Reductive Iron Assimilation and Intracellular Siderophores Assist Extracellular Siderophore-Driven Iron Homeostasis and Virulence

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Iron is an essential nutrient and prudent iron acquisition and management are key traits of a successful pathogen. Fungi use nonribosomally synthesized secreted iron chelators (siderophores) or reductive iron assimilation (RIA) mechanisms to acquire iron in a high affinity manner. Previous studies with the maize pathogen *Cochliobolus heterostrophus* identified two genes, *NPS2* and *NPS6*, encoding different nonribosomal peptide synthetases responsible for biosynthesis of intra- and extracellular siderophores, respectively. Deletion of *NPS6* results in loss of extracellular siderophore biosynthesis, attenuated virulence, hypersensitivity to oxidative and iron-depletion stress, and reduced asexual sporulation, while *nps2* mutants are phenotypically wild type in all of these traits but defective in sexual spore development when *NPS2* is missing from both mating partners. Here, it is reported that *nps2nps6* mutants have more severe phenotypes than both *nps2* and *nps6* single mutants. In contrast, mutants lacking the *FTR1* or *FET3* genes encoding the permease and ferroxidase components, respectively, of the alternate RIA system, are like wild type in all of the above phenotypes. However, without supplemental iron, combinatorial *nps6ftr1* and *nps2nps6ftr1* mutants are less virulent, are reduced in growth, and are less able to combat oxidative stress and to sporulate asexually, compared with *nps6* mutants alone. These findings demonstrate that, while the role of RIA in metabolism and virulence is overshadowed by that of extracellular siderophores as a high-affinity iron acquisition mechanism in *C. heterostrophus*, it functions as a critical backup for the fungus.

Iron is a vital nutrient and its redox activity has a central role in many enzymatic reactions (Kaplan and Kaplan 2009). Bioavailable iron, however, is limited, as iron is largely insoluble in the presence of oxygen (Haas et al. 2008; Lindsay and Schwab 1982). Iron's capacity as a redox mediator can also generate reactive oxygen species (ROS) and cause subsequent cellular damage via the Fenton reaction (Fenton 1894; Winterbourn 1995). All organisms must, therefore, balance iron acquisition,

chelation, and storage with detoxification of the oxygen radicals free iron can produce (Kaplan and Kaplan 2009).

Fungi, including fungal phytopathogens, have two known methods for high-affinity iron acquisition, i.e., reductive iron assimilation (RIA) and siderophore assisted (Haas et al. 2008; Johnson 2008). RIA, originally characterized in *Saccharomyces cerevisiae*, is an extracellular membrane-bound process whereby ferric iron is reduced by ferric reductase Fer1 and then oxidized by the iron multicopper oxidase Fet3 while being transported across the plasma membrane by the high-affinity iron permease Ftr1 (Kosman 2003, 2010). Ftr1 and Fet3 are intimately linked and require one another to co-localize to the plasma membrane (Singh et al. 2006; Stearman et al. 1996). Alternatively, fungi can utilize siderophores, small cyclic peptides produced by nonribosomal peptide synthetases (NRPS) (Schwarzer et al. 2003), which are found only in fungi and bacteria and absent from animals and plants (Finking and Marahiel 2004).

Iron is important for host-microbe interactions, as pathogens must gain access to host-chelated iron to survive. This paradigm is well-established for fungal pathogens of humans. *Aspergillus fumigatus*, for example, requires both intra- and extracellular siderophores for full virulence on a variety of hosts (Hissen et al. 2005; Schrettl et al. 2004, 2007). Conversely, deletion of the RIA-affiliated permease gene *ftrA* in *A. fumigatus* has no effect on virulence in mice (Schrettl et al. 2004). Double deletion mutants of the *ftrA* gene and the *sidA* gene, which encodes N⁵-ornithine-monooxygenase essential for biosynthesis of both intra- and extracellular siderophores, are unable to grow on most media without siderophore (ferrirocen) supplementation, demonstrating that the RIA mechanism does contribute to iron acquisition in the absence of siderophores (Schrettl et al. 2004).

Early studies of iron acquisition by plant pathogens found that the biotrophs *Ustilago maydis* and *Microbotryum violaceum* do not require siderophores for virulence, suggesting iron acquisition was not vital for phytopathogens (Birch and Ruddat 2005; Mei et al. 1993). Later studies demonstrated that *U. maydis* instead requires RIA, as RIA mutants are strongly reduced in virulence (Eichhorn et al. 2006). In the necrotroph *Fusarium graminearum*, RIA (iron permease) mutants are like wild type (WT) for both growth and virulence (Park et al. 2006). These mutants show increased *SIDA* expression comparable to that seen in iron-depleted WT strains, however, suggesting RIA is, in fact, utilized to acquire iron in *F. graminearum* (Park et al. 2006).

A positive role for siderophores in fungal pathogenesis to plants was demonstrated when *Cochliobolus heterostrophus*,

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Cochliobolus miyabeanus, *Alternaria brassicicola*, and *F. graminearum* mutants deleted for the *NPS6* gene, encoding an NRPS responsible for biosynthesis of the extracellular siderophore coprogen, all showed reduced virulence on their respective hosts (corn, rice, Arabidopsis, wheat) (Oide et al. 2006). Deletion of *NPS6* affects more than virulence in all of these species; it also results in hypersensitivity to ROS and hypersensitivity to iron depletion (Lee et al. 2005; Oide et al. 2006).

In contrast, deletion of *C. heterostrophus* *NPS2*, encoding an NRPS for biosynthesis of the intracellular siderophore ferrirocen, responsible for iron storage, impairs sexual reproduction but not virulence. The *nps2* mutants cannot make ascospores in homozygous *nps2* × *nps2* crosses, although these crosses form pseudothecia and are hermaphroditic like WT strains (Oide et al. 2007). A similar sexual development phenotype was found for homothallic *F. graminearum*; deletion of the *NPS2* ortholog results in loss of ferrirocen biosynthesis and defects in ascospore development (Oide et al. 2007).

Pathogens apparently rely on either RIA or siderophores as their primary means of iron acquisition but not both. The underlying mechanism for the reduction of virulence in either RIA or siderophore mutants is unclear, however. *C. heterostrophus* *nps6* mutants are sensitive to both oxidative and iron stress, both of which could be encountered in planta (Oide et al. 2006). Prussian blue staining has shown that Fe³⁺ accumulates at infection sites of the biotroph *Blumeria graminis* f. sp. *tritici* on various monocots (Liu et al. 2007). For the necrotroph *Botrytis cinerea*, virulence increases in synchrony with the intensity of ROS, contributed both by the host and the fungus (Temme and Tudzynski 2009). Similarly, starving *Arabidopsis* for iron by washing roots with an iron chelator reduces susceptibility to *B. cinerea*. Increased fungal siderophore production suggested this reduction in disease was related to iron starvation as opposed to another mechanism such as antimicrobial activity (Kieu et al. 2012).

Additionally, ROS link iron to many biological processes, such as sexual development, signal transduction, and stress responses in fungi as well as plants (Aguirre et al. 2005; Foyer and Noctor 2005). Plant-generated ROS mediate activation of defense genes or additional defenses by redox control of transcription factors or through other signaling components, such as phosphorylation cascades (Kotchoni and Gachomo 2006; Mou et al. 2003). The sexual dysfunction phenotype of *C. heterostrophus* *nps2* mutants could be a result of impaired iron or ROS status (Oide et al. 2007).

The extent to which RIA contributes to virulence of the necrotroph *C. heterostrophus* is unknown. Furthermore, RIA and siderophore polymutants have been reported only in *Aspergillus* species and no differentiation between the absence of extracellular versus intracellular siderophores was made. To address these questions, we constructed RIA mutant strains by deleting the high-affinity permease *FTR1* and generated combinatorial *nps2ftr1*, *nps6ftr1*, and *nps2nps6ftr1* mutants. We analyzed the phenotypes of these mutants with regard to nutrition, iron and oxidative stress, and virulence and provide evidence for a secondary role of RIA in *C. heterostrophus*.

RESULTS

Identification of RIA mutants.

BLAST searches using the *S. cerevisiae* proteins as a query identified *FTR1* (Joint Genome Institute [JGI] identification number 104817, 1,778 bp) and *FET3* (JGI identification number 104814, 2,030 bp) orthologs separated by 1,778 bp in *C. heterostrophus* strains C4 and C5. This region was identical in the two strains except for two single nucleotide polymorphisms that do not alter the coding sequence of either *FTR1* or

FET3. Individual *FTR1* and *FET3* deletion mutants were generated in strain C4. Mutants with genes deleted and replaced by selectable marker *HygB* were verified using diagnostic polymerase chain reaction (PCR) primers as described below and in Supplementary Figure S1. All gene deletion and verification primers are listed in Supplementary Table S1.

None of the RIA mutants (*fet3*, *ftr1*, *fet3ftr1*; Table 1) was different from WT with respect to growth, sexual and asexual development, sensitivity to oxidative and iron stress, or virulence.

Identification of high-affinity iron acquisition and iron storage polymutants.

Combinatorial mutants lacking ability to produce intracellular siderophores (*nps2*), extracellular siderophores (*nps6*), or RIA (*ftr1*, *fet3*, *ftr1fet3*) components were generated by crossing single or single and double mutants as described below. The *nps2nps6*, *nps6ftr1*, *nps2ftr1*, *nps6fet3*, *nps6fet3ftr1*, and *nps2nps6ftr1* (the latter shown in Figure 1) mutants were collected in this way and were confirmed by PCR with the primer combinations used to identify the original mutants among transformants. All strains used in this study are listed in Table 1 and all crosses are listed in Table 2.

Iron combinatorial mutants require supplemental iron for growth.

Previously, we showed that mutants lacking extracellular (*nps6*) or intracellular (*nps2*) siderophores grow at WT rates on complete medium with xylose (CMX) (Oide et al. 2006; 2007). The *ftr1*, *fet3*, and *fet3ftr1* RIA mutants generated here also displayed WT morphology and growth rates. Double mutants lacking both *NPS2* and *NPS6* also grew like WT (Fig. 2). In contrast, all mutants lacking both extracellular siderophores and RIA (*nps6ftr1*, *nps6fet3*, *nps6fet3ftr1*) grew at a reduced rate on CMX and were less pigmented than WT strains (Fig. 2). Growth and pigmentation of these mutants could be restored to WT levels by providing 100 μM supplemental ferric citrate (Fig. 2). The *nps2nps6ftr1* triple mutants, lacking not only extracellular siderophore and RIA iron acquisition mechanisms but also intracellular siderophores and, thus, the ability to store iron, were almost completely unable to grow on CMX and did not sporulate (Fig. 2). Even with supplemental iron, close examination of colony morphology revealed significant alterations compared with *nps6ftr1*.

On minimal medium (MM), the reduced growth and pigmentation phenotype of *nps6ftr1*, *nps6fet3*, and *nps6fet3ftr1* mutants was even more pronounced (Fig. 2). These mutants were all similarly reduced in growth, which suggests that deleting either *FTR1* or *FET3* is sufficient to disrupt RIA. Subsequently, most experiments reported in this manuscript used *ftr1* mutants to represent RIA deficiency. The *nps2nps6* and *nps2ftr1* mutants grew like WT strains on MM (Fig. 2); however, *nps2nps6* mutants, like *nps6* mutants, were reduced in pigmentation. The *nps2nps6ftr1* triple mutants were unable to grow on MM, and while iron supplementation slightly restored growth, it did not do so to the extent that it restored growth of *nps6ftr1* mutants (Fig. 2). These data demonstrate that, while RIA is dispensable for growth and development in the presence of *NPS6*, it functions as a backup mechanism in *nps6* mutants.

To determine if iron mutants are better able to utilize one form of iron over another, we tested growth on CMX with various concentrations of chelated and unchelated ferric and ferrous iron (Supplementary Fig. S2). There was no difference among iron sources used for supplementation; a threshold concentration between 2.5 and 25 μM of ferric or ferrous salt (ferric chloride and ferrous sulfide, respectively) as well as chelated ferric iron (ferric citrate) was able to supplement growth.

Examination of *nps6ftr1* and *nps2nps6ftr1* in CM liquid cultures revealed that hyphal growth is abnormal without supplemental iron (Supplementary Fig. S3). The *nps6ftr1* cultures grew poorly and with an abnormal wavy morphology, while *nps2nps6ftr1* growth was extremely limited and cultures contained senescent melanized hyphae. Supplemental iron alleviated the wavy growth defect of *nps6ftr1* cultures, but abundance of hyphae was still reduced when compared with WT. The *nps2nps6ftr1* cultures also improved in growth with supplemental iron but with defective wavy morphology comparable to that seen in *nps6ftr1* cultures without iron.

***nps2nps6* and *nps2nps6ftr1* strains are affected in sexual development.**

C. heterostrophus nps2 strains fail to form asci and ascospores in homozygous *nps2* crosses on MM, although the number of pseudothecia formed per square centimeter of leaf surface and the morphology of these pseudothecia are like WT crosses (Oide et al. 2007). The *nps6* strains are not affected in sexual development (Oide et al. 2007). *C. heterostrophus nps2nps6* strains, when crossed to a WT strain of opposite mating type, showed WT numbers of pseudothecia and WT

fertility (Fig. 3A and C). No significant reduction in the number of pseudothecia per square centimeter of leaf area or in the number of asci per pseudothecium was observed as compared with WT crosses, whether *nps2nps6* strains acted as male or female (Supplementary Fig. S4). Furthermore, regardless of mating type of the *nps2nps6* strains, crosses between WT and *nps2nps6* strains were as fertile as WT $\times$ WT crosses. Homozygous *nps2nps6* crosses did not form pseudothecia (Fig. 3C and D), in contrast to homozygous *nps2* crosses, which form pseudothecia with WT morphology but no asci (Fig. 3E and F) (Oide et al. 2007).

Increasing concentrations of iron partially restored ability of the *nps2nps6* strains to develop pseudothecia (Fig. 3E), indicating that the defect in pseudothecium development of *nps2nps6* strains is at least partially due to iron starvation. Asci and ascospores were observed in these pseudothecia, although the number per pseudothecium was much less than that of WT crosses and homozygous *nps2* mutant crosses supplemented with iron (Fig. 3F). Homozygous *nps2ftr1* crosses were like *nps2* $\times$ *nps2* crosses, producing barren pseudothecia, while homozygous *ftr1* $\times$ *ftr1* and *nps6ftr1* $\times$ *nps6ftr1* crosses were like WT crosses (data not shown). RIA, therefore, has no obvious

Table 1. *Cochliobolus heterostrophus* strains used in this study.

Strain ^a	Genotype ^b	Comments and References
C4 (ATCC 48331)	<i>Tox1;MAT1-2</i>	Race T, inbred laboratory strain, wild type (WT); Leach et al. 1982
C2	<i>Tox1;MAT1-1;alb1</i>	As above
C9	<i>Tox1;MAT1-1</i>	As above
CB11	<i>Tox1;MAT1-2;alb1</i>	Race T, inbred laboratory strain, WT (Bronson 1988)
ChDsred	<i>RFP;hygB;MAT1-2</i>	Dsred (Matz et al. 1999) expressed under the citrate lyase promoter in pIGREDPAPA
Chnps2-1	<i>nps2;hygB;MAT1-2</i>	Oide et al. 2007
Chnps2-2	<i>nps2;hygB;MAT1-2</i>	As above
Ch1449-T1-1	<i>nps2;hygB;MAT1-2</i>	As above
Ch1449-T1-5	<i>nps2;hygB;MAT1-1;alb1</i>	As above
Chnps6-1	<i>nps6;hygB;MAT1-2</i>	Oide et al. 2006
Chnps6-1R2	<i>nps6;hygB;MAT1-1</i>	As above
Chftr1-1	<i>ftr1;hygB;MAT1-2</i>	This study, iron permease <i>FTR1</i> deletion
Chftr1-2	<i>ftr1;hygB;MAT1-2</i>	As above
Ch1721-R1	<i>ftr1;hygB;MAT1-1;alb1</i>	This study, progeny of cross Chftr1-1 $\times$ C2
Ch1721-R3	<i>ftr1;hygB;MAT1-2;alb1</i>	As above
Chfet3-1	<i>fet3;hygB;MAT1-2</i>	This study, <i>FET3</i> deletion
Chfet3-2	<i>fet3;hygB;MAT1-2</i>	As above
Chfet3ftr1-1	<i>fet3;ftr1;hygB;MAT1-2</i>	This study, deletion of both <i>FET3</i> and <i>FTR1</i>
Chfet3ftr1-2	<i>fet3;ftr1;hygB;MAT1-2</i>	As above
Ch1495-T1-1	<i>nps2;nps6;hygB;MAT1-2</i>	This study, progeny of Chnps2-1 $\times$ Chnps6-1R2
Ch1495-T1-6	<i>nps2;nps6;hygB;MAT1-1</i>	As above
Ch1495-T1-8	<i>nps2;nps6;hygB;MAT1-2</i>	As above
Ch1496-T1-1	<i>nps2;nps6;hygB;MAT1-1</i>	This study, progeny of Chnps2-2 $\times$ Chnps6-1R2
Ch1496-T1-8	<i>nps2;nps6;hygB;MAT1-1</i>	As above
Ch1722-R1	<i>nps6;ftr1;hygB;MAT1-1</i>	This study, progeny of Chnps6-1R2 $\times$ Chftr1-1
Ch1722-R3	<i>nps6;ftr1;hygB;MAT1-2</i>	As above
Ch1722-R4	<i>nps6;ftr1;hygB;MAT1-2</i>	As above
Ch1723-T2-5	<i>nps2;ftr1;hygB;MAT1-2</i>	This study, progeny of Chftr1-1 and 1449-T1-5
Ch1731-T6-3	<i>nps2;nps6;ftr1;hygB;MAT1-1</i>	This study, progeny of Ch1722-R3 $\times$ Ch1449-T1-5
Ch1743-T6-1	<i>nps2;nps6;ftr1;hygB;MAT1-2</i>	This study, progeny of Ch1721-R3 $\times$ Ch1495-T1-6
Ch1744-T6-6	<i>nps2;nps6;ftr1;hygB;alb1</i>	Repeat of above cross
Ch1754-R6	<i>nps6;fet3;hygB</i>	This study, progeny of Chnps6-1R2 $\times$ Chfet3-1
Ch1754-R7	<i>nps6;fet3;hygB</i>	As above
Ch1755-R11	<i>nps6;fet3;ftr1;hygB</i>	This study, progeny of Chnps6-1R2 $\times$ Chfet3ftr1
Ch1755-R18	<i>nps6;fet3;ftr1;hygB</i>	As above
Ch1761-R7	<i>RFP;hygB;MAT1-1</i>	This study, progeny of Dsred $\times$ C2
Ch1761-R9	<i>RFP;hygB;MAT1-1</i>	As above
Ch1762-T1-1	<i>RFP;nps6;hygB</i>	This study, progeny of Chnps6-1 $\times$ Ch1761-R-7
Ch1764-T1-1	<i>RFP;nps6;ftr1;hygB</i>	This study, progeny of 1722-R-3 $\times$ Ch1761-R-7
Ch1765-T2-3	<i>RFP;nps2;nps6;hygB</i>	This study, progeny of 1495-T1-1 $\times$ Ch1761-R-7
Ch1765-T3-5	<i>RFP;nps2;nps6;hygB</i>	As above
Ch1766-R2	<i>RFP;nps2;nps6;ftr1;hygB</i>	This study, progeny of Ch1743-T6-1 $\times$ Ch1761-R-7
Ch1768-T1-7	<i>nps2;ftr1;hygB;MAT1-1</i>	This study, progeny of Ch1723-T2-5 $\times$ CB11
Ch1768-T5-7	<i>nps2;ftr1;hygB;MAT1-1</i>	As above

^a Chnps2-1 is a strain in which *NPS2* is deleted, '-1' indicates transformant number 1; 1449-T1-1 is cross number 1449; T1-1 is tetrad number 1, ascospore 1. 1766-R2 is the second random progeny recovered (rather than part of a tetrad) from cross number 1766.

^b Strains are pigmented unless denoted *alb1* in genotype. If mating type is not included, it was not tested and is unknown.

role in sexual development, whereas the NPS6 siderophore has a role secondary to NPS2 siderophore. This suggests that the phenotype observed in *nps2nps6* mutants compared with *nps2* mutants is not related to the role of coprogen in iron acquisition (because *nps6ftr1* mutants, which are totally impaired in iron acquisition, display WT sexual development) but, instead, to its contribution to intracellular iron or to ROS status.

***nps6ftr1* and *nps2nps6ftr1* strains are affected in asexual development.**

Asexual spore (conidium) production was assessed on CMX and MM with and without supplemental iron for all strains (Fig. 4). On CMX, WT, *nps2*, *nps6*, *ftr1*, *nps2ftr1*, and *nps2nps6* strains all produced 1×10^6 to 7×10^6 conidia per plate with or without 150 μ M supplemental ferric citrate. The *nps6ftr1*, *nps6fet3*, and *nps6fet3ftr1* strains, however, produced approximately 50-fold fewer conidia than this on CMX; supplementation with ferric citrate restored conidium production to WT levels. The *nps2nps6ftr1* strains failed to produce conidia on CMX, but conidium production could be restored with supplemental iron, albeit to 75-fold fewer conidia per plate than WT. WT and strains *nps2*, *ftr1*, and *nps2ftr1* produced similar numbers of conidia on MM as they did on CMX, while *nps6* and *nps2nps6* strains produced 20- to 40-fold fewer than WT. The *nps6ftr1*, *nps6fet3*, *nps6ftr1fet3*, and *nps2nps6ftr1* strains did not produce conidia on MM with or without supplemental iron. High-affinity iron acquisition is required, therefore, for conidial development.

Iron acquisition mutants can be fed iron by other strains.

High-performance liquid chromatography (HPLC) analyses revealed that neither intracellular (ferricrocin, synthesized by

Nps2 and found in mycelium) nor extracellular (coprogen, synthesized by Nps6 and secreted into the broth) siderophores were detectable in the *nps2nps6* sample while both were detected in WT (Fig. 5). As reported earlier (Oide et al. 2006, 2007), no ferricrocin was detected in the *nps2* sample, although coprogens were detected. In contrast, no coprogens were detected in the *nps6* sample, although a small amount of ferricrocin was detected in the mycelial fraction (Fig. 5). Quantitative analyses of siderophore biosynthesis (Table 3) revealed that the *nps2* mutant produced two to six times more coprogens (milligrams per liter of broth) compared with WT, while the *nps6* mutant produced approximately twice as much ferricrocin (milligrams per gram of mycelium) as WT did, suggesting that the N⁵-hydroxyornithine precursor shared by the two pathways is diverted to producing more ferricrocin when Nps6 is not present to synthesize coprogen.

When plated alone, *nps2nps6ftr1* is incapable of growing on CMX (without iron supplement). When plated alongside other strains, such as when tetrad progeny are plated together (Fig. 1), these strains were able to grow, albeit in abnormal patterns. Noticing this, we developed an assay to test whether this phenomenon was due to the ability of the *nps2nps6ftr1* strain to utilize compounds (such as siderophores) produced by other strains. Mycelia and conidia of *nps2nps6ftr1* were applied to complete medium without salt solution (CMNS) plates with hygromycin B at varying distances from hygromycin-sensitive WT mycelia and conidia (Fig. 6). After 2 days, *nps2nps6ftr1* mutants closer to the WT strain were able to grow but not those plated farther away from the WT strain. Furthermore, colony growth habit was not radial; mutants grew towards the

Fig. 1. Identification of triple mutants by plating tetrads on differential media. Eight ascospores from a single ascus isolated from a cross between *nps6ftr1* (strain Ch1722-R3) and *nps2* (albino strain Ch1449-T1-5) mutants were plated on complete medium with xylose (CMX), minimal medium (MM), CMX + iron, and complete medium without salt solution (CMNS) + hygromycin B. Position of strains is the same in each plate. Wild-type (WT) progeny are indicated with closed arrows, and triple *nps2nps6ftr1* mutant progeny with open arrows. All eight progeny grow on CMX + iron. The *nps2nps6ftr1* progeny do not grow on CMX and MM, indicating that they require iron. WT progeny do not grow on medium containing hygromycin B. The *ALB1* gene controlling pigmentation segregates 1:1 and serves as a control for single gene segregation.

Table 2. Crosses performed in this study

Cross	Parent strains ^a
Wild type (WT) × WT	C4 × C2
WT × WT	C4 × C9
<i>ftr1</i> × WT	Chftr1-1 × C2
<i>ftr1</i> × <i>nps6</i>	Chftr1-1 × Chnps6-1R2
<i>ftr1</i> × <i>nps2</i>	Chftr1-1 × Ch1449-T1-5
<i>fet3</i> × WT	Chfet3-1 × C2
<i>nps2</i> × <i>nps6</i>	Chnps2-1 × Chnps6-1R2
<i>nps2</i> × <i>nps6</i>	Chnps2-1 × Chnps6-1R2
<i>ftr1nps6</i> × <i>nps2</i>	Ch1722-R-1 × Chnps2-1
<i>ftr1</i> × <i>nps2nps6</i>	Ch1721-R-3 × Ch1495-T1-6
<i>fet3</i> × <i>nps6</i>	Chfet3-1 × Chnps6-1R2
<i>fet3ftr1</i> × <i>nps6</i>	Chfet3ftr1-1 × Chnps6-1R2
Dsred × WT	ChDsred × C2
Dsred × <i>nps6</i>	Chnps6-1 × Ch1761-R7
Dsred × <i>nps2nps6</i>	Ch1495-T1-1 × Ch1761-R7
Dsred × <i>nps6ftr1</i>	Ch1722-R3 × Ch1761-R7
Dsred × <i>nps2nps6ftr1</i>	Ch1743-T6-1 × Ch1761-R7
<i>ftr1</i> × <i>ftr1</i>	Chftr1-1 × Ch1721-R2
<i>nps2</i> × <i>nps2</i>	Ch1499-T1-1 × Ch1449-T1-5
<i>nps2nps6</i> × WT	Ch1495-T1-1 × C2
<i>nps2nps6</i> × WT	Ch1495-T1-8 × C2
<i>nps2nps6</i> × WT	Ch1496-T1-1 × C4
<i>nps2nps6</i> × WT	Ch1496-T1-8 × C4
<i>nps2nps6</i> × <i>nps2nps6</i>	Ch1495-T1-1 × Ch1496-T1-1
<i>nps2nps6</i> × <i>nps2nps6</i>	Ch1495-T1-1 × Ch1496-T1-8
<i>nps2nps6</i> × <i>nps2nps6</i>	Ch1495-T1-8 × Ch1496-T1-1
<i>nps2nps6</i> × <i>nps2nps6</i>	Ch1495-T1-8 × Ch1496-T1-8
<i>nps6ftr1</i> × <i>nps6ftr1</i>	Ch1722-R1 × Ch1722-R3
<i>nps6ftr1</i> × <i>nps6ftr1</i>	Ch1722-R1 × Ch1722-R4
<i>nps6ftr1</i> × WT	Ch1722-R1 × C4
<i>nps2ftr1</i> × <i>nps2ftr1</i>	Ch1723-T2-5 × Ch1768-T1-7
<i>nps2ftr1</i> × <i>nps2ftr1</i>	Ch1723-T2-5 × Ch1768-T5-7
<i>nps2ftr1</i> × WT	Ch1723-T2-5 × C9

^a Chnps2-1 is a strain in which NPS2 is deleted, '-1' indicates transformant number 1; 1449-T1-1 is cross number 1449; T1-1 is tetrad number 1, ascospore 1. 1766-R2 is the second random progeny recovered (rather than part of a tetrad) from cross number 1766.

WT strain more vigorously than they did in the opposite direction. The *nps2nps6ftr1* strain is, therefore, able to utilize one or more factors biosynthesized by WT, if close by, and to grow in the direction of the supplier.

To investigate further, plates were set up with culture filtrates from WT, *nps6*, *nps2*, and *nps2nps6* strains placed in wells in the agar at the four corners of the experiment (Fig. 6). The *nps2nps6ftr1* scrapings that were adjacent to WT filtrate were

Fig. 2. Double and triple iron acquisition mutants are impaired in growth on complete medium with xylose (CMX) and minimal media (MM). The *ftr1* (strain Chftr1-1), *fet3* (strain Chfet3-1), and *fet3ftr1* (strain Chfet3ftr1-1) reductive iron assimilation (RIA) mutants and the *nps2* (strain Chnps2-1), *nps6* (strain Chnps6-1), and *nps2nps6* (strain Ch1495-T1-1) siderophore mutants grow like wild type (WT) (strain C4) on CMX and MM and do not benefit from supplemental iron. Combined RIA and extracellular siderophore mutants *nps6ftr1* (strain Ch1722-R1), *nps6fet3* (strain Ch1754-R6), and *nps6fet3ftr1* (strain Ch1755-R11), however, are reduced in growth and pigmentation on both CMX and MM. Adding supplemental iron (100 μ M ferric citrate) to CMX restores colony growth, although it remains sparse on MM. The *nps2nps6ftr1* (strain Ch1731-T6-3) triple mutants are extremely reduced in growth on CMX, extending only a few millimeters past the inoculum plug. Supplemental iron restores growth to resemble that of *nps6ftr1*. The *nps2nps6ftr1* mutants are completely unable to grow on MM without supplemental iron, which only partially supplements growth.

well-restored in growth. Those next to *nps2* filtrate also grew, however, *nps2nps6ftr1* scrapings adjacent to *nps6* and *nps2nps6* filtrates were unable to grow. We hypothesize that coprogen siderophores biosynthesized by Nps6 in WT and *nps2* mutants diffuse in the medium and can be used by the *nps2nps6ftr1* mutant to complement its growth defect. Both *nps6* and *nps2nps6* strains lack coprogens (Fig. 5) and, therefore, cannot feed *nps2nps6ftr1* strains.

Iron acquisition mutants are sensitive to oxidative and iron stress.

Deletion of *NPS6* leads to hypersensitivity to oxidative stress and iron depletion in *C. heterostrophus* (Oide et al. 2006). De-

letion of *NPS2*, however, does not affect sensitivity to these stresses (Oide et al. 2007). The RIA and combinatorial mutants generated in this study were assayed for sensitivity to oxidative stress, using hydrogen peroxide (H_2O_2) and the superoxide generator KO_2 , and also to iron stress, using the membrane-permeable iron chelator 2,2-dipyridyl (2DP) and the membrane-impermeable iron chelator bathophenanthroline disulfonate (BPS) (Fig. 7). The *ftr1* mutants grew like WT at each stressor concentration tested.

As previously described (Oide et al. 2006), *nps6* mutant growth was more inhibited than WT for each stress condition, when stress concentrations allowed a distinction (Fig. 7). The *nps2ftr1* sensitivity largely mirrored that of WT and *ftr1* mu-

Fig. 3. Without supplemental iron, *nps2nps6* strains fail to develop pseudothecia in homozygous crosses. **A**, Corn leaf substrate bearing pseudothecia, 21 days after crossing wild type (WT) pigmented strain C4 and WT albino strain C2. Both pigmented and albino pseudothecia form, indicating that each parent is hermaphroditic. **B**, Contents of a crushed pseudothecium from a WT $\times$ WT cross, demonstrating typical fertility (many asci [arrow] and ascospores). **C**, Corn leaf substrate bearing pseudothecia 21 days after crossing *nps2nps6* $\times$ WT strains (Ch1495-T1-1 $\times$ C2). When crossed to a WT strain, *nps2nps6* strains develop pseudothecia as well as WT strains do. **D**, In homozygous *nps2nps6* crosses (strain Ch1495-T1-1 $\times$ Ch1496-T1-1), *nps2nps6* strains fail to develop pseudothecia. **E**, The average number of pseudothecia per square centimeter of leaf is shown for homozygous WT and *nps2nps6* crosses. Ten replicates were set up for each cross and for each condition. Application of ferric EDTA restores ability to develop pseudothecia to the *nps2nps6* strains in homozygous *nps2nps6* crosses (abbreviated 2;6 $\times$ 2;6), although the number of pseudothecia formed is less than homozygous WT crosses. A reduction in the number of pseudothecia per square centimeter of corn leaf was observed in WT crosses when iron was applied, implying an adverse effect of iron overload. **F**, The average number of asci per pseudothecium is shown for homozygous WT, *nps2*, or *nps2nps6* crosses. Twenty pseudothecia were opened for each cross and for each condition. Error bars indicate 95% confidence intervals. Application of iron restores ability to develop asci and ascospores to the *nps2* and *nps2nps6* strains (abbreviated 2 $\times$ 2 and 2;6 $\times$ 2;6), although the number of asci per pseudothecium is smaller compared with that of WT crosses. The number of asci per pseudothecium developed by homozygous *nps2nps6* crosses is smaller than that of homozygous *nps2* crosses, further demonstrating that iron metabolism during sexual development is more severely affected in the *nps2nps6* strains than in the *nps2* strains.

tants, suggesting lack of intracellular siderophores, and the RIA mechanism does not impact resistance to these stresses. Although *nps6ftr1* mutant growth was delayed on MM without stress, growth was comparable to that of the *nps6* strain on H_2O_2 and KO_2 . Growth of *nps2nps6* was more sensitive to oxidative stress than *nps6* and *nps6ftr1* strains, when stress concentrations allowed a distinction. The *nps2nps6ftr1* strain was not included, as it is not capable of growing on MM. Deletion of *NPS2*, therefore, affects oxidative stress only in the absence of *NPS6*, suggesting that intracellular siderophores do play a role in this process. RIA, on the other hand, does not play a clear role in oxidative stress sensitivity, even in the absence of *NPS6*, since *nps6ftr1* strains showed the same sensitivity as *nps6* strains (Fig. 7; Supplementary Fig. S5).

Low concentrations (75 μ M) of 2DP did not significantly impair the growth of any strain and, in some cases, growth was greater than on MM. At high concentrations (150 μ M) of 2DP, WT, *ftr1*, and *nps2ftr1* strains were able to grow, but *nps6*, *nps2nps6*, and *nps6ftr1* strains were not. Growth of WT, *ftr1*, and *nps2ftr1* strains at the highest concentrations (300 μ M) of BPS was comparable to that of the corresponding strain's growth on MM, suggesting they were not affected by this stressor. The *nps6*, *nps2nps6*, and *nps6ftr1* strains, on the other hand, were significantly reduced in growth at all concentrations tested as compared with WT, but double mutants were not consistently more sensitive than *nps6* single mutants (Fig. 7; Supplementary Fig. S6).

Iron acquisition and iron storage mutants are affected in virulence.

It was determined previously that deletion of *C. heterostrophus* *NPS6* leads to reduction in virulence to maize, while deletion of *NPS2* does not affect virulence (Lee et al. 2005; Oide et al. 2006). Virulence assays done in the current study confirmed the reduced virulence of the *nps6* mutant but indicated that *ftr1* and *nps2ftr1* mutants are not reduced in virulence compared with WT (Fig. 8A). The *nps2nps6* and *nps6ftr1* strains caused further reductions in virulence compared with *nps6* strains; *nps2nps6ftr1* mutants were extremely reduced in virulence, forming atypical small white lesions rather than expanding lesions in various stages of necrosis. This reduction in virulence is not due to morphological defects of the *nps2nps6ftr1* conidia, as they germinated as well as WT conidia when spore suspensions were placed on glass slides (Supplementary Fig. S7). Adding either exogenous iron (Fig. 8B) or the antioxidant ascorbic acid (data not shown) or both to the inoculum and leaf surface did not significantly increase average lesion size for any strain used. The average lesion size of the *nps2nps6ftr1* mutant was slightly (but not significantly) complemented by exogenous iron (Fig. 8A). The presence of *NPS6* is, therefore, of primary importance for virulence, but the absence of *NPS2* and *FTR1* can each affect virulence in the absence of *NPS6*.

A key goal of this study was to differentiate between the roles iron and oxidative stress play in the virulence phenotypes of iron acquisition mutants. To this end, we applied the ROS-staining dye 3,3'-diaminobenzidine (DAB) to infected leaves. At 24 h, DAB accumulation (Fig. 9, arrow) could be seen in WT- and *nps6*-infected leaves, presumably immediately surrounding points of infection, indicating the presence of ROS. Leaves challenged with *nps6ftr1* and *nps2nps6* mutants had smaller, more discrete spots of DAB accumulation, and those challenged with the *nps2nps6ftr1* mutant looked more like uninoculated controls, lacking DAB accumulation. At 5 days after infection, when lesions are plainly visible on the leaf, DAB staining was profuse in WT- and *nps6*-mutant challenged leaves. Leaves challenged with *nps6ftr1* and *nps2nps6* mutants

had discrete lesion-shaped DAB stains, often with stain accumulating at the edge without staining in the middle. The *nps2nps6ftr1*-inoculated and uninoculated leaves did not show DAB accumulation. The tiny lesions on leaves (Fig. 8) and the lack of DAB staining (Fig. 9) suggest that *nps2nps6ftr1* mutants elicit a different response from the plant than all other strains used. Loss of DAB staining in *nps2nps6*, *nps6ftr1*, and *nps2nps6ftr1* infections suggests iron acquisition mutants may be deficient in producing virulence-promoting ROS. Alternatively, DAB accumulation may be a product of host cell death and iron mutants are less able to kill host tissue, resulting in less DAB accumulation.

Iron-acquisition mutants have impaired in planta growth.

To further investigate how virulence is impaired in these mutants, Dsred (red fluorescent protein)-expressing strains were observed in planta at 24 h and 5 days after inoculation. At 24 h, no clear difference in spore germination or appressorium formation was observed between WT and the *nps6*, *nps6ftr1*, or *nps2nps6* mutants (not shown). The extent of colonization by both *nps6ftr1* and *nps2nps6* mutants at this time, however, was reduced compared with WT or *nps6*. For all strains, sites of fungal entry were often accompanied by autofluorescence originating from the challenged epidermal cell. After 5 days, *nps6ftr1* and *nps2nps6* mutant growth had halted after spreading 100 to 500 μ m, whereas comparable regions were extensively colonized by WT and *nps6* strains (Fig. 10). Combined with lesion measurements (Fig. 8B) and DAB staining (Fig. 9) data, this suggests that both *nps2nps6* and *nps6ftr1* mutants are able to enter the leaf and initiate infection, but they fail to colonize extensively. Patches of accompanying autofluorescence likely correspond to host defense response (Fig. 10, bottom row).

Fig. 4. The *nps6ftr1* and *nps2nps6ftr1* mutants are impaired in asexual spore production. On complete medium with xylose (CMX), wild type (WT) (strain C4), *nps2* (strain Chnps2-1), *nps6* (strain Chnps6-1), *ftr1* (strain Chftr1-1), *nps2ftr1* (strain Ch1723-T2-5), and *nps2nps6* (strain Ch1495-T1-1) mutants all produced between 1,280,000 to 6,960,000 conidia per plate with or without supplemental iron. While *nps6ftr1* (strain Ch1722-R1), *nps6fet3* (strain Ch1754-R6), and *nps6fet3ftr1* (strain Ch1755-R11) mutants produced between 32,000 and 136,000 conidia (50- to 100-fold fewer). *nps2nps6ftr1* mutants did not produce conidia on CMX. With 50 μ M supplemental iron, *nps6ftr1*, *nps6fet3*, and *nps6fet3ftr1* conidia production was restored to WT levels. The *nps2nps6ftr1* mutants were also able to produce conidia (but 10-fold fewer than WT) with supplemental iron. On minimal medium (MM), conidia production was reduced 20- to 60-fold for both *nps6* and *nps2nps6* compared with WT, with or without supplemental iron. The *nps6ftr1*, *nps6fet3*, *nps6fet3ftr1*, and *nps2nps6ftr1* mutants did not produce conidia on MM with or without supplemental iron.

Fig. 5. The *nps2nps6* mutants do not produce siderophores. High-performance liquid chromatography analyses of wild-type (WT) (strain C4), *nps2nps6* (strain Ch1495-T1-1), *nps2* (strain Chnps2-1), and *nps6* (strain Chnps6-1) strains. Samples were extracted from broth (left) and mycelium (right) of fungal cultures grown under iron-depleted conditions. Coprogen and ferricrocin standards are shown in the bottom panel. WT produces coprogens and ferricrocin as extra- and intracellular siderophores, respectively. In contrast, no coprogens or ferricrocin were detected in the *nps2nps6* sample, demonstrating that double deletion of *NPS2* and *NPS6* completely abolished siderophore biosynthesis.

For the *nps2nps6ftr1* triple mutant, there were fewer conidia to sample at both 24 h and 5 days. Those that could be observed were able to germinate and form appressoria (Fig. 10), as seen in vitro. No in planta fungal growth beyond appressorium formation and the initial site of penetration could be observed at 24 h or 5 days. Combined with the atypical tiny lesions observed on whole leaves (Fig. 8) and the lack of DAB staining (Fig. 9), this suggests that *nps2nps6ftr1* does not advance as a pathogen beyond appressorium formation and initial penetration.

DISCUSSION

Fungal pathogens appear to favor one mode of high-affinity iron acquisition over the other. The mammalian pathogen *A. fumigatus* requires siderophores but not RIA for full virulence (Schrettl et al. 2004, 2007). The same has been demonstrated for the necrotroph *F. graminearum* in its interaction with maize (Greenshields et al. 2007) and implied for *Epichloë festucae* in its mutualistic interaction with ryegrass (Johnson et al. 2013). The biotroph *U. maydis*, on the other hand, requires RIA for virulence, whereas siderophores are dispensable (Eichhorn et al. 2006; Mei et al. 1993). The necrotroph *C. heterostrophus* was previously shown to require extracellular siderophores for full virulence to maize (Oide et al. 2006). Because RIA *ftr1*, *fet3*, and *fet3ftr1* mutants have no abnormal

phenotypes, we can conclude that *C. heterostrophus* prefers high affinity siderophore iron acquisition over RIA.

In contrast to RIA single mutants, however, the combined RIA and siderophore mutants *nps6ftr1*, *nps6fet3*, and *nps6fet3ftr1* are all impaired in growth without supplemental iron, indicating that the RIA mechanism is an important iron-gathering backup system when extracellular siderophores are absent. Note that deletion of *FTR1*, *FET3*, or *FTR1* and *FET3* was sufficient to disrupt RIA. Growth was further disrupted in *nps2nps6ftr1*, suggesting that iron acquisition and storage both contribute to proper iron homeostasis.

Removal of both RIA and extracellular siderophores impairs growth and asexual development and only by supplying high quantities of iron (much higher than would be found naturally) can growth be restored. Neither *nps6* nor *ftr1* mutants have this phenotype, suggesting that one iron acquisition system can substitute for the other in vitro (but not in planta). Unlike *NPS6*, *FTR1* expression was not repressed by the iron-responsive transcription factor Sre1 (Zhang et al. 2013). This, coupled with our finding that *ftr1* phenotypes occur only in the absence of *NPS6*, supports a model wherein the extracellular siderophore coprogen is favored for high-affinity iron acquisition. The RIA system functions well enough to allow growth of *nps6* mutants in standard culture but not in the presence of iron or oxidative stress or in planta. Further removal of intracellular siderophores (ferricrocin) results in extremely limited growth without supplementation, likely due to an already iron-limited fungus being extra-sensitive to disruption of iron and oxidative stress homeostasis.

There is only one other study thus far that we are aware of in which RIA/siderophore double mutants were generated (Schrettl et al. 2004). *A. fumigatus* *sidA/ftrA* double mutants lacked RIA plus extracellular and intracellular siderophores because SidA is required for biosynthesis of both types of siderophore. These mutants were highly defective in growth, as we have demonstrated here for *C. heterostrophus* *nps2nps6ftr1*. Our study is unique, however, in distinguishing between extracellular and intracellular siderophore mutations, in combination with RIA mutations (i.e., we generated *nps2ftr1*, *nps6ftr1*, and *nps2nps6 ftr1* mutants). Interestingly, loss of intracellular siderophores and RIA (*nps2ftr1*) does not affect stress sensitiv-

Table 3. Quantitative analysis of *Cochliobolus heterostrophus* intra- and extracellular siderophores

Siderophore	Wild type ^a	<i>nps2</i> ^{a,b}	<i>nps6</i> ^{a,b}	<i>nps2nps6</i> ^b
Broth (mg/liter)				
Neocoprogen II	2.76 (0.8)	5.50 (0.85)	nd	nd
Neocoprogen I	11.91 (6.21)	32.86 (8.05)	nd	nd
Coprogen	4.14 (2.87)	27.07 (8.04)	nd	nd
Mycelium (mg/g of dry weight)				
Ferricrocin	0.82 (0.12)	nd	1.60 (0.39)	nd
Neocoprogen II	0.98 (0.42)	1.10 (0.29)	nd	nd
Neocoprogen I	5.45 (1.78)	7.71 (2.02)	nd	nd
Coprogen	2.06 (0.62)	6.18 (1.68)	nd	nd

^a Value in parentheses = standard error.

^b nd = none detected.

Fig. 6. Growth of *nps2nps6ftr1* mutants is restored by proximity to WT and *nps2* strains or filtrates but not by *nps6* or *nps2nps6* culture filtrates. Left: Small amounts of wild-type (WT) (C4) mycelia with conidia (without agar medium) were placed in a vertical line on the right side of a plate of complete medium without salt solution (CMNS) + hygromycin B. WT is unable to grow because it is sensitive to hygromycin B. Similar amounts of hygromycin B-resistant triple mutant *nps2nps6ftr1* (strain Ch1731-T6-3) were placed on the diagonal with patches progressively closer to the WT strain. The *nps2nps6ftr1* mutant is unable to grow unless it is in close proximity to WT, in which case it grows towards the WT culture on the plate. Right: When *nps2nps6ftr1* is grown adjacent to filtrate from WT, *nps6* (strain Chnps6-1), *nps2* (strain Chnps2-1), and *nps2nps6* (strain Ch1495-T1-1) liquid cultures, only WT and *nps2* filtrate can restore growth.

ity, growth, or virulence. In the presence of a functional *NPS6* gene, *nps2*, *ftr1*, and *nps2ftr1* mutants are all WT for these phenotypes.

The oxidative and iron stress findings in this study demonstrate that, although deletion of *NPS2* alone has no effect on sensitivity to ROS or to low iron (Oide et al. 2007), deletion of *NPS2* has a clear effect when combined with *nps6*. Growth of *nps6ftr1* mutants, on the other hand, was not reduced relative to *nps6* on media containing oxidative stressors. This confirms that coprogen is sufficient for dealing with both oxidative and iron stress, while ferricrocin but not RIA plays a secondary role.

The *nps2ftr1* mutants were like WT in terms of virulence on maize, demonstrating that *C. heterostrophus* relies first and foremost on extracellular siderophore-mediated iron acquisition for virulence. Both *nps6ftr1* and *nps2nps6* mutants were further reduced in virulence compared with *nps6*, with *nps2nps6ftr1* mutants almost completely unable to proceed past appressorium formation. The reduction in disease seen for *nps6ftr1* could be due to its reduced growth without excess iron but is

not a product of increased sensitivity to oxidative or iron stress, given its growth on media containing iron or oxidative stressors. Conversely, *nps2nps6* mutants are not impaired in in vitro growth but are more sensitive to oxidative stress than *nps6* mutants. This suggests that both iron limitation and oxidative stress may be the cause of the reduced virulence of *nps6* strains.

MATERIALS AND METHODS

Fungal strains and culture conditions.

C. heterostrophus WT race T strain C4 (*Tox1+*; *MAT1-2*, American Type Culture Collection [ATCC] number 48331) was used for all transformations and strain C2 (*Tox1+*; *MAT1-1*; *alb1*, ATCC 48329) was used as an albino tester for initial crosses. All strains used in this study are listed in Table 1. Strains were stored at -80°C in liquid CM (Leach et al. 1982) containing 25% glycerol and were plated onto CMX (Tzeng et al. 1992) for growth and optimal conidiation. Fungi were grown at 24°C in an alternating 16-h-light and 8-h-dark cycle

Fig. 7. Iron acquisition and storage mutants are sensitive to oxidative and iron stress. Wild type (WT) (strain C4), *nps6* (strain Chnps6-1), *ftr1* (strain Chftr1-1), *nps2ftr1* (strain Ch1723-T2-5), *nps6ftr1* (Ch1722-R1), and *nps2nps6* (strain Ch1495-T1-1) were plated on minimal medium (MM) containing increasing concentrations of the oxidative stressors H_2O_2 and KO_2 , the membrane permeable iron chelator 2,2-dipyridyl (2DP), and membrane impermeable iron chelator bathophenanthroline disulfonate (BPS). Average colony diameter was measured from leading edge to leading edge of hyphal growth. The *ftr1* and *nps2ftr1* mutants are similar to WT in response to all stressors, suggesting lack of intracellular siderophores and the reductive iron assimilation mechanism does not impact resistance to these stresses. As reported previously (Oide 2007), *nps6* is more sensitive than WT and *nps2nps6* is more sensitive than *nps6* on H_2O_2 and KO_2 . In these experiments, *nps6* and *nps2nps6* responded similarly to iron stress, however stress conditions chosen were not optimal for a distinction to be drawn. The growth of *nps6ftr1* mutants is comparable to that of *nps6* on both types of oxidative stress and on BPS, although its reduced growth on MM is a complicating factor. Within a graph, different letters above bars indicate values that are statistically significantly different as determined by one-way analysis of variance and Tukey's range test.

under fluorescent light (Watt-Miser F34 WW/RS/WM, Warm White; General Electric, Home Depot, Ithaca, NY, U.S.A.).

MM (Leach et al. 1982) was used for iron and oxidative stress assays. CMNS was used for selection for resistance to hygromycin B. CMX+Fe and MM+Fe were CMX or MM with 100 μ M ferric citrate (Sigma, St. Louis) added prior to autoclaving, unless stated otherwise. Sach's (Luttrell 1958) medium or MM (Oide et al. 2007) was used for crosses.

Iron and fungal growth.

Iron source supplementation was tested on solid CMX with ferric citrate, ferrous sulphate 7-H₂O (Mallinckrodt), and ferric chloride 6-H₂O (Fluka). Iron, from aliquots of 1 mM sterilized stock solutions, was added to CMX after autoclaving to final concentrations of 0.25, 2.5, and 25 μ M. Cultures were grown for 6 days in the dark and were then photographed.

For liquid cultures, spore suspensions were prepared by applying 2 to 3 ml of CM to culture plates, rubbing the surface to dislodge conidia, and filtering the slurry through sterile cheesecloth. Conidia (10^5) were added to 10 ml of liquid CM, with or without 100 μ M ferric citrate. Cultures were grown for 18 h at room temperature at 150 rpm, and aliquots were observed under a Leica DM5500 Epifluorescence microscope using differential interference contrast at the Boyce Thompson Institute Plant Cell Imaging Center (Ithaca, NY, U.S.A.). Images were acquired using Qcapture Pro 6.0 (Qimaging, Surrey, Canada).

Identification of RIA associated genes.

Orthologs of the *S. cerevisiae* iron permease *FTR1* and multicopper oxidase *FET3* in the RIA pathway (Table 1) were identified initially by BLAST search (Altschul et al. 1990), using the *S. cerevisiae* proteins as a query against the *C. heter-*

Fig. 8. *FTR1* and *NPS2* affect virulence in a manner secondary to *NPS6*. **A**, Representative fourth true leaves from N-cytoplasm W64-A corn 5 days after inoculation with wild type (WT) and iron mutants are shown. On leaves inoculated with WT (strain C4) and *nps2ftr1* (strain Ch1723-T2-5), lesions are dark, long, and necrotic. The *nps6* (strain Chnps6-1) mutants cause smaller brown lesions, while *nps6ftr1* (strain Ch1722-R1) and *nps2nps6* (strain Ch1495-T1-1) cause much smaller, lighter lesions with small dark centers. The *nps2nps6ftr1* (strain Ch1731-T6-3) mutants cause the smallest lesions, observable as white flecks. All leaf images are applications without supplemental iron, with the exception of the control water-inoculated leaves and the triple mutant-inoculated leaves, which are shown without and with supplemental iron. **B**, Average lesion size of fourth true leaves from N-cytoplasm W64-A maize inoculated with WT and iron mutants. The *nps2ftr1* (and *ftr1*, not shown) mutant lesion sizes are like WT. As shown previously, *nps6* mutants are reduced in virulence and *nps2nps6* mutants are further reduced. The *nps6ftr1* mutant is comparable to *nps2nps6*, while *nps2nps6ftr1* mutants are extremely reduced in virulence compared with WT. Iron supplementation slightly increased lesion size for all inoculated plants. This difference was not significantly different as determined by one-way analysis of variance (ANOVA) and Tukey's range test for any mutant or WT. Letters above bars indicate strains that were statistically significantly different for standard (lacking supplemental iron) treatments as determined by one-way ANOVA and Tukey's range test. Means not connected by the same letter are significantly different.

ostrophus C5 gene catalog (Condon et al. 2013). *FTR1* (JGI identification number 104817, 1,778 bp) and *FET3* (JGI identification number 104814, 2,030 bp) were both located on scaffold 3, separated by 1,778 bp. Deletions were made in the sister inbred strain C4.

DNA manipulations and fungal transformations.

Fungal genomic DNA was prepared using the Ultraclean Microbial DNA isolation kit (MO BIO, Carlsbad, CA, U.S.A.). PCR reactions were carried out with Phusion (New England Biolabs, Ipswich, MA, U.S.A.) DNA polymerase and mix for generating transformation constructs or GoTaq (Promega, Madison, WI, U.S.A.) for screening mutants, following the manufacturer's recommendations.

Transformations were performed using split marker-based homologous integration (Catlett et al. 2003; Turgeon et al. 2010). The 700- to 1,000-bp DNA flanking (5' and 3' flanks) coding sequences were amplified, using primer pairs with extensions complementary to M13Rhyg for the reverse primer of one flanking region and M13Fhyg for the forward primer of the other flanking region. *FTR1* was deleted using the upstream primer pair *ftrupf/ftrupr* and the downstream pair *ftrdnf/ftrdnr* to amplify the 5' and 3' flanks, respectively. *FET3* was deleted using the upstream pair *fet3upf/fet3upr* and the downstream pair *fet3dnf/fet3dnr*, and *FTR1* and *FET3* were deleted together using the upstream (relative to *FET3*) pair *fet3upf2/fet3upr2* and the downstream pair *fet3dnf/fet3dnr*. *NPS2* and *NPS6* gene deletions were described previously (Oide et al. 2006, 2007).

Protoplasting and transformation of strain C4 was performed as described by Turgeon and associates (2010). Candidate transformants recovered from the hygromycin B overlay were transferred approximately 1 week after plating to fresh selective medium (CMNS containing 50 µg hygromycin B per milliliter) to confirm resistance. Conidia were streaked on 5% water agar to separate individual conidia and eliminate heterokaryons, and individual germinating conidia were transferred to CMX. Purified candidates were then patched onto CMNS hygromycin B (50 µg hygromycin B per milliliter) and CMX to confirm antibiotic resistance and for storage in glycerol, respectively.

DNA was prepared from candidate transformants and was subjected to diagnostic PCR to verify gene deletion. A set of primers internal to the deleted gene and two sets of primer pairs, in which one primer was internal to the introduced selectable marker and the other was external to either the 5' or the 3' flanking region used to delete the gene, were used. DNA from candidates with a gene of interest deleted and replaced with the selectable marker showed a band of predictable size when a primer external to the 5' or the 3' flanking region was combined with either NLC37 or NLC38 (internal to *HygB*), while WT DNA did not. Additionally, a band was amplified from WT DNA when a primer pair internal to the gene targeted for knockout was used, whereas no band was amplified from candidate deletion mutant DNA.

The *ftr1* candidates were confirmed using internal primer pair *ftrinf/ftrinr* to verify gene deletion and external primer pairs *fet3upr/NLC37* and *fet3extfarupf/NLC38* to verify cor-

Fig. 9. Reactive oxygen species production as indicated by 3,3'-diaminobenzidine (DAB) staining is reduced in maize leaves inoculated with iron mutants. Inoculated and DAB-stained leaves at 1 (left in each pair) and 5 (right in each pair) days after inoculation. In wild type (strain C4) and *nps6*-challenged (strain ChChnps6-1) leaves, DAB precipitates form at discrete locations after 24 h (arrow) and ubiquitously throughout the leaf by 5 days. The *nps6ftr1* (strain Ch1722-R1) and *nps2nps6* (strain Ch1495-T1-1) mutants form fewer localized DAB precipitates at 24 h and much smaller, contained DAB-stained areas at 5 days. The *nps2nps6ftr1* (strain Ch1731-T6-3) mutants do not form visible DAB precipitate at 1 or 5 days.

Fig. 10. Iron mutants are progressively impaired in colonizing maize leaves. Top and middle rows: Dsred-expressing strains of wild type (WT) (strain Ch1761-R7), *nps6* (strain Ch1762-T1-1), *nps6ftr1* (strain Ch1764-T1-1), *nps2nps6* (strain Ch1765-T2-3), and *nps2nps6ftr1* (strain Ch1766-R2) mutants were inoculated onto maize and leaves were visualized by confocal microscopy at 5 days. The scale bar is set to 50 μ m and is equivalent for each image. WT and *nps6* mutants extensively colonized the leaf; *nps6ftr1* and *nps2nps6* growth was less prolific and caused smaller autofluorescent patches of dead plant tissue; *nps2nps6ftr1* was able to germinate and form appressoria (insert image taken at 24 h) but did not typically penetrate or grow inside the leaf. When it did, it did not progress beyond the initial cell colonized. Bottom row: At 24 h, all strains had attached and formed appressoria (only *nps2nps6* and *nps6ftr1* shown). Conidia are still attached (open white arrows) and the precise site of penetration can be identified (closed arrows). Autofluorescence at the perimeter of penetrated epidermal and surrounding cells occurs in an emission spectrum indistinguishable from that of Dsred.

rect integration into the 5' and the 3' target flanks, respectively. The *fet3* candidates were confirmed using the internal primer pair *fet3inf/fet3inr* and the external primer pairs *fet3extupf/NLC38* and *fet3extdnr/NLC37*. The *fet3* and *ftr1* double deletion candidates were confirmed using the internal primer pair *fet3inf/fet3inr* and the external primer pairs *fet3extdnr/NLC37* and *fet3extfarupf/NLC38*.

Construction of combinatorial mutants.

Transformants of strain C4 (*MAT1-2*) were initially crossed to strain C2 (*MAT1-1*) to generate albino and pigmented strains of both mating types. All WT genes were replaced with the *hygB* gene, conferring resistance to hygromycin B. Tracking hygromycin B resistance, therefore, also tracked gene deletion (assuming *hygB* has correctly replaced the targeted gene, as confirmed by diagnostic PCR of candidate transformants). Single and polymutants were recovered from crosses by collecting random or tetrad progeny. For tetrads, the full set of progeny from a given ascus was collected, in isolation from other asci to ensure each ascospore was from the same ascus. In crosses between two *hygR* parents, if hygromycin B-sensitive progeny are observed in a tetrad, the tetrad must contain WT progeny and combinatorial mutants containing each mutation carried by both parents. Diagnostic PCR reactions with the same primer pairs used to confirm successful gene deletions were used to verify the genotype of mutants with multiple gene deletions collected from both random and tetrad isolated ascospores.

nps2nps6 strains were tetrad progeny of *Chnps2-1* × *Chnps6-1R2* crosses and *nps2ftr1* strains were tetrad progeny of *Chftr1-1* × 1449-T1-5 crosses. Additional *nps2ftr1* strains were generated by crossing the *nps2ftr1* strain Ch1723-T2-5 to WT (CB11) and collecting whole tetrads. The *nps6fet3* strains were random progeny from *Chnps6-1-R2* × *Chfet3-1* crosses; *nps6fet3ftr1* strains were random progeny of *Chnps6-1R2* × *Chfet3ftr1-1* crosses; *nps6ftr1* strains were random progeny of *Chnps6-1R2* × *Chftr1-1*; *nps2nps6ftr1* were tetrad progeny of *nps6ftr1* strain 1722-R1 × *Chnps2-1* and *ftr1* strain 1721-R3 × *nps2nps6* strain 1495-T1-6 (Table 2).

Evaluation of fertility.

Crosses (Table 2) were set up using autoclaved senescent maize leaves placed on MM as described previously (Oide et al. 2007). Crosses between WT strains C4 (*MAT1-2*) and either C2 (for *nps2*, *nps6*, *nps2nps6*) or C9 (for *ftr1*, *nps2ftr1*, *nps6ftr1*) were set up as a control for all experiments. Fertility was evaluated based on the average number of pseudothecia per square centimeter of senescent corn leaf and the average number of asci per pseudothecium.

Asexual development.

Asexual sporulation of WT (C4) and *nps6* (*Chnps6-1*), *ftr1* (*Chftr1-1*), *nps6ftr1* (*Ch1722-R1*), *nps2ftr1* (*Ch1723-T2-5*), *nps2nps6* (*Ch1495-T1-6*), and *nps2nps6ftr1* (*Ch1731-T6-3*) mutant strains was quantified. Strains were grown on CMX with and without exogenous application of iron (100 μ M ferric citrate) for 7 days at 24°C under 16 h of light and 8 h of dark fluorescent light and were photographed. The entire area of each plate was scraped with 0.2% Tween 20 in H₂O to collect conidia and the slurry was filtered through sterile cheesecloth and was suspended in 10 ml of 0.2% Tween 20. The concentration was determined by counting spores under a stereomicroscope with a haemocytometer and the number per plate was estimated for each plate.

Feeding plates.

Triple high-affinity iron mutant *nps2nps6ftr1* (*Ch1731-T6-3* or *Ch1743-T6-1*) was grown for 5 days on CMX + 100 μ M

ferric citrate. Small scrapings of the culture (approximately 1 × 1 mm) were transferred to CMNS with hygromycin B. Care was taken to collect mycelium and conidia only, without any of the iron-rich growth medium. For experiments with the WT hygromycin B-sensitive culture, scrapings were set up in a vertical row on one side of the plate and the *nps2nps6ftr1* triple mutant scrapings were placed on a diagonal line opposite, with each scraping progressively closer to the WT row.

When supplementing with hygromycin B-resistant strains *nps2*, *nps6*, or *nps2nps6*, culture filtrate was used instead of mycelial scrapings, to prevent the supplementing culture from overgrowing the *nps2nps6ftr1* mutant. For this, flasks containing 50 ml of CM were inoculated with a 5 × 5 mm aliquot of scraped conidia and were shaken overnight. Cultures were centrifuged for 5 min at 10,000 RPM at room temperature in a tabletop centrifuge to pellet mycelium. A 20- μ l supernatant was transferred to wells in the CMNS with hygromycin B plates carved into the agar with a cork borer. Plates were grown for 2 days at 24°C under a 16-h-light and 8-h-dark alternating fluorescent light cycle and were photographed.

Isolation, identification, and quantitative analysis of siderophores.

Fungal cultures for HPLC analyses were grown and prepared as described previously (Oide et al. 2006, 2007). Quantitative analysis of siderophore production was performed as described previously (Oide et al. 2006). Coprogen and ferri-crocin, used as reference standards, were purified as described (Oide et al. 2006, 2007).

Stress sensitivity assays.

Sensitivity to H₂O₂, the superoxide-generator KO₂, the membrane-permeable iron chelator 2DP, and the membrane-impermeable iron chelator BPS was assessed. A fresh stock solution of each stress agent was prepared for each experiment (1 M KO₂, 10 mM 2DP, and 100 mM BPS solutions in Millipore water), and the stress agents were added to MM after autoclaving and cooling to approximately 48°C. Fresh MM plates with the stress agents were prepared for each experiment. All experiments were carried out in the dark at room temperature and were allowed to grow for 6 days before being photographed. Colony diameters were measured from leading tip to leading tip of hyphal growth; the thickness of growth within that diameter was not considered. Each strain was plated on each condition in triplicate, and the entire experiment was repeated three times.

Plant cultivars and growth conditions.

Corn cultivar W64A with N cytoplasm was used in this study. Corn was grown in a growth chamber under a 16-h-light and 8-h-dark cycle at 24°C. Seed was planted three plants per #6 standard pot, in Cornell Mix soil (3 × 3.8 cubic feet compressed bales of peat moss, 2 × 6 cubic feet bags of vermiculite, 20 pounds of dolomitic limestone, 6 pounds 11-5-11 fertilizer; Cornell University New York State Agricultural Experiment Station website).

Virulence.

Virulence assays of *C. heterostrophus* on maize were carried out after emergence of the fourth true leaf (approximately three weeks after planting), as described previously (Oide et al. 2006). At least three replicates (i.e., inoculation to three independent plants) were set up for each strain and experiments were repeated at least three times in all cases.

All fungal strains were recovered from -80°C glycerol stocks on CMX with 100 μ M supplemental ferric citrate (if experiment included *nps6ftr1* or *nps2nps6ftr1*; otherwise, plain CMX was used) for inoculation. After strains were grown for

10 to 14 days, conidia were harvested by scraping plates in 0.02% Tween 20 and filtering through cheesecloth to remove hyphal debris. Conidia were counted with a haemocytometer and were adjusted with 0.02% Tween 20 to 2×10^4 conidia per milliliter. Conidia were spray-inoculated onto maize plants using Preval sprayers, at the rate of 2 ml per plant. Plants were placed overnight in a mist chamber and were then moved to the growth chamber. Third or fourth true leaves were collected after 5 days and were photographed. At least 50 independent lesions were measured for each strain for each experiment. Statistical analyses were performed using one-way analysis of variance with a Tukey's range test using JMP software (SAS Institute, Cary, NC, U.S.A.).

Viability of *nps2nps6ftr1* conidia was confirmed by harvesting conidia as above and placing 1-ml aliquots of spore suspension on glass microscope slides. Conidia were incubated in a moist chamber for 90 min, and germination rates were counted for 50 conidia, each, on three glass slides. The entire experiment was carried out twice. WT conidia were used as a positive control.

For iron supplementation, maize leaves were rubbed with 1 mM ferric citrate solution containing 1 g of gluconate 215 CS UP (Fluka, Milwaukee, WI, U.S.A.) per liter, 1 h prior to inoculation. The iron-treated plants were incubated in a growth chamber under continuous light until inoculation. Fungal inoculation was carried out in the same way as the standard inoculation assay, except that spore suspensions with or without 0.5 mM ferric citrate were prepared. Control plants inoculated with 0.5 mM ferric citrate without fungal spores were set up as a control. A fresh stock solution of ferric citrate (10 mM in water) was prepared for each experiment.

DAB staining and confocal microscopy.

Inoculation and growth conditions for confocal microscopy and DAB staining were the same as above, except fungal spores were as concentrated as possible. For this, conidia from an entire 100×15 petri dish were scraped into 50 ml of 0.02% Tween 20 in H_2O , and all 50 ml was used per inoculation. For DAB staining, leaf squares were submerged in DAB solution (1 mg/ml, pH 3.7), were shaken for 4 h at 50 rpm, were then transferred to 3:1 ethanol/acetic acid, and were shaken overnight at 50 rpm to clear leaves.

Prior to confocal microscopy, fungal mutants were crossed to a red fluorescent Dsred (Matz et al. 1999) strain previously transformed using the vector pIGREDPAPA (a modification of pIGPAPA) (Horwitz et al. 1999), with the open reading frame of Dsred replacing green fluorescent protein. Tetrads were collected from *nps6*, *nps2nps6*, and *nps6ftr1* $\times$ Dsred crosses; random progeny were collected for WT $\times$ Dsred and *nps2nps6ftr1* $\times$ Dsred crosses. Progeny were assayed for fluorescence under a Leica DM5500 Epifluorescence microscope, and were screened for hygromycin B resistance on CMNS with hygromycin B (50 μ g/ml) plates. Complete tetrads that contained hygromycin B-sensitive, nonfluorescent progeny (and, therefore, the intended fluorescent, *hygB*-resistant mutant in the same ascus) were screened by PCR to verify genotype. Fluorescent, *hygB^R nps2nps6ftr1* progeny were collected by phenotype (inability to grow on MM and CMX without supplemental iron) and fluorescence.

Inoculation was carried out as above for DAB staining. Fourth leaves were collected 1 day (24 h) and 5 days after inoculation and were trimmed with a scalpel to approximately 1×1 mm squares along either side of the major vein. For microscopy, leaves were examined in a Leica TCS SP5 laser scanning confocal microscope at the Boyce Thompson Institute Plant Cell Imaging Center. Images were collected by exciting samples at 488 nm with an argon laser and collecting emissions at 566

nm (Dsred) and 675 nm (chloroplasts). Images were processed in ImageJ (Schneider et al. 2012).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

Cornell University New York State Agricultural Experiment Station website: www.nysaes.cornell.edu/cals/nysaes/bp/shops/greenhouse/potting-mix.cfm