactin in virulence (Enard et al., 1988; Neema et al., 1993; Masclaux and Expert, 1995), and a recent study reported that another siderophore produced by *E. chrysanthemi*, achromobactin, also contributes to its virulence (Franza et al., 2005). This body of work on *E. chrysanthemi* and the work on the closely related apple pathogen, *Erwinia amylovora* (Dellagi et al., 1998, 2005), remain the only clear demonstrations of the contribution of siderophores to infection in plants. Early studies with the basidiomycete maize pathogen *Ustilago maydis* showed that siderophores produced by this species do not play an essential role in virulence to maize (Mei et al., 1993).

Here, we demonstrate that the NRPS encoded by *NPS6* is responsible for extracellular siderophore biosynthesis and that *NPS6* is functionally conserved among diverse species of filamentous ascomycetes. We propose that extracellular siderophores play a role in fungal infection of plants by supplying an essential nutrient, iron, to their producers in planta.

RESULTS

Deletion of *C. heterostrophus* *NPS6* Leads to Hypersensitivity to Superoxide and to Iron Depletion, in Addition to Reduction in Virulence and Hypersensitivity to H_2O_2

Deletion of *C. heterostrophus* *NPS6* causes a reduction in virulence to maize and hypersensitivity to H_2O_2 (Lee et al., 2005), whether part of *NPS6* (Lee et al., 2005) or the entire *NPS6* gene is deleted (see Supplemental Figures 1A to 1C online). To further characterize the role of *NPS6*, *C. heterostrophus* $\Delta nps6$ strains (Δ = partial or complete deletion of *NPS6*) were examined for sensitivity to different types of stress, including oxidative stress caused by superoxide and *tert*-butyl hydroperoxide, nitrosative stress, osmotic stress, and iron depletion. No differences in sensitivity to *tert*-butyl hydroperoxide, nitrosative, or osmotic stress were detected between the *C. heterostrophus* wild-type and $\Delta nps6$ strains. The $\Delta nps6$ strains, however, showed increased sensitivity to oxidative stress, caused by the superoxide generator KO_2 , and to iron depletion, generated by the iron chelator

2,2'-dipyridyl (2DP) (Figures 1A and 1B), as well as to H_2O_2 . The minimal inhibitory concentrations (MIC) of these stress agents to *C. heterostrophus* wild-type and $\Delta nps6$ strains are listed in Table 1. Hypersensitivity of the $\Delta nps6$ strain to iron depletion was further confirmed using a second iron chelator, bathophenanthroline disulfonic acid (BPS). A statistically significant difference ($P < 0.01$) in growth was observed between the wild-type and $\Delta nps6$ strains on minimal medium (MM) supplemented with 100 μM BPS but not on MM (Figure 1C). Reintroduction of wild-type *C. heterostrophus* *NPS6* into a *C. heterostrophus* $\Delta nps6$ strain restored wild-type virulence to maize, and wild-type tolerance to stress (oxidative and iron), to the $\Delta nps6$ strain (data not shown).

C. heterostrophus $\Delta nps6$ strains show reduction in pigmentation (see Supplemental Figure 1D online) and in asexual sporulation (data not shown) on MM, but not on nutrient-rich media, such as complete medium (CM) or CM with xylose (CMX); the latter is the optimal medium for asexual sporulation of *C. heterostrophus*. Reintroduction of wild-type *C. heterostrophus* *NPS6* into $\Delta nps6$ strains restores wild-type pigmentation and wild-type levels of asexual sporulation on MM (data not shown). Percentage asexual spore germination was approximately the same for *C. heterostrophus* wild-type and $\Delta nps6$ strains in vitro (see Supplemental Table 1 online).

The growth of *C. heterostrophus* wild-type and $\Delta nps6$ strains carrying green fluorescent protein (GFP) was monitored and compared in planta using epifluorescence microscopy. Both strains germinated and formed appressoria and penetrated successfully into the host. By 72 h after inoculation, the extent of colonization by the $\Delta nps6$ strain was less than that of the wild-type strain (see Supplemental Figure 2 online). Expression of *C. heterostrophus* *NPS6* in the wild-type strain in planta was detectable at 24, 48, and 72 h after inoculation at approximately the same levels (data not shown). There was no expression of *NPS6* with the control $\Delta nps6$ sample, although a control glyceraldehyde phosphate dehydrogenase signal was detected.

These observations, coupled with the spore germination data (see Supplemental Table 1 online), demonstrate that deletion of *NPS6* causes a defect in colonization in planta, but not in prepenetration growth or in penetration.

Function of *NPS6* Is Conserved among Filamentous Ascomycetes

In contrast with most other genes encoding fungal NRPSs, *NPS6* is widely conserved among filamentous ascomycetes (Lee et al., 2005). To examine the functional conservation of *NPS6*, *NPS6* orthologs were deleted (see Supplemental Methods, Supplemental Figure 3, and Supplemental Table 2 online) in different species of plant pathogenic fungi, including the rice pathogen, *Cochliobolus miyabeanus*, the pathogen of *Arabidopsis thaliana*, *Alternaria brassicicola*, and the wheat (*Triticum aestivum*)/maize/barley (*Hordeum vulgare*) pathogen, *Fusarium graminearum*. As observed for the $\Delta nps6$ strain of *C. heterostrophus*, deletion of *NPS6* orthologs in *C. miyabeanus* and *A. brassicicola* led to a statistically significant reduction ($P < 0.01$) in virulence to each host, as measured by lesion size (Figures 2A to 2D). Similarly, the $\Delta nps6$ strains of *F. graminearum* were reduced in virulence ability compared with wild-type strains in point-inoculation assays

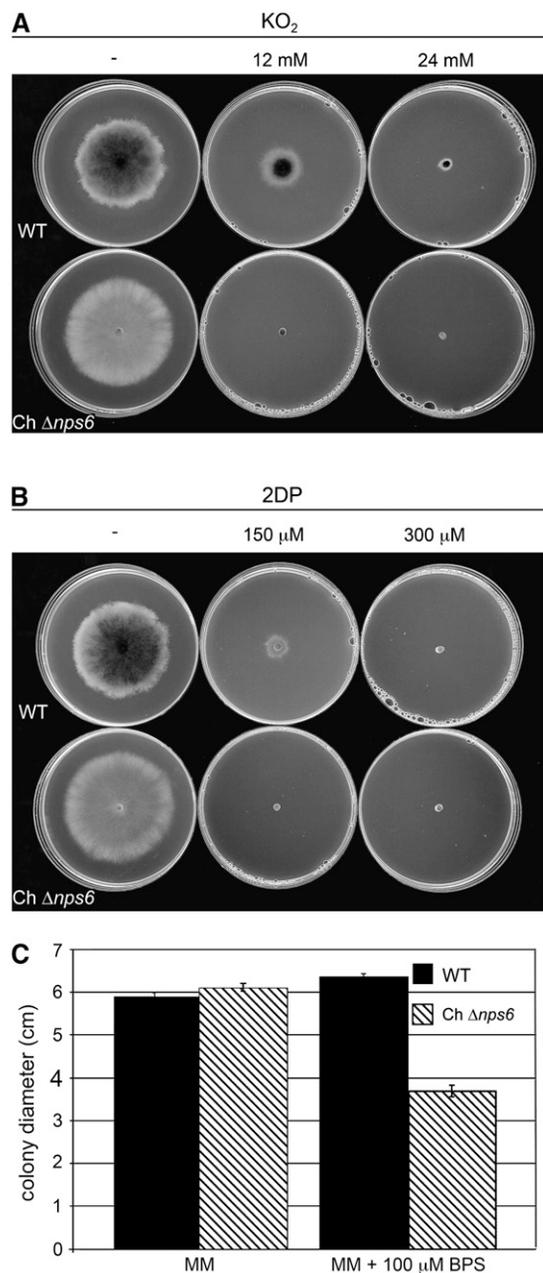


Figure 1. The *C. heterostrophus* $\Delta nps6$ Strain Is Hypersensitive to the Superoxide Generator KO_2 and to Iron Depletion.

(A) Hypersensitivity of *C. heterostrophus* $\Delta nps6$ strains to KO_2 . Five-day-old cultures of wild-type (top row) and $\Delta nps6$ (bottom row) strains of *C. heterostrophus*. Growth of the $\Delta nps6$ strain is completely inhibited on MM supplemented with 12 mM KO_2 (bottom row, middle), whereas the wild type is still able to grow on MM with 24 mM KO_2 (top row, right).

(B) Hypersensitivity of *C. heterostrophus* $\Delta nps6$ strains to the iron chelator 2DP. Five-day-old cultures of wild-type (top row) and $\Delta nps6$ (bottom row) strains of *C. heterostrophus*. Growth of the $\Delta nps6$ strain is completely inhibited on MM supplemented with 150 μ M 2DP (bottom row, middle), whereas the wild type still grows under the same conditions.

(C) Hypersensitivity of the *C. heterostrophus* $\Delta nps6$ strain to the iron chelator BPS. Average colony diameters of wild-type and $\Delta nps6$ strains of *C. heterostrophus* grown on MM or MM supplemented with 100 μ M

of wheat spikes and also developed symptoms more slowly (Figure 3A). At each time point, spikes infected by *F. graminearum* $\Delta nps6$ strains were, overall, not as severely spoiled as those infected by wild-type strains (Figure 3A). At 20 d after inoculation, almost all of the kernels in spikes infected by the wild-type strain were completely brown and shrunken, whereas several round, greenish kernels were always found in spikes challenged by the $\Delta nps6$ strain (Figure 3B). Survival analyses of wheat spikes challenged by *F. graminearum* wild-type and $\Delta nps6$ strains indicated a statistically significant reduction ($P < 0.05$) in the virulence of $\Delta nps6$ strains (see Supplemental Figure 4 online). All $\Delta nps6$ strains also showed increased sensitivity to H_2O_2 , KO_2 , and iron depletion (Table 1; see Supplemental Figure 5 online).

To further examine the functional conservation of *NPS6*, the *NPS6* ortholog from the saprobe *Neurospora crassa* (Nc *NPS6*) was introduced (see Supplemental Methods and Supplemental Figure 6 online) to the $\Delta nps6$ strain of *C. heterostrophus*. Nc *NPS6* rescued the wild-type virulence ability of the *C. heterostrophus* $\Delta nps6$ strain to maize (Figures 2E and 2F) and tolerance to stress (data not shown). Together, these data demonstrate that the function of *NPS6* is conserved among diverse species of filamentous ascomycetes, including plant (monocot and dicot) pathogenic and saprobic species.

***NPS6* Encodes an NRPS Involved in Extracellular Siderophore Biosynthesis**

The phenotype of the $\Delta nps6$ strains (i.e., increased sensitivity to iron depletion and sensitivity to oxidative stress), phylogenetic conservation of *NPS6*, and the fact that *NPS6* encodes an NRPS suggested that the NRPS encoded by *NPS6* might be involved in siderophore biosynthesis. Supporting this hypothesis, *C. heterostrophus*, *A. brassicicola*, and *F. graminearum* *NPS6* genes were upregulated under iron-depleted conditions (Figure 4) as reported previously for genes involved in siderophore biosynthesis in other fungi (Yuan et al., 2001; Yamada et al., 2003; Welzel et al., 2005). HPLC analyses of *C. heterostrophus* broth and mycelial fractions from wild-type and $\Delta nps6$ strains indicated that *NPS6* is indeed involved in siderophore biosynthesis, specifically in extracellular siderophore biosynthesis. HPLC analyses of broth extracts from wild-type *C. heterostrophus* showed three major peaks with absorption at 435 nm, indicative of ferrated siderophores (Konetschny-Rapp et al., 1988) (Figure 5, top left). Low-resolution electrospray ionization mass spectroscopy (LRESI-MS) analyses of the crude extracts indicated the presence of at least three major ferrated components with molecular masses of 821, 751, and 681 D, which are consistent with the known siderophores coprogen, one or both of the isomeric pair neocoprogen I and isoneocoprogen I, and neocoprogen II, respectively (Hossain et al., 1987) (Figure 6). These mass peaks were absent from broth extracts of the *C. heterostrophus* $\Delta nps6$ mutant (Figure 5, bottom left), indicating

BPS for 5 d. Error bars indicate 95% confidence intervals. A statistically significant difference ($P < 0.01$) in growth was observed on MM with BPS between the wild-type and *C. heterostrophus* $\Delta nps6$ strains. Note that there was no difference in their growth on MM.

Table 1. MIC of H₂O₂, KO₂, and 2DP for Wild-Type and Mutant Strains

Species	Strain	Assay		
		H ₂ O ₂ (mM)	KO ₂ (mM)	2DP (μM)
<i>C. heterostrophus</i>	Wild type	16	>24	300
	$\Delta nps6$	8	12	150
<i>C. miyabeanus</i>	Wild type	16	>24	300
	$\Delta nps6$	8	12	150
<i>A. brassicicola</i>	Wild type	>12	>20	300
	$\Delta nps6$	6	10	150
<i>F. graminearum</i>	Wild type	16	40	400
	$\Delta nps6$	8	20	200
<i>F. graminearum</i> (+Fe) ^a	Wild type	16	50	ND ^b
	$\Delta nps6$	8	25	ND

^a For *F. graminearum*, sensitivity to each stress agent was determined on MM supplemented with and without 200 μM ferric citrate (see Methods).

^b ND, not determined.

a loss of ability to produce extracellular siderophores; thus, the NRPS encoded by *NPS6* is involved in extracellular siderophore biosynthesis.

After preparative isolation of the three main siderophore components by HPLC, the identification of the 821-D compound as coprogen was confirmed by cochromatography with an authentic standard (8.8 mg, brownish red oil; high-resolution electrospray ionization mass spectroscopy [HRESI-MS] m/z [M+H]⁺ = 822.3083 [calculated for C₃₅H₅₄N₆O₁₃Fe, 822.3098]; HPLC retention time = 13.29 min). Similarly the molecular formula deduced for the 751-D compound was consistent with the isomers neocoprogen I and isoneocoprogen I and no other known fungal siderophores. After deferritation of the 751-D compound, ESI-MS-MS spectra of the deferritated compound showed fragment ions consistent with neocoprogen I but not isoneocoprogen I (see Supplemental Figure 7 online). The elution order of three major components agreed with published data for the known siderophores on various reverse-phase HPLC systems (Konetschny-Rapp et al., 1988), adding further support to the proposed identifications of the minor components as neocoprogens I and II.

Quantitative HPLC analyses yielded estimates of extracellular siderophore production by the wild type that ranged from 1 to 4 mg/L broth for the coprogens (see Supplemental Table 3 online). These compounds were not present at a detectable level (1 μg/L broth) in the broth extracts of the *C. heterostrophus* $\Delta nps6$ strain (see Supplemental Table 3 online), further demonstrating that the *C. heterostrophus* *NPS6* protein is responsible for extracellular siderophore biosynthesis.

Detectable levels of coprogens were present, also, in the mycelial extracts (Figure 5, top right). The components were identified by matching HPLC retention times and LRESI mass spectra of preparatively isolated HPLC fractions with the compounds isolated from the broth extracts. Importantly, quantitative HPLC analyses showed that the estimated amount of coprogens in mycelial extracts was >100-fold less than that of extracellular coprogens (see Supplemental Table 3 online). Ferricrocin, an intracellular siderophore, was identified similarly (Figure 5, bot-

tom right) by cochromatography with an authentic standard and by LRESI-MS analyses of the preparatively isolated component with an HPLC retention time matching that of authentic ferricrocin (see Supplemental Methods online).

Estimates of mycelial siderophore content showed that the coprogens as well as ferricrocin were present in wild-type cells, whereas only ferricrocin was detectable in the *C. heterostrophus* $\Delta nps6$ strain (see Supplemental Table 3 online). Ferricrocin cell content was higher in the mutant strain than in the wild type, indicating that the NRPS encoded by *NPS6* is not involved in ferricrocin biosynthesis.

Siderophore production of the wild-type and $\Delta nps6$ strains of *A. brassicicola* and *F. graminearum* was examined essentially in the same way as for *C. heterostrophus*. HRESI-MS data (see Supplemental Methods online) suggested *N*^α-dimethylcoprogen, previously identified as a siderophore from *Alternaria longipes* (Jalal et al., 1988), as the major extracellular siderophore of *A. brassicicola*. Triacetyl fusarinine C (Diekmann and Krezdorn, 1975; Hossain et al., 1987) was definitively identified as the major extracellular siderophore of *F. graminearum* by HRESI-MS and cochromatography with an authentic standard (Figure 6; see Supplemental Methods online). The *A. brassicicola* and *F. graminearum* $\Delta nps6$ strains were defective in the production of these siderophores (see Supplemental Figure 8 online), further demonstrating the role of *NPS6* in extracellular siderophore biosynthesis and its functional conservation.

Exogenous Application of Iron Enhances or Restores the Virulence of the $\Delta nps6$ Strains to the Hosts

To test whether the increased sensitivity of the $\Delta nps6$ strains to iron depletion causes the defect in colonization in planta, the virulence of the $\Delta nps6$ strains to their hosts supplied with iron was examined. Exogenous application of ferric citrate enhanced the virulence of *C. heterostrophus* $\Delta nps6$ strains to maize, compared with that of the same strain without iron application (Figures 7A and 7B). Application of Fe(III) EDTA restored the virulence ability of the *C. miyabeanus* $\Delta nps6$ strain to rice (Figures 7C and 7D).

Similarly, *F. graminearum* $\Delta nps6$ strains performed more like wild-type strains when iron was applied. *F. graminearum* $\Delta nps6$ strains developed symptoms more quickly on wheat spikes pretreated with ferric citrate than on untreated spikes (Figures 8A and 8B), and the kernels in spikes pretreated with iron were more severely damaged compared with those in the spikes not pretreated (Figure 8B). Application of iron did not significantly affect the virulence ability of wild-type strains (see Supplemental Figure 9 online).

Exogenous application of iron also restored the wild-type virulence of the *A. brassicicola* $\Delta nps6$ strains to *Arabidopsis* (Figures 9A, top two panels, and 9B [cf. water control and ferric citrate applications]). There was a minor amount of stress on *Arabidopsis* caused by the application of ferric citrate (Figure 9A, second panel from top, white arrows); however, these symptoms were easily distinguished from symptoms caused by fungal infection (Figure 9A, second panel from top, red arrow).

Overall, these data demonstrate that the reduced virulence of $\Delta nps6$ strains is at least partly attributable to their defect in iron gathering and the resulting starvation for iron in planta.

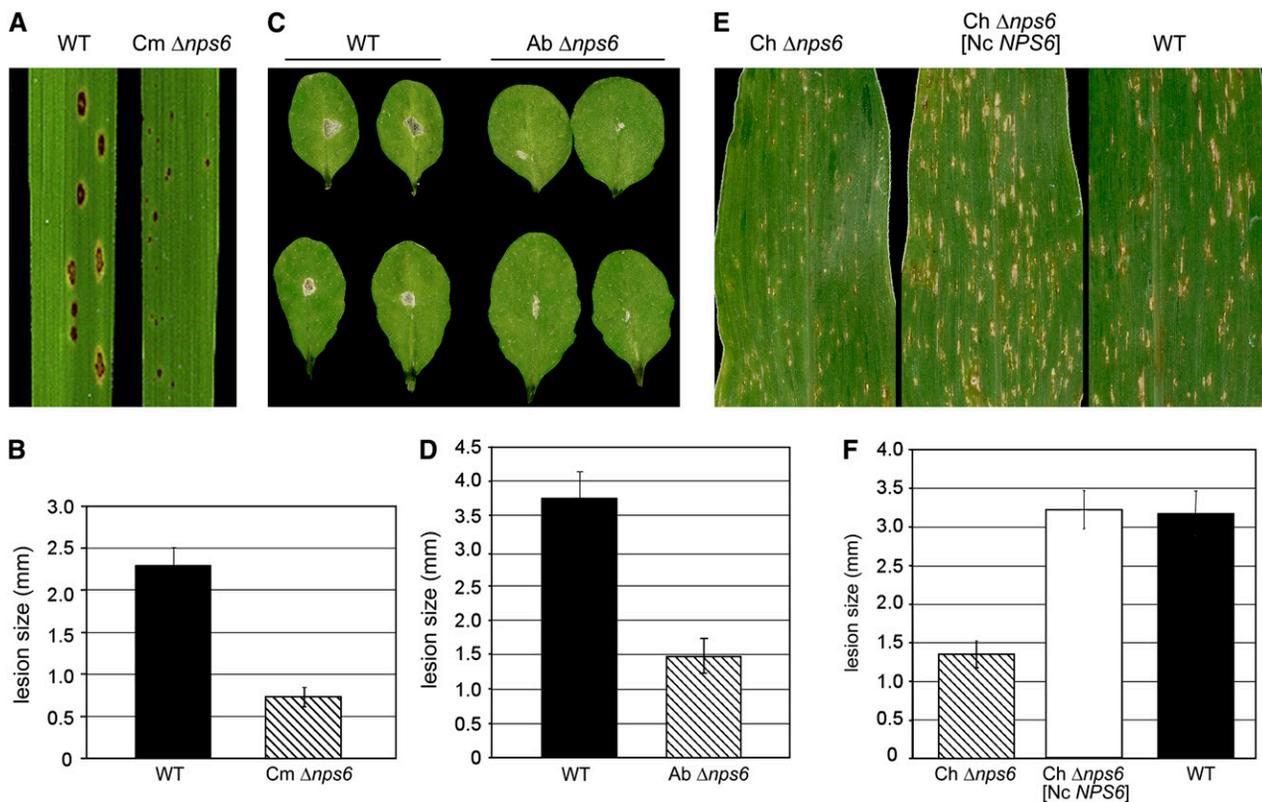


Figure 2. The Function of NPS6 Is Conserved among Filamentous Ascomycetes.

(A) and (B) Deletion of *NPS6* in *C. miyabeanus* leads to reduction in virulence to rice.

(A) Third true leaves of rice cv Nipponbare inoculated with the wild-type or $\Delta nps6$ strain (*Cmnp6-2*) of *C. miyabeanus*, 5 d after inoculation.

(B) Average length of lesions formed by wild-type and *C. miyabeanus* $\Delta nps6$ strains. Error bars indicate 95% confidence intervals. A statistically significant difference ($P < 0.01$) in size was detected (lesion size [mm]: wild type = 2.30 ± 0.21 ; $\Delta nps6$ = 0.73 ± 0.11).

(C) and (D) Deletion of *NPS6* in *A. brassicicola* causes reduction in virulence to *Arabidopsis* ecotype Di-G.

(C) Second (top row) and third (bottom row) pairs of true leaves of *Arabidopsis* ecotype Di-G inoculated with the wild-type or $\Delta nps6$ (*Abnps6-9*) strain of *A. brassicicola*, 4 d after inoculation.

(D) Average vertical lengths of lesions formed by wild-type and $\Delta nps6$ strains. Error bars indicate 95% confidence intervals. A statistically significant difference in size ($P < 0.01$) was observed (lesion size [mm]: wild type = 3.78 ± 0.38 ; $\Delta nps6$ = 1.48 ± 0.25).

(E) and (F) Introduction of the *NPS6* ortholog of *N. crassa* to the $\Delta nps6$ strain of *C. heterostrophus* restores wild-type virulence to maize.

(E) Third true leaves of maize cv W64A-N cytoplasm infected by $\Delta nps6$ (*Chnps6-1*), *C. heterostrophus* $\Delta nps6$ carrying *N. crassa* *NPS6* (*Chnps6-1 [NcNPS6-1]*), and wild-type *C. heterostrophus*, 5 d after inoculation.

(F) Average vertical lengths of lesions formed by each strain. Error bars indicate 95% confidence intervals. No significant difference in size was observed between the lesions formed by wild-type and *Chnps6*[*NcNPS6-1*] strains (lesion size [mm]: wild type = 3.18 ± 0.30 ; *C. heterostrophus* $\Delta nps6$ = 1.35 ± 0.18 ; *Chnps6*[*NcNPS6-1*] = 3.23 ± 0.25).

Extracellular Siderophores Play a Role in Fungal Virulence to Plant Hosts

The roles of siderophores and iron in fungal virulence to plants were further investigated by examining the virulence of the *A. brassicicola* $\Delta nps6$ strains on *Arabidopsis* treated with siderophores.

In the first experiment, we tried application of desferrioxamine (DFO). DFO is a commercially available siderophore (Desferal; deferoxamine) produced by various species of bacteria (Meyer and Abdallah, 1980). Certain fungi, such as *Saccharomyces cerevisiae* and *Candida albicans*, can use DFO to acquire extracellular iron, although they themselves do not produce this

siderophore (Howard, 2004). Through in vitro characterization of the *A. brassicicola* $\Delta nps6$ strains growing under iron-depleted conditions generated by 2DP, we found that *A. brassicicola*, but not the other species tested, could use DFO (see Supplemental Figure 10 online, bottom row [cf. right and middle plates]).

With the in vitro data as background, we next asked whether DFO could restore the wild-type virulence ability of the *A. brassicicola* $\Delta nps6$ strain to *Arabidopsis*. Both iron-saturated and nonsaturated forms of DFO restored the ability of the $\Delta nps6$ strains to infect *Arabidopsis* as successfully as the wild-type strain (Figure 9A, cf. top row, middle leaves with bottom two rows, middle leaves). No significant difference was observed in

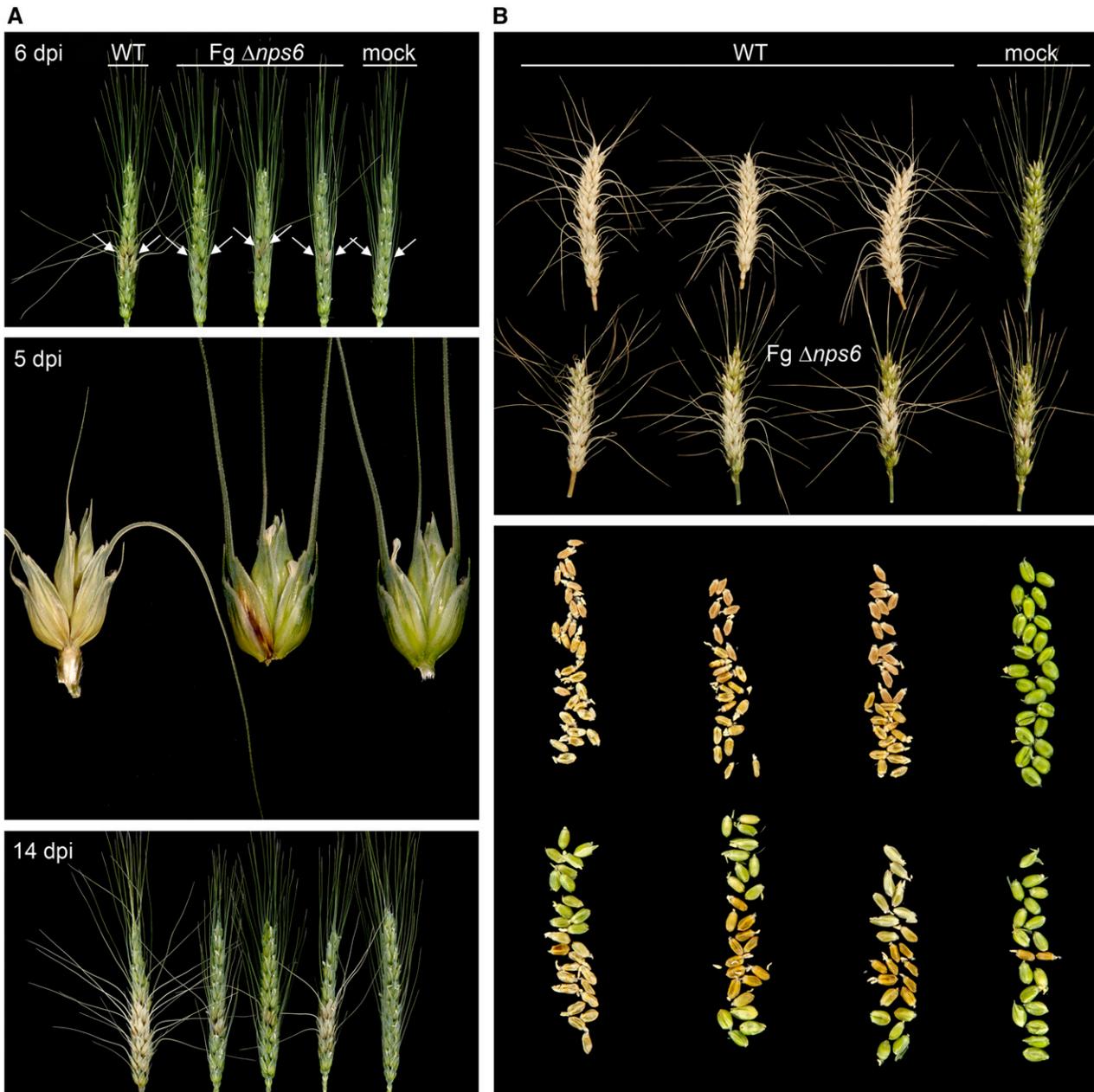


Figure 3. Deletion of *NPS6* in *F. graminearum* Leads to a Reduction in Virulence to Wheat.

(A) Symptom development of wheat spikes in cv Norm point-inoculated with wild-type and $\Delta nps6$ strains (Fgnps6-3-2 and Fgnps6G1-2) of *F. graminearum* shown at 6 d (top panel) and 14 d (bottom panel) after inoculation. The mock control was inoculated with water. Arrows in the top panel indicate inoculation points. The middle panel shows close-up views of the spikelets to which inoculum was applied (i.e., eighth from the bottom), 5 d after inoculation. Left, wild type; middle, $\Delta nps6$; right, mock. Note that the spikelets inoculated with the wild-type strain are completely bleached, whereas disease is localized in those inoculated with the $\Delta nps6$ strain. In general, wheat spikes infected by $\Delta nps6$ strains developed symptoms slowly, compared with those infected by the wild-type strain, although some variation was observed among those challenged by the $\Delta nps6$ strains.

(B) Top panel, wheat spikes infected by the wild-type (top row, left three) or $\Delta nps6$ (bottom row) strains of *F. graminearum*, 20 d after inoculation. The mock control was inoculated with water. Bottom panel, kernels from the spikes shown in the top panel. The positions of the kernels correspond to the original positions on the spikes in the top panel. Generally, kernels in spikes infected by the wild-type strain were more severely damaged than those in spikes infected by the $\Delta nps6$ strains. Note also that most awns on the wheat heads inoculated with the wild-type strain were bent downward, whereas most of the upper awns on the heads inoculated with the mutant strains were still pointing upward, as on the mock-inoculated heads.

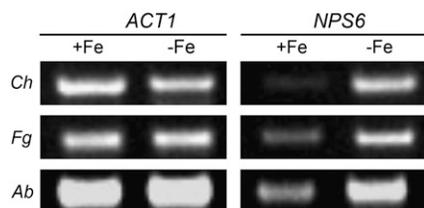


Figure 4. *NPS6* Is Upregulated under Iron Depletion.

Expression of *NPS6* is upregulated under iron-depleted (–Fe) compared with iron-replete (+Fe) conditions in *C. heterostrophus* (*Ch*), *F. graminearum* (*Fg*), and *A. brassicicola* (*Ab*). Expression of the control actin gene (*ACT1*) is not affected by the presence or absence of iron.

the ability of the *A. brassicicola* $\Delta nps6$ strains to cause wild-type virulence on plants treated with iron-saturated DFO versus nonsaturated DFO (Figure 9B). A subtle increase in virulence upon application of DFO was observed also for the wild-type strain, but there was no statistical difference at the 95% level of confidence.

Application of DFO caused minor stress on *Arabidopsis*, as observed with ferric citrate treatment (Figure 9A, bottom two rows), but these symptoms were easily distinguished from those caused by fungal infection.

Finally, we examined whether the actual siderophores produced by *A. brassicicola* wild-type strains could restore the

wild-type virulence of the $\Delta Abnps6$ strain to the host. As shown in Figures 9C and 9D, application of the Amberlite XAD-16 resin fraction from a culture of the *A. brassicicola* wild-type strain grown under iron-depleted conditions enhanced the virulence of the $\Delta nps6$ strain, whereas a similar fraction prepared from the $\Delta nps6$ strain itself did not. HPLC and HRESI-MS analyses indicated that the major metabolite in the wild-type XAD-16 resin fraction was *N* $^{\alpha}$ -dimethylcoprogen, biosynthesis of which is abolished in the $\Delta nps6$ strain (see Supplemental Figure 8 online). Virulence of the $\Delta nps6$ strain was reexamined with or without the application of purified *N* $^{\alpha}$ -dimethylcoprogen. Two hundred micromolar *N* $^{\alpha}$ -dimethylcoprogen fully restored the wild-type virulence of the $\Delta nps6$ strain to *Arabidopsis* (Figure 9D).

Together, these data demonstrate that reduction in the virulence of the $\Delta nps6$ strains is attributable to the loss of extracellular siderophore biosynthesis and that siderophores play a role in fungal virulence to plants.

DISCUSSION

NPS6: From Gene to the Extracellular Siderophores Biosynthesized by Its Encoded NRPS

NPS6, encoding an NRPS, was identified previously as a virulence determinant in *C. heterostrophus* (Lee et al., 2005). In this

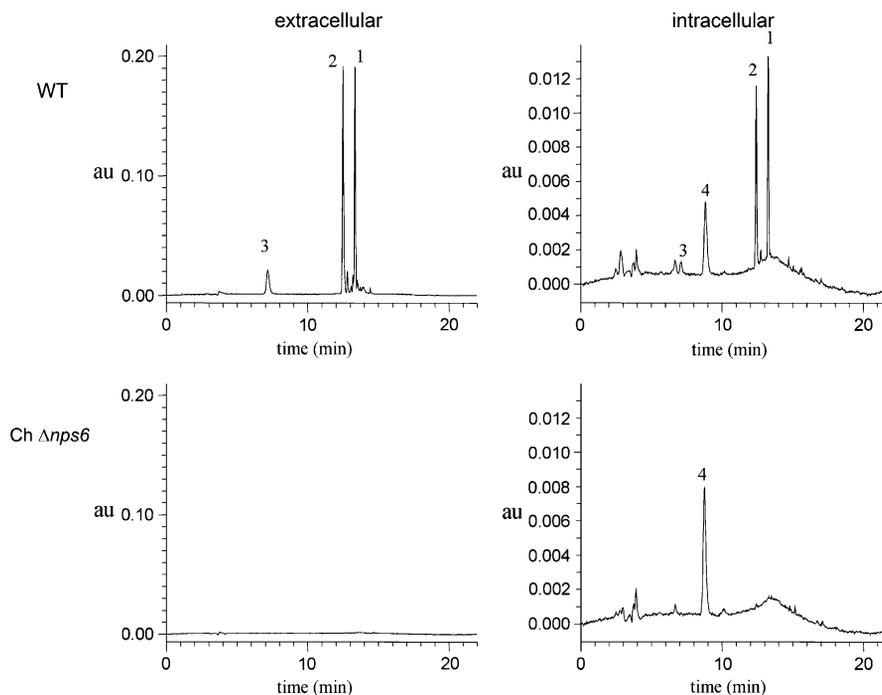


Figure 5. The NRPS Encoded by *C. heterostrophus* *NPS6* Is Responsible for the Biosynthesis of Extracellular Siderophores.

HPLC analyses of broth (left; extracellular) and mycelial (right; intracellular) extracts from cultures of wild-type and *C. heterostrophus* $\Delta nps6$ strains. Analyses within each column represent the same proportion of the total extract injected in a 10- μ L aliquot, 1-mL equivalent for the broth extracts and 10-mL equivalents for the mycelial extracts. Siderophore peaks are labeled as follows: coprogen (1), neocoprogen I (2), neocoprogen II (3), and ferricrocin (4).

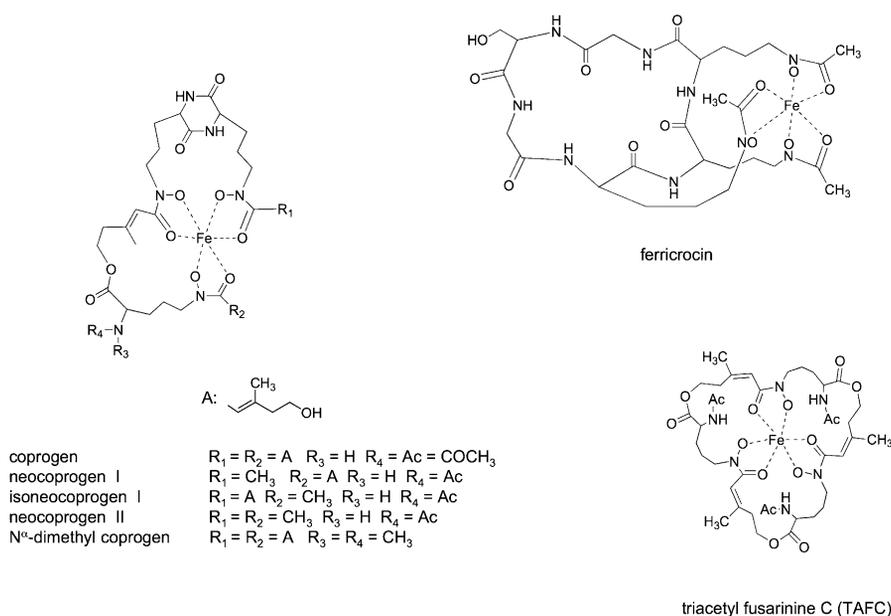


Figure 6. Chemical Structures of the Siderophores of *C. heterostrophus*, *A. brassicicola*, and *F. graminearum*.

study, we further characterized *NPS6* with the aim of revealing its actual role(s) in virulence and identifying the metabolite product of the NRPS it encodes. Starting from the *NPS6* gene, through detailed analyses of strains deleted for the gene, and expression studies, we learned that *NPS6* is involved in iron metabolism and we identified the products of the NRPS encoded by the *NPS6* gene as extracellular siderophores.

In our HPLC analyses, coprogens/fusarinines were detected in both broth and mycelial extracts; however, the estimated amount of intracellular, compared with extracellular, coprogens/fusarinines was negligible. Those detected in the mycelial extracts could come from culture broth, which may have contaminated the mycelial fraction during sample preparation. Alternatively, intracellular coprogens/fusarinines could be newly synthesized siderophores, caught before secretion to the extracellular space, or siderophores that had returned to the cell, carrying iron. Several mechanisms for the uptake of iron bound to siderophores have been reported in fungi (reviewed in Howard, 1999; Renshaw et al., 2002). According to one mechanism (known as the shuttle mechanism), intact siderophore–iron complexes enter cells and iron is then transferred from the extracellular to the intracellular siderophores. Note that we would expect to detect extracellular siderophores intracellularly if this mechanism were operational. This type of mechanism has been described for the uptake of the coprogen family of siderophores in *N. crassa* (Ernst and Winkelmann, 1977) and the fusarinine family of siderophores in *Aspergillus nidulans* (Haas et al., 2003). Overall, we propose that coprogens/fusarinines function as extracellular siderophores in *C. heterostrophus*, *A. brassicicola*, and *F. graminearum*. This report connects a *NPS* gene (*NPS6*) to the production of a coprogen/fusarinine-type siderophore and demonstrates that extracellular siderophores are involved in fungal virulence to plants.

Functional Conservation of *NPS6* among Filamentous Ascomycetes

Production of coprogens, one of the major families of hydroxamate-type siderophores (Renshaw et al., 2002), has been reported for diverse species of ascomycetes, including the close relative of *C. heterostrophus*, *Curvularia lunata* (teleomorph *Cochliobolus lunata*) (Hossain et al., 1987), several species of the sister genus, *Alternaria* (i.e., *A. alternata*, *A. longipes*, and *A. cassiae*) (Jalal et al., 1988; Jalal and van der Helm, 1989; Ohra et al., 1995), and also the model saprophyte *N. crassa* (Winkelmann and Huschka, 1987). Production of fusarinines has also been reported in species of diverse genera, including *Aspergillus*, *Penicillium*, *Fusarium*, and *Trichoderma* (summarized in Renshaw et al., 2002).

Consistent with the wide distribution of coprogen/fusarinine-type siderophores, *NPS6* is conserved among filamentous ascomycetes. As observed for *C. heterostrophus*, deletion of *NPS6* orthologs in the rice pathogen, *C. miyabeanus*, the wheat/maize/barley pathogen, *F. graminearum*, and the pathogen of cruciferous plants, *A. brassicicola*, resulted in a reduction in virulence to their respective hosts and sensitivity to reactive oxygen species and to low iron. In addition, introduction of the *NPS6* ortholog from the saprobe *N. crassa* restored wild-type virulence capability to the $\Delta nps6$ strain of *C. heterostrophus*. Together, these data demonstrate functional conservation of *NPS6* among diverse species of filamentous ascomycetes, including several pathogens and a saprobe.

Role of Extracellular Siderophores in Fungal Virulence to Plants

The role of siderophores in virulence to plants was first demonstrated for the bacterial pathogen *E. chrysanthemi*; disruption of

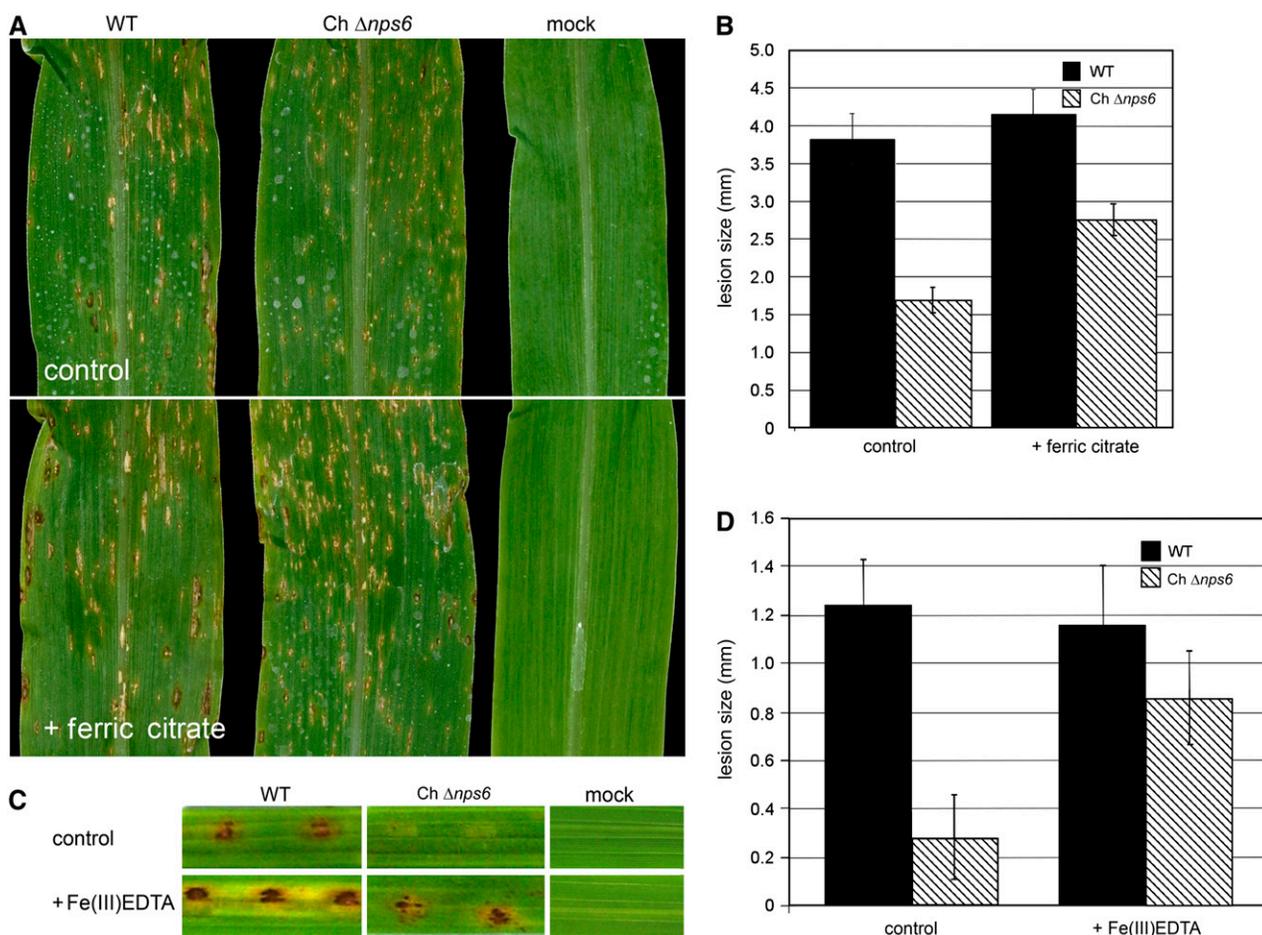


Figure 7. Exogenous Application of Iron Enhances the Virulence of $\Delta nps6$ Strains to Each Host.

(A) and **(B)** Application of ferric citrate partially restores the virulence of the *C. heterostrophus* $\Delta nps6$ strain to maize.

(A) Third true leaves of maize cv W64A-N inoculated with the wild-type or $\Delta nps6$ (*Chnps6-1*) strains of *C. heterostrophus* without (top row) or with (bottom row) 1 mM ferric citrate, 5 d after inoculation. Mock control (right leaves, both panels) were treated with 0.02% Tween 20 solution without (top row) or with (bottom row) ferric citrate.

(B) Average vertical lengths of lesions are shown for each strain and for each treatment. Error bars show 95% confidence intervals. Application of iron led to a statistically significant enhancement of virulence of the $\Delta nps6$ strain ($P < 0.05$) (lesion size [mm]: $\Delta nps6 = 1.68 \pm 0.17$; $\Delta nps6$ with iron = 2.75 ± 0.21).

(C) and **(D)** Application of Fe(III) EDTA enhances the virulence of the *C. miyabeanus* $\Delta nps6$ strain to rice.

(C) Rice leaves drop-inoculated with wild-type (left) and $\Delta nps6$ (*Cmnps6-2*) strains of *C. miyabeanus*, with and without exogenous application of 0.5 mM Fe(III) EDTA. A mock-inoculated control is shown at right.

(D) Average vertical lengths of lesions formed by the wild-type or $\Delta nps6$ strains with or without iron treatment. Error bars indicate 95% confidence intervals. A statistically significant change in the virulence of the $\Delta nps6$ strain was observed when iron was supplied ($P < 0.05$).

the siderophore chrysoactin-mediated iron transport system led to a reduction in virulence to African violet (*Saintpaulia ionantha*) (Enard et al., 1988). Recently, a second siderophore in *E. chrysanthemi*, achromobactin, was also demonstrated to play a role in virulence (Franza et al., 2005). DFO B, a siderophore produced by the closely related apple pathogen *E. amylovora*, is essential for the successful infection of apple flowers (Dellagi et al., 1998). By contrast, siderophores seem not to play any essential role in virulence in another closely related species, *E. carotovora* subsp. *carotovora*, or in the crown gall pathogen *Agrobacterium tumefaciens* (Leong and Neilands, 1981; Bull

et al., 1996), indicating differences in the role of siderophores in plant-microbe interactions among different pathosystems.

As mentioned in the Introduction, the initial attempt to elucidate the role of siderophores in fungal virulence to plants was made with the basidiomycete maize pathogen, *U. maydis* (Mei et al., 1993). In this study, a mutant defective in L-ornithine N5-monooxygenase, the enzyme responsible for the first committed step of fungal siderophore biosynthesis, was as virulent to maize as the wild type, demonstrating that the siderophores by themselves do not play an essential role in the virulence of *U. maydis*. A recent report on another plant pathogenic

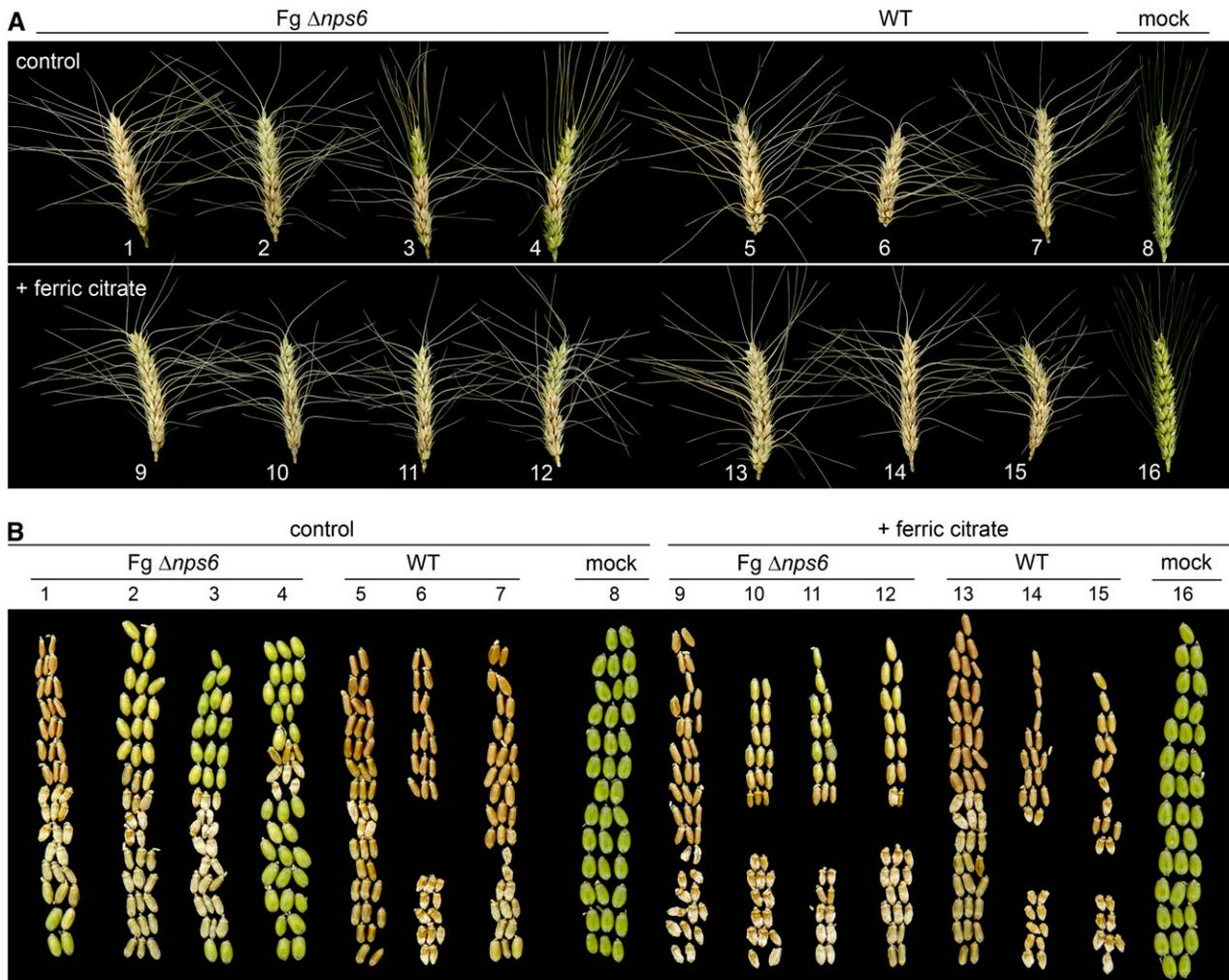


Figure 8. Application of Ferric Citrate Enhances the Virulence of the *F. graminearum* $\Delta nps6$ Strain to Wheat.

(A) Wheat spikes inoculated with $\Delta nps6$ strain Fg $nps6$ -3-2 or the wild-type strain of *F. graminearum*, with or without application of 1 mM ferric citrate, 20 d after inoculation. Mock-inoculated controls are shown at right in both rows.

(B) Kernels from the spikes shown in **(A)**. Numbers correspond to those in **(A)**. Blanks indicate that the kernels were not recovered as a result of the severity of infection.

basidiomycete, *Microbotryum violaceum*, also noted that rhodotorulic acid, the hydroxamate-type siderophore produced by this species, does not contribute to virulence on *Silene latifolia* (Birch and Ruddat, 2005). By contrast, this study demonstrates that the siderophores produced by the NRPS encoded by *NPS6* play a role in virulence in at least four different species of plant pathogenic ascomycetes.

The $\Delta nps6$ strain of *C. heterostrophus* shows reduced pigmentation, indicating reduction in the accumulation of 1,8-dihydroxynaphthalene (DHN)-melanin, which is responsible for the dark green color of this species. A similar phenotype was also observed for the $\Delta nps6$ strains of *C. miyabeanus* and *A. brassicicola* (data not shown). DHN-melanin plays an essential role in virulence in certain species of phytopathogenic fungi (Chumley and Valent, 1990; Kubo et al., 1991; Perpetua et al., 1996). Previous studies, however, clearly demonstrated that

DHN-melanin does not contribute to the virulence of *C. heterostrophus*, *C. miyabeanus*, or *A. alternata*, a close relative of *A. brassicicola* (Guillen et al., 1994; Kawamura et al., 1999; Moriwaki et al., 2004); hence, the reduced virulence of the $\Delta nps6$ strains is not likely attributable to their reduction in pigmentation.

Our observations of in planta growth of the *C. heterostrophus* $\Delta nps6$ strains show that these strains are defective in colonizing plants, compared with wild-type strains, but are normal in terms of germination of asexual spores, prepenetration growth, and penetration. Hypersensitivity of the $\Delta nps6$ strains to low iron and the strong iron-chelating activity of siderophores prompted us to hypothesize that extracellular siderophores of plant pathogenic ascomycetes contribute to colonization in planta by supplying an essential nutrient, iron, to their producers. Supporting this idea, exogenous application of iron restored the virulence of the $\Delta nps6$

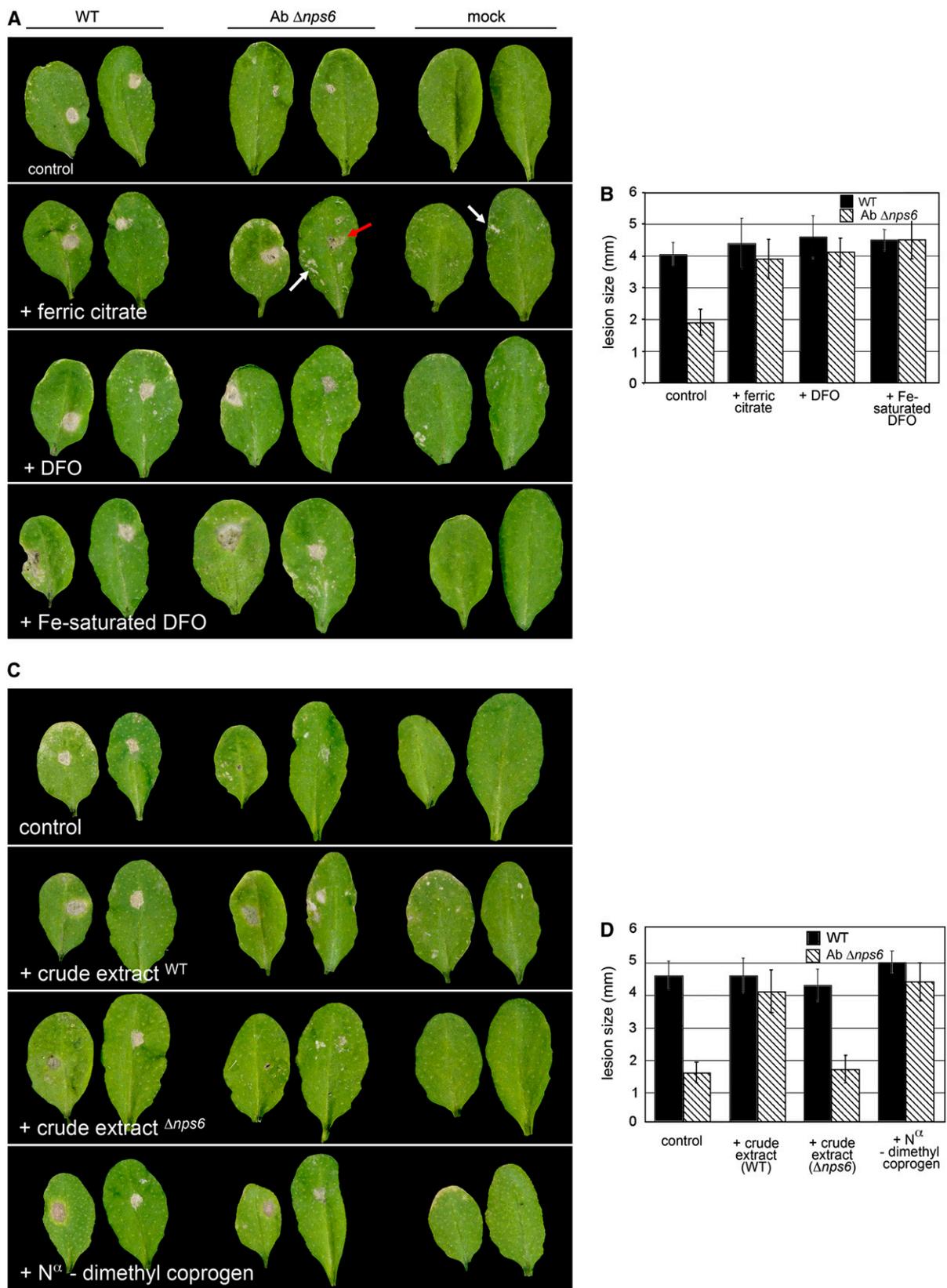


Figure 9. Application of Iron or Siderophores Restores the Virulence of the *A. brassicicola* $\Delta nps6$ Strain to the Host.

strains to each host in all of the pathosystems tested. One idea competing with our hypothesis is that siderophores act as phytotoxins. Previously characterized NRPSs involved in fungal virulence to plants (e.g., the NRPSs encoded by *HTS1* in race 1 of *C. carbonum*, *AMT1* in the apple pathotype of *A. alternata*, and *Esyn1* in *F. scirpi* and some other *Fusarium* species) are all responsible for the production of phytotoxins. Phytotoxicity of fungal siderophores was reported in a study of *A. cassiae*, in which coprogen and ferricrocin, the siderophores it produces, caused necrosis on cowpea (*Vigna unguiculata*) leaves in a leaf-wounding assay (Ohra et al., 1995). These data showed that ferricrocin had much higher phytotoxicity to cowpea than did coprogen. Ferricrocin has been reported as an intracellular siderophore in *N. crassa* and *A. nidulans* (Charlang et al., 1981), and it appears from initial HPLC and MS analyses that ferricrocin is an intracellular siderophore of *A. brassicicola*, a close relative of *A. cassiae*. The conserved intracellular locale of ferricrocin among ascomycetes suggests that it is also likely to be an intracellular siderophore in *A. cassiae*, raising doubts about its role as a phytotoxin. Although coprogen showed weak phytotoxicity when applied at 10 mM, the highest concentration tested by Ohra et al. (1995), we question whether this concentration is physiologically relevant in nature (for discussion, see Walton, 1996). It is important to note that no significant difference was observed between iron-saturated and nonsaturated DFO in their restoration of virulence to the *A. brassicicola* $\Delta nps6$ strain. The former is not likely to scavenge iron from the host, because it was saturated with iron. Collectively, we propose that the role of the extracellular siderophores in the virulence of plant pathogenic ascomycetes is to supply iron to pathogens for their survival in hosts, but not to destroy host cells as a phytotoxin.

Siderophore-Mediated Iron Metabolism, Oxidative Stress, and Virulence

Coupling of siderophore-mediated iron metabolism, oxidative stress, and virulence has been reported in a study of the bacterial

plant pathogen *E. amylovora*, in which the *E. amylovora* mutant deficient in DFO B siderophore biosynthesis showed reduced virulence to apple in a flower infection assay (Dellagi et al., 1998). The protective effect of the iron-free form of DFO B to the bacteria against H_2O_2 was observed in vitro. A study by Subramoni and Sonti (2005) on a *fur* (for *ferric uptake regulator*) mutant of *Xanthomonas oryzae* pv *oryzae* is another example of a connection among siderophore-mediated iron metabolism, oxidative stress, and virulence, although the observations are the opposite of those reported for *E. amylovora* (Dellagi et al., 1998). The *fur* mutant of *X. oryzae* pv *oryzae*, which overproduces extracellular siderophores, showed, concomitantly, a reduction in virulence to rice and increased sensitivity to H_2O_2 .

As we reported previously (Lee et al., 2005), deletion of *NPS6* leads to a reduction in virulence to maize and hypersensitivity to H_2O_2 in *C. heterostrophus*. In this study, we found that the $\Delta nps6$ strain also showed hypersensitivity to the superoxide generator KO_2 . These phenotypes were also observed in the $\Delta nps6$ strains of the other three species tested. Interestingly, exogenous application of ferric citrate enhanced tolerance to reactive oxygen species of the $\Delta nps6$ strains of *A. brassicicola*, *C. heterostrophus*, and *F. graminearum* (see Supplemental Figure 11A online, for *A. brassicicola*), suggesting that hypersensitivity of the $\Delta nps6$ strains is not likely to be explained by the Fenton reaction. As described in the Introduction, the Fenton reaction is catalyzed by intracellular free iron; hence, application of iron would be expected to promote the reaction. Both iron-saturated and non-saturated DFO restored the wild-type level of tolerance to reactive oxygen species to the $\Delta nps6$ strain of *A. brassicicola*, further demonstrating the connection between oxidative stress and siderophore-mediated iron metabolism in filamentous ascomycetes (see Supplemental Figure 11B online). Although still unsettled, direct antifungal activity of reactive oxygen species generated in planta has been proposed in several studies (Vanacker et al., 2000; De Rafael et al., 2001). Our initial attempts to characterize a protective role of fungal siderophores against reactive oxygen species in planta were inconclusive. The $\Delta nps6$

Figure 9. (continued).

(A) and (B) Exogenous application of ferric citrate or DFO enhances the virulence of $\Delta nps6$ strains of *A. brassicicola* to *Arabidopsis*.

(A) Second and third true leaves, in pairs, of *Arabidopsis* ecotype Di-G drop-inoculated with the wild-type strain and $\Delta nps6$ strain Abnps6-9 of *A. brassicicola*, 4 d after inoculation. Pairs of mock-inoculated control leaves are shown at right in each row. Plants were pretreated with 0.5 mM ferric citrate, 0.25 mM DFO, or 0.25 mM iron-saturated DFO. Iron and DFO were dissolved in 1 g/L Glucopon 215 CS UP solution, and control plants (top row) were pretreated with Glucopon 215 CS UP solution alone.

(B) Average vertical lengths of lesions formed by wild-type and $\Delta nps6$ strains of *A. brassicicola* are shown for each treatment. A statistically significant enhancement in virulence of the *A. brassicicola* $\Delta nps6$ strain was observed when iron, DFO, or iron-saturated DFO (right) was applied ($P < 0.01$). Error bars indicate 95% confidence intervals (lesion size [mm]: $\Delta nps6 = 1.90 \pm 0.41$; wild type = 4.05 ± 0.37 ; $\Delta nps6$ with iron = 3.90 ± 0.62 ; $\Delta nps6$ with DFO = 4.1 ± 0.46 ; $\Delta nps6$ with iron-saturated DFO = 4.5 ± 0.37).

(C) and (D) Application of N^α -dimethylcoprogen, the extracellular siderophore produced by *A. brassicicola*, restores wild-type virulence to *Arabidopsis* to the $\Delta nps6$ strain.

(C) Second and third true leaves, in pairs, of *Arabidopsis* ecotype Di-G infected by the wild-type strain and $\Delta nps6$ strain Abnps6-9 of *A. brassicicola*, 4 d after inoculation. Mock-inoculated controls are shown at right in all rows. Plants were pretreated with crude extracts prepared from cultures of wild-type and $\Delta nps6$ strains grown under iron-depleted conditions or 0.2 mM N^α -dimethylcoprogen purified from the crude extract of the wild-type culture. Control plants were pretreated with 1 g/L Glucopon 215 CS UP solution.

(D) Average vertical lengths of lesions formed by wild-type and *A. brassicicola* $\Delta nps6$ strains are shown for each treatment. Application of crude extract from wild-type culture or N^α -dimethylcoprogen led to a statistically significant enhancement in virulence of the *A. brassicicola* $\Delta nps6$ strain ($P < 0.01$). Error bars indicate 95% confidence intervals (lesion size [mm]: $\Delta nps6$ with wild-type crude extract = 4.00 ± 0.65 ; $\Delta nps6$ with $\Delta nps6$ crude extract = 1.60 ± 0.43 ; $\Delta nps6 = 1.50 \pm 0.33$; wild type = 4.50 ± 0.44 ; $\Delta nps6$ with siderophore = 4.3 ± 0.59).

strain of *C. miyabeanus* showed enhanced virulence to rice plants pretreated with the NADPH oxidase inhibitor diphenyleneiodonium (W. Moeder and K. Yoshioka, unpublished data). On the other hand, treatment with diphenyleneiodonium did not change the virulence of the *A. brassicicola* $\Delta nps6$ strain to *Arabidopsis*. A *rboh* D/F double mutant of *Arabidopsis*, defective in NADPH oxidase, shows enhanced susceptibility to the wild-type strain of *A. brassicicola*, indicating that reactive oxygen species, indeed, play a role in host defense against this pathogen (W. Moeder and K. Yoshioka, unpublished data). The *Arabidopsis rboh* D/F mutant, however, did not show any enhanced susceptibility to the *A. brassicicola* $\Delta nps6$ strain, raising a question about the direct connection between hypersensitivity to reactive oxygen species and the reduction in virulence of the $\Delta nps6$ strains. Further investigation is required to draw a conclusion regarding the role of siderophore/siderophore-mediated iron metabolism in virulence through the protection of fungal pathogens against reactive oxygen species generated in planta.

METHODS

Fungal Strains, Plant Materials, and General Growth Conditions

The following fungal strains were used: *Cochliobolus heterostrophus* C4 [MAT1-2; American Type Culture Collection 48331], *Cochliobolus miyabeanus* WK-1C (American Type Culture Collection 44560), *Alternaria brassicicola* Tf383 (a kind gift from Hiroshi Otani, Tottori University), *Fusarium graminearum* (*Gibberella zeae*) Gz3639 (Northern Regional Research Laboratory 29169), and *Neurospora crassa* 74-OR23-1A (Fungal Genetics Stock Center 987). Unless mentioned otherwise, all strains were grown on CM (Leach et al., 1982) at 24°C under continuous fluorescent light. To induce asexual sporulation, *C. heterostrophus* and *C. miyabeanus* were grown on CMX (Tzeng et al., 1992) and *F. graminearum* was grown on CM without salts. Fungal strains used for this work are listed in Supplemental Table 4 online.

The following plant cultivars were used: *Arabidopsis thaliana* ecotype Di-G (ABRC stock number CS910), *Oryza sativa* cv Nipponbare, *Triticum aestivum* cv Norm (spring wheat; a generous gift from Gary Bergstrom, Cornell University), and *Zea mays* cv W64A-N. Wheat was grown in a greenhouse in 14 h of light/10 h of dark at 25°C. Maize, rice, and *Arabidopsis* were grown in a growth chamber with light regimens of 16 h of light/8 h of dark at 24°C, 16 h of light/8 h of dark at 29°C, and 14 h of light/10 h of dark at 22°C, respectively.

DNA Manipulations, Fungal Transformation, and Crosses

Fungal genomic DNA was extracted from mycelia ground in liquid nitrogen as described previously (Edwards et al., 1991). PCR amplification was performed with PCR master mix (Promega), according to the manufacturer's recommendations.

Orthologs of the *C. heterostrophus* and *F. graminearum* *NPS6* genes were amplified by PCR from *C. miyabeanus* and *A. brassicicola* genomic DNA using the following degenerate primer pair, 5'-TTYGARAARTCING-3' and 5'-GAYACGYTGTCRCGIAGSTTGA-3', corresponding to the first adenylation domain of the NRPS encoded by Ch *NPS6*. PCR products of ~1 kb were cloned and sequenced. The sequences of *C. miyabeanus* and *A. brassicicola* *NPS6* were further extended by inverse PCR (Ochman et al., 1988). For targeted deletion of each *NPS6* gene, split marker constructs were prepared, as described by Catlett et al. (2003) and in Supplemental Figure 3 online. All constructs contained ~2-kb homologous

sequence in total. For *C. heterostrophus*, the *NPS6* open reading frame was deleted completely. For *C. miyabeanus* and *A. brassicicola*, portions of *NPS6* open reading frame (380 and 312 bp, respectively) corresponding to the first thiolation domain (Lee et al., 2005) of the NRPS encoded by Ch *NPS6* were deleted. For *F. graminearum*, the *NPS6* open reading frame was deleted both completely and partially. In the latter case, a portion of the *NPS6* open reading frame (1060 bp), corresponding to part of the adenylation domain, the first thiolation domain, and part of the first condensation domain, was deleted. Complete deletion of the *F. graminearum* *NPS6* open reading frame was performed with the *hygB* gene as a drug resistance marker, and partial deletion was performed with the *nptII* gene, which confers resistance to G418. *nptII* under the control of the *Aspergillus nidulans* *trpC* promoter and terminator was amplified by PCR from pII99 (a generous gift from S.-H. Yun, Seoul National University). 5' and 3' flanking sequences of the target region were amplified by PCR and fused to the PCR product described above containing *nptII* by fusion PCR. Approximately 5 μ g of the resulting PCR product (5' flank-*nptII*-3' flank) was used for gene deletion. Deletion of *NPS6* in transformants was confirmed by PCR as described in the Supplemental Methods online (see Supplemental Figure 3 and Supplemental Table 2 online).

A construct for reintroduction of the wild-type Ch *NPS6* allele into the $\Delta nps6$ strain of *C. heterostrophus* was prepared as described in the Supplemental Methods online. A PCR fragment carrying the wild-type *C. heterostrophus* *NPS6* allele was cotransformed into the $\Delta nps6$ strain of *C. heterostrophus* along with p314. p314 (*C. Schoch* and B.G. Turgeon, unpublished data) is a plasmid carrying the *Streptomyces noursei* *NAT* gene, which encodes nourseothricin *N*-acetyl transferase, under the control of the *A. nidulans* *trpC* promoter (Cullen et al., 1987) and confers resistance to the antibiotic nourseothricin (clonNAT; Werner BioAgents) when expressed in *Cochliobolus* species. Procedures for screening of transformants are described in the Supplemental Methods online (see Supplemental Figure 3 and Table 2 online).

Procedures for fungal transformation and crosses are described in the Supplemental Methods online.

Examination of Asexual Sporulation In Vitro

Asexual spore (conidial) germination of *C. heterostrophus* was examined by suspending spores of strains C4, Chnps6-1 ($\Delta nps6$), and Chnps6-3 ($\Delta nps6$), grown on CMX medium, at a concentration of 10^5 /mL in water, then 300 μ L of each suspension was incubated (in a 1.5-mL tube) at 30°C for 1, 2, or 3 h. A drop of spore suspension was transferred to the well of a glass slide (catalog number 71878-04; Electron Microscopy Sciences), and the number of germinated spores was recorded at each time point (50 spores/sample) using bright-field optics.

Virulence Assays

For evaluation of the virulence of *C. heterostrophus*, 3-week-old W64A-N cytoplasm maize plants (three plants per assay) were spray-inoculated with 2 mL of spore suspension (5×10^4 /mL) in 0.02% Tween 20. Inoculated plants were kept in a mist chamber for 24 h, then further incubated in a growth chamber for 4 d under 16 h of light/8 h of dark at 24°C. Virulence was evaluated at 5 d after inoculation. For statistical treatment, see Statistical Analyses below. The virulence of *C. miyabeanus* was evaluated similarly, except for the following. For the infection assay of *C. miyabeanus*, 3-week-old rice plants (three plants per assay) were spray-inoculated with 5 mL of spore suspension (10^4 /mL) containing 0.1% gelatin. For the infection assay of *A. brassicicola*, second and third pairs of true leaves of 25-d-old *Arabidopsis* ecotype Di-G (six plants per assay) were drop-inoculated with 5 μ L of spore suspension (1×10^5 /mL) and 0.05% Tween 20. Inoculated plants were kept in a mist chamber for 24 h, then further incubated in a growth chamber for an additional 3 d.

During incubation in the growth chamber, the plants were kept in a moist plastic box to keep humidity high. Virulence was evaluated at 4 d after inoculation.

Point-inoculation of wheat spikes at anthesis was performed according to the method of Pritsch et al. (2001) with minor modifications. Briefly, 5 μ L of spore suspension (10^5 /mL) in water was placed between the palea and lemma of two basal florets of seventh and eighth spikelets from the bottom of wheat spikes (5 to 10 per assay). Control plants were mock-inoculated with water without fungal spores. The inoculated plants were incubated in a mist chamber for 24 h, then further incubated in a growth chamber for an additional 19 d. Symptoms were visually evaluated daily, and the number of completely bleached spikes was recorded for each spike. For statistical treatment, see Statistical Analyses below. Twenty days after inoculation, kernels were taken from each spike and their symptoms were evaluated visually, as described by Voigt et al. (2005).

Experiments were repeated at least three times for each pathosystem.

Complementation of the *C. heterostrophus* $\Delta nps6$ Strain with *N. crassa* NPS6

A construct for introduction of the wild-type *N. crassa* NPS6 allele into the $\Delta nps6$ strain of *C. heterostrophus* was prepared as described in the Supplemental Methods online. Protoplasts of the *C. heterostrophus* $\Delta nps6$ strain (*nps6*; *hygB^R*) were cotransformed with the pair of split constructs (which overlap in the *N. crassa* NPS6 open reading frame) described in the Supplemental Methods online and p314 carrying the *NAT* gene. *NAT^R* transformants were examined for their sensitivity to hygromycin B, which would indicate replacement of the *hygB* gene in the *C. heterostrophus* $\Delta nps6$ strain with the wild-type *N. crassa* NPS6 gene (see Supplemental Figure 6 online). Correct integration of *N. crassa* NPS6 at the native *C. heterostrophus* NPS6 locus was confirmed by PCR in *NAT^R*; *hygB^S* transformants, as described in Supplemental Figure 6 online. Virulence and sensitivity to H_2O_2 of *C. heterostrophus* $\Delta nps6$ strains carrying *N. crassa* NPS6 were examined as described above. Finally, cosegregation of *N. crassa* NPS6 and restoration of the wild-type phenotype were confirmed by analyzing the progeny of crosses between strain ChNcNPS6-1 (*nps6*; *N. crassa* NPS6; *NAT^R*; *MAT1-2*) and strain Chnps6-1R2 (*nps6*; *hygB^R*; *MAT1-1*).

Construction of Strains for the Observation of Fungal Growth in Planta

C. heterostrophus wild-type strain C4 and $\Delta nps6$ strains, both carrying GFP, were prepared as follows. Strain C4 was transformed with pIGPAPA (Horwitz et al., 1999) carrying *hygB* and sGFP, the latter under the control of the *N. crassa* isocitrate lyase gene promoter. *HygB^R* transformants were examined microscopically for fluorescence. *C. heterostrophus* strain C4pG-1 (*GFP+*; *hygB^R*; *MAT1-2*) was crossed to strain Chnps6-1R2 (*nps6*; *hygB^R*; *MAT1-1*), and *hygB^R* progeny were isolated and screened for *GFP*. *GFP+*; *hygB^R*; *nps6* progeny were identified by microscopic examination for GFP fluorescence and by PCR confirmation of the deletion of *C. heterostrophus* NPS6 as described in the Supplemental Methods online (see Supplemental Figure 3 and Supplemental Table 2 online).

Strains C4pG-1 and 1504-T1-4 (*nps6*; *hygB^R*; *GFP+*) were inoculated on maize independently, as described below. Infected plants were harvested at 24, 48, and 72 h after inoculation. Leaf pieces were cut out and observed microscopically using epifluorescence optics.

Stress Sensitivity Assays

Each strain was grown on solid MM with or without the stress agent. Sensitivity to each stress was scored by measuring the colony radius of 5-d-old cultures on plates with the stress agent. To evaluate the sensi-

tivity of the $\Delta nps6$ strains, three to four independent transformants were examined and the tests were repeated at least three times for each condition. All chemicals used in stress assays were purchased from Sigma-Aldrich.

Three different oxidants, H_2O_2 (3%), *tert*-butyl hydroperoxide (70%), and the superoxide generator KO_2 , were used. Final concentrations of these oxidants were 5, 0.5, and 10 mM, respectively. Iron-depleted conditions were created using an iron chelator, 2DP, at a final concentration of 50 μ M. Sodium nitrite was used to produce nitrosative stress (final concentration, 15 mM). Sensitivity to osmotic stress was examined using sorbitol (final concentration, 1 M). After the initial tests, sensitivities to H_2O_2 , KO_2 , and 2DP (iron depletion) were examined in detail and the MIC of each stress agent was determined as follows. Wild-type and $\Delta nps6$ strains were incubated on MM with twofold increasing concentrations of each stress agent, in the dark, for 4 d for *F. graminearum*, 5 d for *C. heterostrophus* and *C. miyabeanus*, and 7 d for *A. brassicicola*. For H_2O_2 , MM with 1, 2, 4, 8, and 16 mM H_2O_2 was prepared. For KO_2 , a fresh stock solution (1 M in water) was prepared for each assay. MM with 3, 6, 12, and 24 mM KO_2 was prepared for *C. heterostrophus* and *C. miyabeanus*, and MM with 2.5, 5, 10, 20, and 40 mM KO_2 was prepared for *A. brassicicola* and *F. graminearum*. For 2DP, a fresh stock solution (10 mM in water) was prepared for each assay, and MM with 25, 50, 100, 200, and 400 μ M 2DP was used. Growth of each strain on each plate was visually examined, and the concentration of a stress agent at which fungal growth was completely inhibited was recorded as the MIC of the stress agent to the strain. MIC was obtained for several independent $\Delta nps6$ strains, triplicate cultures were set up for each strain, and the experiments were repeated three times. *F. graminearum* $\Delta nps6$ strains show a growth defect on MM (see Supplemental Figure 5B online); however, this growth defect is complemented by supplying ferric citrate (for details, see below). MIC of each stress agent to *F. graminearum* wild-type and $\Delta nps6$ strains was determined on MM supplemented with 200 μ M ferric citrate as well as on MM.

In contrast with 2DP, which is membrane-permeable, BPS stays in the extracellular space (Kicic et al., 2001). Hence, BPS causes iron depletion only extracellularly and does not inhibit fungal growth completely. Note that fungi store iron in the intracellular space. Sensitivity to BPS was evaluated for *C. heterostrophus* and *A. brassicicola* as follows. A fresh stock solution of BPS (10 mM in water) was prepared for each assay. Wild-type and $\Delta nps6$ strains were grown on MM with or without 100 μ M BPS for 5 d for *C. heterostrophus* and for 7 d for *A. brassicicola*. Six replicates were set up for each strain under each condition, and two independent $\Delta nps6$ strains were examined. After incubation, the colony diameter of each plate was recorded, and the data were analyzed by one-way analysis of variance (ANOVA).

As noted, the $\Delta nps6$ strain of *F. graminearum* shows reduced growth on MM. Because $\Delta nps6$ strains are hypersensitive to iron depletion, we thought that the growth defect of the $\Delta nps6$ strain on MM was attributable to starvation for iron. Growth of the $\Delta nps6$ strains of *F. graminearum* on MM with 50, 100, and 200 μ M ferric citrate was examined, and we found that increasing concentrations of iron promoted the growth of the $\Delta nps6$ strain (data not shown). Taking advantage of this phenotype, sensitivity to iron depletion was examined in *F. graminearum* as follows. Wild-type and $\Delta nps6$ strains were grown on MM with or without 200 μ M ferric citrate for 4 d. Six replicates were set up for each strain under each condition, and two independent $\Delta nps6$ strains were examined. The colony diameter of each plate was recorded, and the data were analyzed by one-way ANOVA.

Fungal RNA Extraction and Expression Analyses

For in vitro expression analyses, total RNA was extracted from mycelia as described by Sokolovsky et al. (1990). cDNA was prepared using SuperScript III reverse transcriptase (Invitrogen). Serially diluted cDNA samples

were prepared, and concentrations of these samples were normalized based on expression of the *C. heterostrophus* actin gene or of the gene encoding glyceraldehyde phosphate dehydrogenase.

To analyze the in vitro expression of *NPS6* under iron depletion, $\sim 10^6$ conidia were inoculated into 30 mL of either MM or MM without any iron source in 125-mL flasks. Both cultures were shake-incubated (150 rpm) at room temperature for 72 h. Mycelia were harvested from each culture, and total RNA was extracted. For in planta expression analyses, total RNA was extracted, using the RNeasy plant mini kit (Qiagen), from maize leaves at 24, 48, and 72 h after inoculation with *C. heterostrophus*. Synthesis and normalization of cDNA were performed as described above.

Isolation, Identification, and Quantitative Analysis of Siderophores

For preparative isolation of siderophores, *C. heterostrophus* wild-type strain C4 and the $\Delta nps6$ strain Chnps6-1 were grown in liquid culture by inoculating 500 mL of MM (iron-replete) or MM without FeSO_4 (the sole iron source in MM; therefore, iron-depleted) (Simonart et al., 2001) with $\sim 10^7$ to 10^8 conidia in a 1-liter flask and shake-incubating (175 rpm) at room temperature ($22 \pm 3^\circ\text{C}$). After 120 h, the cultures were separated into mycelial and broth fractions by vacuum filtration through filter paper (Whatman No. 1). For quantitative analysis of siderophore production, 3×100 -mL cultures were grown in 250-mL flasks for 72 h. After harvesting, FeCl_3 was added to filtered culture broth to a final concentration of 1.5 mM. The ferrated broth was loaded onto a column of Amberlite XAD-16 resin (1:10 ratio of column volume to broth volume) preequilibrated in 50 mM KPO_4 buffer, pH 7.5. The column was rinsed with 4 column volumes of 50 mM KPO_4 . Siderophores were eluted with methanol until all red coloration was removed from the column (~ 3 column volumes), then this fraction was dried in vacuo and stored at 4°C .

The crude fraction from XAD-16 was then further purified by semipreparative HPLC on a 250×10 -mm, 5- μm , 100 Å, RPC18 Prodigy ODS(3) column (Phenomenex) using a binary mobile phase consisting of (A) acetonitrile and (B) 15 mM acetate buffer, pH 4.2, with gradient elution at a flow rate of 4 mL/min (10% A for 6 min, linear ramp to 60% A in 10 min, 60% A for 2 min, and linear ramp to 10% A in 1 min). Detection was by absorption at 215 and 435 nm. Fractions containing peaks with absorption at 435 nm, indicative of siderophore ligand- Fe^{3+} complexes (Konetschny-Rapp et al., 1988), were concentrated to dryness in vacuo.

For isolation of intracellular siderophores, mycelial mats were washed twice with 100 mL of water to minimize contamination with extracellular products. The mycelium was ground in liquid nitrogen, suspended in 20 mL of 50 mM KPO_4 , and sonicated for ~ 10 min. The suspension was centrifuged at $5000g$ for 5 min, then the supernatant was chromatographed on XAD-16 by semipreparative HPLC as described above for the extracellular fraction.

Electrospray mass spectra were obtained by infusion of solutions at 5 $\mu\text{L}/\text{min}$, via a syringe pump (Harvard Apparatus), into a Micromass ZMD 4000 spectrometer (capillary, 3.5 kV; cone, 40 and 90 V) for LRESI-MS and an ABI-Sciex Q-Trap 2000 spectrometer (declustering potential, 100 V; collision energy, 30, 40, and 50 eV) for MS/MS experiments. HRESI-MS data were acquired on a Micromass QTOF Ultima spectrometer (capillary, 3 kV; cone, 30 V).

For quantitative analysis of siderophore production, the crude siderophore fractions from XAD-16 extraction of 3-d-old cultures were dissolved in a minimum of water and loaded onto an RPC18 solid-phase extraction cartridge (300 mg; Alltech). The red siderophore fraction was then eluted in methanol, concentrated to dryness, and reconstituted in 1 mL of a 1:9 mixture of the HPLC mobile phase constituents A and B, and a 10- μL aliquot was analyzed by HPLC. The analytical HPLC method was identical to the semipreparative method described above except for the column dimensions (250×4.6 mm), the flow rate (1 mL/min), and the

gradient (10% A for 2 min, linear ramp to 60% A in 8 min, 60% A for 2 min, and linear ramp to 10% A in 1 min).

Quantitative estimates of siderophore production in liquid cultures were made by HPLC after determination of standard curves. One- and 10-mL broth equivalents were analyzed for each extracellular and mycelial extract, respectively. Limits of detection were established conventionally at a signal-to-noise ratio of 3.

The coprogen used as a reference standard was purchased from EMC Microcollections. Ferricrocin and triacetyl fusarinine C used as reference standards were purified as described by Oberegger et al. (2001). Deferriated siderophores were obtained by treatment with 8-hydroxyquinoline as described previously (Jalal et al., 1988).

Virulence Assays with Fe or DFO treatment

For application of iron to maize, the method described by Fernandez et al. (2004) was followed. Briefly, ~ 1.5 mL of iron solution (1 mM ferric citrate solution in 1 g/L Glucocon 215 CS UP [alkyl-polyglucoside; Fluka]) was applied with a brush to the first, second, and third true leaves of maize plants. Both adaxial and abaxial surfaces of leaves were treated. Glucocon 215 CS UP has been reported to facilitate iron uptake by plants (Fernandez et al., 2004). Plants were incubated in a growth chamber under continuous fluorescent light for 4 h. After incubation, plants were subjected to virulence assays. Fungal inoculation was performed as described in Virulence Assays above, except that spore suspensions with or without 0.5 mM ferric citrate were prepared. Control plants were inoculated with 0.02% Tween 20 solution with or without 0.5 mM ferric citrate. Virulence was evaluated 5 d after inoculation. For statistical tests, see the following section.

For the application of exogenous iron to rice, pieces of rice leaves were sprayed with 0.5 mM Fe(III) EDTA solution in 0.05% Tween 20. The leaves were held at room temperature for 2 h before inoculation. Five microliters of *C. miyabeanus* conidial solution ($1 \times 10^4/\text{mL}$) with or without 500 μM Fe(III) EDTA was drop-inoculated onto the leaves, and the leaves were kept in Petri dishes on wet filter paper. After 24 h, the droplets were removed with laboratory tissue (Jia et al., 2003). Control leaves were mock-inoculated with Tween 20 solution with or without 0.5 mM Fe(III) EDTA. Photographs were taken 4 d after inoculation.

Application of iron to *Arabidopsis* was performed essentially as described for maize except that ~ 1 mL/pot of 0.5 mM ferric citrate solution in 1 g/L Glucocon 215 CS UP was applied by spraying, instead of brushing. Note that one pot contained three to four plants. Spore suspensions with or without 0.5 mM ferric citrate were prepared. Virulence was evaluated at 4 d after inoculation. Application of DFO was performed as described for iron application (0.25 mM DFO and 0.25 mM iron-saturated DFO were prepared). Iron-saturated DFO was prepared by adding FeCl_3 to the DFO solution (1:1 ratio) immediately before use (Simonart et al., 2001).

For the *F. graminearum* assay, ~ 5 mL/pot of 1 mM ferric citrate solution in Glucocon 215 CS UP (1 g/L) was sprayed on wheat spikes before inoculation. One pot contained 7 to 10 spikes. Plants were kept under continuous fluorescent light for 4 h. Point-inoculation of wheat spikes was performed as described in Virulence Assays above. Spore suspensions were prepared with or without 0.5 mM ferric citrate.

Virulence Assays with Siderophore Treatment

Crude extracts and N^α -dimethylcoprogen were prepared from *A. brassicicola* cultures as described in Isolation, Identification, and Quantitative Analysis of Siderophores above. Crude extract (51.8 and 24.6 mg) was obtained from wild-type and $\Delta nps6$ cultures, respectively. Concentrations of crude extracts from both wild-type and $\Delta nps6$ cultures were adjusted to 0.2 mg/mL (in 1 g/L Glucocon 215 CS UP solution) before application. The concentration of the N^α -dimethylcoprogen solution was

0.2 mM. Application of either crude extract or N^6 -dimethylcoprogen and inoculation of *Arabidopsis* with *A. brassicicola* were performed as described in the previous section.

Statistical Treatment

Unless mentioned otherwise, statistical analyses were performed by one-way ANOVA, followed by multiple range tests. Homogeneity of variance was confirmed by the F_{\max} test for each data set. For evaluation of the virulence of *C. heterostrophus*, third true leaves were harvested from three independent plants that had been inoculated with each strain and given each treatment (i.e., iron application or not). Ten lesions were randomly chosen for each leaf, and the vertical length of each lesion with or without the attendant halo was recorded (a total of 30 lesions per strain per treatment). The data sets with or without halos were analyzed independently by one-way ANOVA.

Statistical evaluation of the virulence of *C. miyabeanus* was performed in essentially the same manner. Twenty lesions on third true leaves were randomly picked for each strain and each treatment, and the vertical length of each lesion was recorded.

The virulence of *A. brassicicola* was examined statistically essentially as described for *C. heterostrophus*. Ten lesions representing each strain and each treatment, on the second and third pairs of true leaves of *Arabidopsis*, were chosen randomly. The vertical length of each lesion was recorded, and the data set was analyzed by one-way ANOVA.

We used survival analysis tests to determine whether deletion of *F. graminearum* NPS6 affected the time required for the completion of systemic infection. Inoculated spikes were checked daily for up to 20 d after inoculation. When the number of completely bleached spikelets reached 16, infection of the spike was considered complete, and the time after inoculation to completion of infection was recorded as the lifetime for each spike. Based on the lifetime data, a survivor function [S(t)], in this case the probability that the lifetime of a spike is greater than t (where t can be any nonnegative number [$t \leq 20$]), was estimated. The smaller the S(t) value, the greater the likelihood that a spike is completely infected by the pathogen at t. The survival distribution function is the proportion of spikes with a lifetime greater than t among the total number of spikes tested. The survival curve describes the change of the survival distribution function with time. The null hypothesis that the survivor functions of the spikes challenged by the wild-type and *F. graminearum* $\Delta nps6$ strains are the same was examined with the log-rank test. Estimation of survivor function and the log-rank test were both performed with SAS 9.1 (SAS Institute). For evaluation of the reduced virulence of the $\Delta nps6$ strains, wild-type and two independent $\Delta nps6$ strains were examined, and six replicates (six spikes) were set up for each strain. The effect of iron application on the virulence of the $\Delta nps6$ strains was examined with survival analyses essentially as described above. Five replicates were set up for the wild-type strain for each condition, and 10 replicates were set up for the $\Delta nps6$ strains for each condition.

Accession Numbers

Sequence data for nucleotide and protein sequences of *C. miyabeanus* and *A. brassicicola* NPS6 can be found in the GenBank data library under accession numbers DQ860090 and DQ860091, respectively. *C. heterostrophus* (AY884191), *F. graminearum* (XM_383923), and *N. crassa* (NCU8441.1) NPS6 sequences and *C. heterostrophus* (AY748990) actin and glyceraldehyde phosphate dehydrogenase (X63516) sequences, deposited previously in GenBank, were also used in this study.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Methods.

Supplemental Table 1. Percentage of *C. heterostrophus* Spore Germination in Vitro.

Supplemental Table 2. PCR Diagnosis of Type of Integration of Transforming DNA.

Supplemental Table 3. Quantitative Analysis of *C. heterostrophus* Intracellular and Extracellular Siderophore Production.

Supplemental Table 4. Strains Used in This Study.

Supplemental Figure 1. The *C. heterostrophus* $\Delta nps6$ Strain Shows Reduced Virulence to Maize and Hypersensitivity to H_2O_2 .

Supplemental Figure 2. The $\Delta nps6$ Strain of *C. heterostrophus* Shows a Defect in Colonization in Planta.

Supplemental Figure 3. Strategy for and Confirmation of Gene Deletion.

Supplemental Figure 4. Survival Curves of Wheat Spikes Challenged by Wild-Type and Two $\Delta nps6$ Strains of *F. graminearum*.

Supplemental Figure 5. Hypersensitivity of $\Delta nps6$ Strains of *A. brassicicola* and *F. graminearum* to Iron Depletion.

Supplemental Figure 6. Confirmation of Integration of Nc NPS6.

Supplemental Figure 7. Structures of Deferriated Coprogens, Depicting Cleavages That Explain Fragment Ions at m/z 285 and 397 That Were Observed in ESI-MS-MS Spectra of Coprogen and Isotriornicin.

Supplemental Figure 8. The NRPSs Encoded by *F. graminearum* NPS6 and *A. brassicicola* NPS6 Are Responsible for the Biosynthesis of Extracellular Siderophores.

Supplemental Figure 9. Survival Curves of Wheat Spikes Infected by Wild-Type and $\Delta nps6$ Strains of *F. graminearum* with or without Exogenous Application of Iron.

Supplemental Figure 10. Increased Sensitivity of the $\Delta nps6$ Strain of *A. brassicicola* to Iron Depletion Generated by 2DP, and Restoration of Tolerance to Iron Depletion by in Vitro Application of DFO.

Supplemental Figure 11. Restoration of Tolerance to Reactive Oxygen Species by Application of Ferric Citrate or DFO.

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