Intracellular Siderophores Are Essential for Ascomycete Sexual Development in Heterothallic Cochliobolus heterostrophus and Homothallic Gibberella zeae

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The innate benefits, to their producers, of the preponderance of diverse metabolites biosynthesized by fungal and bacterial nonribosomal peptide synthetases (NRPSs) are largely unknown. In contrast, because of the medicinal, pharmaceutical, or industrial value of some NRPS metabolites, considerable effort has been expended in characterizing these metabolites with respect to their effects (e.g., antibiotic, immunosuppressive) on other organisms, particularly humans.

Our objective is to determine the natural biological functions of NRPS metabolites in the organisms that produce them; for this, we focus on the genetically tractable heterothallic ascomycete pathogen of maize Cochliobolus heterostrophus. Twelve genes that encode NRPSs (NPS1 to NPS12) have been identified in the C. heterostrophus genome (18, 23). Initially, we focused on the role of NRPS metabolites in virulence to the plant host, since the best-known NRPS metabolites in phytopathogenic ascomycetes are phytotoxins, such as AM-toxin, made by the apple pathotype of Cochliobolus heterostrophus, and HC-toxin, made by race 1 of Cochliobolus heterostrophus.

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Although iron is an essential nutrient for virtually all organisms, including fungi, bioavailable forms are scarce in aerobic environments because of oxidation and formation of minimally soluble iron hydroxides (19). When starved for iron, many fungi and bacteria secrete siderophores to acquire iron extracellularly. The strong iron-chelating activity of these low-molecular-weight organic compounds can solubilize iron effectively from the environment (25, 35). Indeed, as noted above, preventing extracellular siderophore biosynthesis by deletion of NPS6 in C. heterostrophus leads to hypersensitivity to iron depletion, which highlights the major contribution of siderophores to iron gathering in this species (23). Similarly, ∆nps6 strains of two other filamentous ascomycetes, a second cereal pathogen, Gibberella zeae/Fusarium graminearum, and the dicot pathogen Alternaria brassicicola, lacking the ability to biosynthesize extracellular siderophores, are hypersensitive to
iron depletion and reduced in virulence to their hosts, indicating that siderophore-mediated iron metabolism is generally important among filamentous ascomycete phytopathogens (25). Except for the polycarboxylates identified in zygomycetes (31), all fungal siderophores are products of NRPSs.

While characterizing the role of C. heterostraphus NPS6 in extracellular siderophore biosynthesis, we identified ferricrocin as the intracellular siderophore of this species and determined also that the NRPS encoded by NPS6 is not involved in its biosynthesis (i.e., the \( \Delta nps6 \) strain produces wild-type [WT] levels of ferricrocin) (23). Intracellular siderophores, in contrast to extracellular ones, have been proposed to play a role in iron storage in mycelia and spores (36) and have been recognized as axenic spor germination factors of Neurospora crassa, Penicillium chrysogenum, and Aspergillus nidulans (9, 15). In these species, treatment of asexual spores with solutions with low water activity (high salinity) led to loss of intracellular siderophores stored in the spores and to inhibition or delay of spore germination. In addition, A. nidulans intracellular siderophores are essential for efficient asexual sporulation (12). In contrast, deletion of C. heterostraphus NPS6, which is responsible for extracellular siderophore production, leads to a reduction of asexual sporulation, as well as to a reduction of virulence to the host (23). Recently, intracellular siderophores have been reported also to play a role in the self-compatible sexual development of A. nidulans (13). In addition to siderophores, other small molecules, such as the oxylinin psi factor, have been demonstrated to alter the ratio of asexual to sexual sporulation in A. nidulans (5, 6, 33) and a small \gamma\-butyrolactone-containing molecule, butyrolactone I, has been reported as an inducer of asexual sporulation in A. nidulans (27).

This close relationship between secondary-metabolite or small-molecule production and fungal development has been documented comprehensively for Aspergillus spp. (reviewed in references 7 and 37). It has been demonstrated that sporulation and sterigmatocystin- aflatoxin production are regulated by a common G-protein/cyclic-AMP/protein kinase A signaling pathway (7).

The primary objective of the present study was to determine if one or more C. heterostraphus NRPS metabolites function in sexual development, by mating assay screening of our collection of mutant strains, each with 1 of the 12 NPS genes deleted. We found that NPS2 is involved in ascospore development. \( \Delta nps2 \) strains fail to form asci and ascospores in crosses in which the \( \Delta nps2 \) mutation is homozygous; otherwise, sexual development is normal up to this stage. Through biochemical analyses of WT and \( \Delta nps2 \) strains, we identified the product of the NRS2 enzyme as the intracellular siderophore ferricrocin, thus connecting the production of this small molecule to a fundamental fungal development process. Furthermore, we demonstrated functional conservation of NPS2 in ferricrocin biosynthesis and in sexual development in the heterothallic ascomycete C. heterostraphus and in the unrelated homothallic ascomycete G. zeae. We propose that iron and intracellular siderophores are essential for successful sexual development in both self-incompatible and self-compatible ascomycetes.

**Materials and Methods**

**Strains and culture conditions.** C. heterostraphus WT strains C4 (MAT1-2; ATCC 48531) and CB7 (MAT1-1 alb1; ATCC 48332) and G. zeae WT strain Gz3639 (NRRL 29169) were used as background strains for all experiments. Unless otherwise mentioned, all cultures were grown on complete medium (CM) (17) at 24°C under continuous fluorescent light illumination. Transgenic strains developed in this study or in the study of Oide et al. (23) and their progeny are listed in Table 1.

**Gene identification.** The 12 C. heterostraphus NPS genes (accession no. AY854186 to AY854197) were annotated as described previously (18, 23). Phylogenetic analysis (see Fig. 7 in reference 18; K. E. Bushey and B. G. Turgeon, unpublished data) indicates that C. heterostraphus NPS2 falls in a group that includes NPS genes that encode NRPSs known to produce siderophores. These include A. nidulans SidC (accession no. AAPS6239/AN0607.3) and Ustilago maydis SidU (accession no. AAB93493/UMU6278).

The G. zeae NPS and NPS2 genes were identified by BLAST query of the G. zeae genome sequence (http://mips.gsf.de/genre/proj/fusarium/) with the four AMP-binding domains that make up the C. heterostraphus NPS2 protein (18) and with AMP-binding domains of A. nidulans SidC and U. maydis SidU. The top G. zeae hits were extracted and annotated, and all of the AMP-binding domains were used for phylogenetic analysis as in Fig. 7 of reference 18 (data not shown).

**DNA manipulations.** Fungal genomic DNA was prepared as described previously (11, 23). Unless otherwise mentioned, all PCR were performed with PCR master mix (Promega) by following the manufacturer’s recommendations.

**Deletion of the complete set of C. heterostraphus NPS genes.** For targeted gene deletion, split marker constructs were prepared (see Table S1 in the supplemental material) as previously described (8, 23). In previous work on NPS genes (18), partial gene deletions were generated for 11 of the NPS genes. NPS12 was not included. For the present study, the open reading frame (ORF) of each of genes NPS1 to -3 and NPS5 to -12 was completely deleted and 94% of that of NPS14 was deleted (see Table S1 in the supplemental material) and replaced with the hygB cassette from pUCATPH (20), which confers resistance to hygromycin B. Transformation of C. heterostraphus was carried out as described previously (23).

**Deletion of G. zeae nps1 and nps2 genes.** We deleted 94% of the G. zeae NPS1 and NPS2 ORFs (see Table S1 in the supplemental material) and replaced the deleted portions with the hygB gene cassette from pUCATPH. G. zeae \( \Delta nps1 \) \( \Delta nps2 \) strains were constructed from strain FgNps2-6-1 (\( \Delta nps2 \) hygB) (Table 1), which carries a deletion of NPS2. The NPS1 ORF was replaced with the hygB gene (23), which confers resistance to G418. Transformation of G. zeae was carried out as described by Oide et al. (23).

**Complementation.** Reintroduction of the WT C. heterostraphus NPS2 ORF was carried out with strain Chnps2-3p (Table 1), which carries a deletion of the first thiolation domain of the ChnPS2 ORF, as described by Lee et al. (18). The construct for reintroduction of the C. heterostraphus NPS2 ORF was prepared by PCR with genomic DNA and Platinum Taq High Fidelity (Invitrogen) by following the manufacturer’s directions. A 2,241-bp DNA fragment including the deleted region (342 bp) and the surrounding flanking sequences (829-bp 5’ flank and 1,070-bp 3’ flank) was amplified. Strain Chnps2-3p was transformed with approximately 5 µg of the PCR product and 10 µg of plasmid pHIP9 (23) carrying the nps2 gene. Initial screening of transformants was carried out by overlaying a 1% agar solution containing 600 µg/ml G418 on the transformants. Secondary screening of transformants was carried out on CM without salts (23) containing 400 µg/ml G418/ml. Transformants resistant to G418 were selected and screened for sensitivity to hygromycin B. Replacement of the hygB gene in G418R hygB transformants, at the nps2 locus, with the NPS2 fragment was confirmed by PCR as described previously for NPS6 (23).

**Croses.** For C. heterostraphus, croses were set up as described previously (17), except that minimal medium (MM) without glucose (MMNOS) was used instead of Sacht’s medium (21). MMNOS is easier to prepare than Sacht’s medium, and to date, no differences in the fertility of any cross tested have been observed when pseudohyphae from each medium type were compared. For G. zeae, crosses were set up as described previously (10) except that carrot medium was replaced with carrot juice (CJ) medium. In our experiments, WT G. zeae showed better fertility on CJ medium than on medium made with fresh carrots. CJ medium is 50 ml of 100% carrot juice (LakeWood, Miami, FL), 3.0 g of CaCO\(_3\), and 950 ml of H\(_2\)O per liter of medium. For mating, growing mycelial tips were placed in the centers of CJ agar plates and the plates were sealed with Parafilm and incubated under continuous black light at 24°C for 7 days. Approximately 1 ml of sterile 2.5% Tween 60 solution was applied to the plate, and mycelia growing on the plate were knocked down with a rubber policeman, which induced sexual development. Excess Tween 60 solution was discarded and the unsealed plates were incubated under the original conditions for an additional 7 days.

**Screening of all C. heterostraphus \( \Delta nps \) strains for a role in sexual development.** (i) Crosses of C. heterostraphus \( \Delta nps \) strains to the WT. The complete set of 12 \( \Delta nps \) strains was screened for a role in sexual development. For all exper-
TABLE 1. Strains used in this study

| Species and strain** | Genotype | Genetic background or reference |
|----------------------|----------|---------------------------------|
| C. heterostrophus    |          |                                 |
| C4                   | MAT1-2   |                                 |
| CB7                  | MAT1-1   |                                 |
| Chnps1-1             | Δps1 MAT1-2 hygB |                                 |
| Chnps1-2             | Δps1 MAT1-2 hygB | C4                              |
| Chnps1-8             | Δps1 MAT1-2 hygB | C4                              |
| 1447-T1-1            | Δps1 MAT1-1 hygB |                                 |
| 1448-T1-2            | Δps1 MAT1-1 hygB |                                 |
| 1448-T1-6            | Δps1 MAT1-2 hygB ab1 |                                 |
| Fgnps2-1             | Δps2 MAT1-2 hygB | C4                              |
| Fgnps2-2             | Δps2 MAT1-2 hygB | C4                              |
| Fgnps2-3**           | Δps2 MAT1-2 hygB | C4                              |
| 1449-T1-5            | Δps2 MAT1-1 hygB ab1 |                                 |
| 1449-T1-7            | Δps2 MAT1-2 hygB |                                 |
| 1451-T1-2            | Δps2 MAT1-1 hygB |                                 |
| 1451-T1-3            | Δps2 MAT1-2 hygB |                                 |
| Chnps3-3             | Δps3 MAT1-2 hygB | C4                              |
| Chnps3-4             | Δps3 MAT1-2 hygB | C4                              |
| 1437-T1-2            | Δps3 MAT1-2 hygB ab1 |                                 |
| 1437-T1-6            | Δps3 MAT1-1 hygB |                                 |
| 1438-T1-2            | Δps3 MAT1-2 hygB |                                 |
| 1438-T1-8            | Δps3 MAT1-1 hygB ab1 |                                 |
| Chnps4-3             | Δps4 MAT1-2 hygB |                                 |
| Chnps4-4             | Δps4 MAT1-2 hygB | C4                              |
| 1466-T1-2            | Δps4 MAT1-1 hygB |                                 |
| 1466-T1-6            | Δps4 MAT1-2 hygB ab1 |                                 |
| 1466-T1-7            | Δps4 MAT1-1 hygB |                                 |
| 1466-T1-8            | Δps4 MAT1-2 hygB ab1 |                                 |
| Chnps5-1             | Δps5 MAT1-2 hygB | C4                              |
| Chnps5-4             | Δps5 MAT1-2 hygB | C4                              |
| Chnps5-5             | Δps5 MAT1-2 hygB | C4                              |
| 1443-T1-3            | Δps5 MAT1-1 hygB |                                 |
| 1443-T1-7            | Δps5 MAT1-1 hygB |                                 |
| Chnps6-1**           | Δps6 MAT1-2 hygB | C4                              |
| Chnps6-2**           | Δps6 MAT1-2 hygB | C4                              |
| Chnps6-1R2           | Δps6 MAT1-1 hygB |                                 |
| Chnps7-2             | Δps7 MAT1-2 hygB | C4                              |
| Chnps7-5             | Δps7 MAT1-2 hygB | C4                              |
| 1435-T1-2            | Δps7 MAT1-1 hygB ab1 |                                 |
| 1436-T1-1            | Δps7 MAT1-1 hygB ab1 |                                 |
| Chnps8-1             | Δps8 MAT1-2 hygB | C4                              |
| Chnps8-4             | Δps8 MAT1-2 hygB | C4                              |
| 1468-T1-2            | Δps8 MAT1-1 hygB ab1 |                                 |
| 1468-T1-3            | Δps8 MAT1-1 hygB ab1 |                                 |
| Chnps9-1             | Δps9 MAT1-2 hygB | C4                              |
| Chnps9-3             | Δps9 MAT1-2 hygB | C4                              |
| 1539-T1-8            | Δps9 MAT1-1 hygB ab1 |                                 |
| Chnps10-2            | Δps10 MAT1-2 hygB | C4                              |
| Chnps10-5            | Δps10 MAT1-1 hygB | C4                              |
| 1479-T1-4            | Δps10 MAT1-1 hygB | C4                              |
| 1480-T1-6            | Δps10 MAT1-2 hygB | C4                              |
| 1480-T1-8            | Δps10 MAT1-2 hygB ab1 |                                 |
| Chnps11-5            | Δps11 MAT1-2 hygB | C4                              |
| Chnps11-7            | Δps11 MAT1-2 hygB | C4                              |
| 1450-T1-2            | Δps11 MAT1-1 hygB ab1 |                                 |
| 1450-T1-3            | Δps11 MAT1-1 hygB ab1 |                                 |
| 1452-T1-1            | Δps11 MAT1-1 hygB | C4                              |
| 1452-T1-2            | Δps11 MAT1-2 hygB ab1 |                                 |
| Chnps12-1            | Δps12 MAT1-2 hygB | C4                              |
| Chnps12-3            | Δps12 MAT1-2 hygB | C4                              |
| 1550-T1-1**          | Δps12 MAT1-1 hygB ab1 |                                 |
| G. zae               |          |                                 |
| Gz3639               | WT       | 2                               |
| Fgnps1-1-1**         | Δps1 hygB | Gz3639                          |
| Fgnps1-5-1           | Δps1 hygB | Gz3639                          |
| Fgnps2-2-5           | Δps2 hygB | Gz3639                          |
| Fgnps2-6-1           | Δps2 hygB | Gz3639                          |
| Fgnps2-6-1Δnps1-2-2-1* | Δps1 Δps2 hygB nptII | Fgnps2-6-1                     |
| Fgnps2-6-1Δnps1-5-1* | Δps1 Δps2 hygB nptII | Fgnps2-6-1                     |

** Nomenclature: Chnps1-1 is a strain in which NPS1 is deleted; -1 indicates transformant 1; 1447-T1-1 is cross no. 1447; T1-1 is tetrad no. 1, ascospore 1; for Fgnps2-6-1, nps2 refers to deletion of NPS2; 6 refers to transformant no. 6, and 1 refers to purified conidial isolate 1 of transformant 6; Fgnps2-6-1Δnps1-2-1 is single conidial isolate 1 of transformant 2 of Fgnps2-6-1Δnps1-2-1, in which the NPS1 gene was deleted in strain Fgnps2-6-1.

* Only a portion of the NPS2 ORF is deleted in this strain.

** See reference 23.

* This strain was obtained as a derivative of a cross aimed to isolate Δnps11 Δnps12, which will be reported in the future.
**FIG. 1.** *C. heterostrophus* Δ*bps2* strains form pseudothecia as well as WT strains do, but homozygous Δ*bps2* crosses fail to develop asci and ascospores. (A, B, D, E, G, H, J, K) Pseudothecia on senescent corn leaves 21 days after cross setup. Images A, D, G, and J were taken at 6.4×. (M, N) Graphs showing the number of pseudothecia and asci per pseudothecium for different crosses.
ments, crosses between C. heterostrophus strains C4 (MAT1-2) and CB7 (MAT1-1 alb1) were set up as WT controls. The fertility of Δnps strains in heterozygous crosses was evaluated by crossing each of the 12 original Δnps strain (all MAT1-2, pigmented) to tester strain CB7 (MAT1-1 alb1). At least two independent transformants were examined for each of the 12 NPS genes deleted, and each cross combination was duplicated. The ability of strains to act both male and female (as hermaphrodites) was tracked by crossing pigmented Δnps strains to albino WT testers of the opposite mating type. If a Δnps strain retains WT hermaphroditic ability, both pigmented and albino pseudothecia should form, as pseudothecium color is determined by the female partner. Thus, the ability of the pigmented Δnps strains to act female was assessed by monitoring for the presence or absence of fertile pigmented pseudothecia, while the ability of each Δnps strain to act male was assessed by monitoring for the presence or absence of fertile albino pseudothecia.

The number of pseudothecia per square centimeter of corn leaf was recorded for each cross. For the initial screening, at least 10 pigmented and 10 albino pseudothecia were opened and the number of asci in each pseudothecium was recorded. A selfed WT strain was set up for all crosses. For the initial screening, at least 10 pigmented and 10 albino WT pseudoclones were examined for each genotypic combination. The ability of each Δnps strain to act male was assessed by monitoring for the presence or absence of fertile albino pseudothecia.

(ii) C. heterostrophus Δnps × Δnps strain crosses. The fertility of C. heterostrophus Δnps strains in homozygous crosses was evaluated in essentially the same way as described for heterozygous crosses. C. heterostrophus Δnps MAT1-1 strains were obtained as follows. hygB homologous recombination were prepared from a cross between the original Δnps MAT1-2 transformants and CB7 (MAT1-1 alb1), and their mating type was determined by crossing them to MAT1-2 (strain C4) and MAT1-1 (strain CB7) testers. For each homozygous Δnps cross, at least two different combinations of independent Δnps strains were set up (e.g., for each Δnps MAT1-1, 1449-T1-1 × 1449-T1-5 and 1451-T1-2 × 1451-T1-3 were examined; see Table S2 in the supplemental material and Table 1). Each cross was duplicated.

Evaluation of the fertility of C. heterostrophus Δnps2 strains and G. zeae Δnps1, Δnps2, and Δnps1 Δnps2 strains. (i) C. heterostrophus. After the initial analyses, the fertility of Δnps2 strains was examined more extensively. The fertility of Δnps2 strains in a heterozygous cross was examined also in a MAT1-1 background in order to exclude the possibility that the observed abnormal sexual development was connected in some way to the mating type. Note that only Δnps2 strains with MAT1-2 backgrounds were examined in the initial characterization of fertility in a heterozygous cross. Ten replicates of each WT × WT, WT × Δnps MAT1-1, WT × Δnps MAT1-2, and Δnps MAT1-1 × Δnps MAT1-2 were set up, and the number and color of pseudothecia per square centimeter of leaf were recorded for each cross. The data were analyzed by analysis of variance (ANOVA). More than 30 pseudothecia were opened for each type of cross, and the number of asci in each pseudothecium was recorded. The data were statistically analyzed by ANOVA (note that no asci are formed in Δnps2 strains; hence, the data were excluded from the statistical test).

(ii) G. zeae. For homothallic G. zeae, each mutant strain was selfed and its fertility was evaluated by counting the perithecia on each plate and determining the number of asci per perithecium. A selfed WT strain was set up for all experiments as a control. For evaluation of perithecium formation, 10 plates were set up for each genotypic class (i.e., Δnps1, Δnps2, Δnps1 Δnps2, and WT) and the number of perithecia formed on each plate was recorded. Data were analyzed by ANOVA. At least 30 perithecia were opened for each genotypic class, and the number of asci was recorded for each perithecium. Data on WT and Δnps1 strains were analyzed by ANOVA.

Evaluation of fertility when iron is supplied. For C. heterostrophus, MMNOS with or without Fe(III)EDTA was prepared. Before autoclaving, stock solutions of Fe(III)EDTA (10 mM) or ferric citrate (10 mM) were added to MM medium such that a final concentration of 125 or 250 mM was achieved. Crosses were set up as described above. Induction of sexual development was carried out with 2.5% Tween 60 with or without 125 mM Fe(III)EDTA or ferric citrate. Fertility was evaluated as described above.

Evaluation of fertility when ferricrocin is supplied. For C. heterostrophus, MMNOS with or without 200 mM ferricrocin was prepared. Ferricrocin (a generous gift from H. Haas, Innsbruck Medical University, Innsbruck, Austria) was applied to the medium after autoclaving. Crosses were set up by the standard method, and fertility was evaluated as described above.

Expression analyses. Expression of C. heterostrophus NPS2 and G. zeae NPS1 and NPS2 under iron-depleted conditions was examined as described previously (23). Briefly, total RNA was extracted from 48-h cultures grown in 30 ml MM or in MM without iron. Reverse transcription-PCR was carried out with SuperScript III (Invitrogen) by following the manufacturer’s recommendations. cDNA samples were normalized on the basis of the expression of the gene that encodes C. heterostrophus glyceraldehyde phosphate dehydrogenase (GPD1) or G. zeae actin (ACT1). The primers used for this study are available on request.

Isolation, identification, and quantitative analysis of siderophores. Fungal cultures for HPLC analyses were prepared as described previously (23). Extractions of extracellular siderophores from culture broth was carried out as described by Oide et al. (23). For isolation of intracellular siderophores, freeze-dried mycelia were ground in approximately 40 ml of 15 mM potassium phosphate buffer (pH 7.5) with a pestle and a spatula and the suspension was subjected to sonication for 10 min. The insoluble fraction was removed by centrifugation, and the supernatant was collected. The insoluble fraction was resuspended in fresh potassium phosphate buffer (~40 ml) and subjected to sonication for 10 min. After centrifugation to remove the insoluble fraction, the soluble fractions (about 80 ml) were combined and subjected to siderophore extraction on XAD-16 resin as described previously (23).

For quantitative analysis of siderophore production, crude siderophore fractions were treated to solid-phase extraction as described previously (23), reconstituted in 200 μl of a 50:50 mixture of methanol and HPLC mobile components (50:50 acetonitrile–15 mM ammonium acetate, pH 4.5), filtered through a 0.4-μm-pore size polyvinylidene difluoride membrane, and then analyzed by HPLC with an injection volume of 10 μl for broth samples and 20 μl for mycelial samples. Analytical HPLC was conducted on a Polaris C18 3-μm column (250 by 4.6 mm) with a binary mobile phase consisting of acetonitrile (A) and 15 mM ammonium acetate buffer (pH 4.5) (B) with gradient elution at a flow rate of 1.0 ml/min (2.5% A for 1 min, a linear ramp up to 60% A in 13 min, 60% A for 2 min, and a linear ramp down to 2.5% A in 1 min). Detection was by absorption at 215 and 435 nm. Quantitative estimates of siderophore production were made by comparison of the areas of the siderophore standards prepared as described above.
RESULTS

Screening of the 12 *C. heterostrophus* nps deletion strains for a role in sexual development. When each of the 12 *C. heterostrophus* Δnps strains (carrying individual deletions of *NPS1* to *NPS12*) was crossed to an albino WT strain of the opposite mating type, each resulting strain was fully fertile (data not shown). Both pigmented and albino pseudothecia formed, as in WT crosses, indicating that each Δnps strain could act either male or female (compare Fig. 1A and B to D and E, data shown for the Δnps2 strain). No clear difference was observed in the average number of asci per pseudothecium of Δnps crosses, compared to that of WT crosses (compare Fig. 1C with F). No significant reduction in the number of pseudothecia formed or in the number of asci per pseudothecium was observed, whether a Δnps strain acted male (as evidenced by the presence and fertility of albino pseudothecia) or female (as evidenced by the presence and fertility of pigmented pseudothecia) (Fig. 1N, left three bars).

*C. heterostrophus* Δnps2 strains are defective in sexual development. When each of the *C. heterostrophus* Δnps strains with one of the 12 NPS genes deleted was homozygous in a cross, one type of cross (Δnps2 × Δnps2) was sterile. When any of the other 11 *C. heterostrophus* Δnps strains participated in a homozygous cross, the resulting strains were as fertile as in crosses to the WT (data not shown).

In the homozygous crosses with Δnps2 strains, WT numbers of both pigmented and albino pseudothecia were produced (Fig. 1J, K, and M, right bar); however, these were barren (Fig. 1L and N, right bar). No mature asc or ascospores developed in these pseudothecia, even when cross plates were incubated for 30 days after cross setup. WT crosses are generally mature by 21 days. Furthermore, results were the same when the deletion of the NPS2 gene was in a *MAT1-1* background (compare Fig. 1G, H, and I to D, E, and F; middle bars in panel M; data not shown for ascus/ascospore development).

Reintroduction of the *C. heterostrophus* WT NPS2 ORF into the Δnps2 strain restored WT fertility capability to the Δnps2 strain (data not shown).

*C. heterostrophus* NPS2 encodes an NRPS responsible for ferricrocin biosynthesis. Previous phylogenetic analyses indicated that *C. heterostrophus* NPS2 is closely related to NPS genes involved in ferrichrome-type siderophore biosynthesis in other fungal species (i.e., sidC of *A. nidulans* or sid2 of *U. maydis*) (18), suggesting that NPS2 also plays a role in ferrichrome-type siderophore biosynthesis in *C. heterostrophus*. In contrast to *C. heterostrophus* Δnps6 strains, which are defective in extracellular siderophore biosynthesis (23), *C. heterostrophus* Δnps2 strains do not show any increase in sensitivity to iron depletion on media including the iron chelator 2DP or BPS, compared to WT strains (Fig. 2A and B). Nevertheless, expression of ChNPS2 was very weakly induced under iron-depleted conditions (Fig. 2C), which suggested to us that *C. heterostrophus* NPS2 may have a role in iron metabolism.

![FIG. 2. Expression of NPS2 is induced by iron depletion, but deletion of NPS2 does not affect sensitivity to iron-depleted conditions.](image-url)

(A) The Δnps2 strain shows WT tolerance to the membrane-permeable iron chelator 2DP. Five-day-old cultures of *C. heterostrophus* WT, Δnps2, and Δnps6 strains are shown. The MIC of 2DP for the WT and Δnps2 strains is 300 μM, while that for the Δnps6 strain is 150 μM. No significant difference in growth on MM with 150 μM 2DP was observed between the WT and Δnps2 strains, demonstrating that the Δnps2 strain is as tolerant to 2DP as the WT is. Note that some of the plates have bubbles in the agar at the edges. (B) The Δnps2 strain shows WT tolerance to the membrane-impermeable iron chelator BPS. The average colony diameter of 5-day-old cultures grown on MM with or without 100 μM BPS is shown for *C. heterostrophus* WT, Δnps2, and Δnps6 strains. Three replicates were set up for each strain under each condition. Error bars indicate 95% confidence intervals. No significant difference in sensitivity to BPS was observed between the WT and Δnps2 strains. The Δnps6 strain shows increased sensitivity to BPS, as reported previously (23). (C) *C. heterostrophus* NPS2 and *G. zeae* NPS1 and NPS2 are up-regulated under iron-depleted conditions. Expression of *C. heterostrophus* NPS2 (ChNPS2) and *G. zeae* NPS1 and NPS2 (GzNPS1 and GzNPS2) in 48-h-old cultures grown under iron-replete (+) or iron-depleted (−) conditions is shown. ChNPS2, GzNPS1, and GzNPS2 are induced by iron depletion, while the expression levels of the controls, ChGPD1 and GzACT1, are approximately the same under both conditions.

HPLC analyses of *C. heterostrophus* WT and Δnps2 strains revealed that the Δnps2 strain lacks the ability to biosynthesize ferricrocin, the intracellular siderophore of *C. heterostrophus* (Fig. 3, peak 2). The *C. heterostrophus* Δnps2 strain, however,
still produces extracellular siderophores (broth extract, data not shown) and their presence is detectable in mycelial extracts (Fig. 3, peaks 1, 3, and 4; see Fig. 5 in reference 23), demonstrating that the NRPS encoded by \( \text{G. zeae NPS2} \) is responsible for coprogen biosynthesis. As observed previously (23), coprogen and its derivatives were detected in mycelial extracts, as well as in culture broth (not shown here). Note that coprogen and the derivatives were present in the \( \Delta nps2 \) sample, demonstrating that NRPS encoded by \( \text{NPS2} \) is not responsible for coprogen biosynthesis. au, absorbance units.

**Identification of the ortholog of \( \text{C. heterostrophus NPS2} \) in \( \text{G. zeae} \).** Of the 12 \( NPS \) genes identified in \( \text{C. heterostrophus} \), \( \text{NPS2} \) is one of the few conserved among diverse species of fungi (18). When the AMP-binding domains of \( \text{C. heterostrophus NPS2} \), \( \text{A. nidulans SidC} \), and \( \text{U. maydis Sid2} \) were used as queries in a BLAST search against the \( \text{G. zeae} \) database (http://mips.gsf.de/genre/proj/fusarium/), two \( \text{G. zeae} \) candidates (FG05372.1/fgd457-680 and FG11026.1/fgd217-330) were identified as top matches. When the three AMP-binding domains from FG05372.1 and the three AMP-binding domains from FG11026.1 were added to the data set used to build the phylogenetic tree shown in Fig. 7 of reference 18, all six AMP domains grouped in the NPS2 siderophore clade (data not shown). FG05372.1 is designated \( \text{G. zeae NPS2} \), and FG11026.1 is designated \( \text{G. zeae NPS1} \) (http://mips.gsf.de/genre/proj/fusarium/). Recent analyses of the NPS2/siderophore clade indicate that there may have been a duplication of an ancestral gene and that \( \text{GzNPS2} \) groups with the ChNPS2 lineage, while \( \text{GzNPS1} \) groups with the \( \text{A. nidulans SidC} \) and \( \text{U. maydis Sid2} \) lineage (Bushley and Turgeon, unpublished).

**NPS2 function is conserved between heterothallic \( \text{C. heterostrophus} \) and homothallic \( \text{G. zeae} \).** To examine the functional conservation of \( \text{NPS2} \), strains carrying a deletion of \( \text{G. zeae NPS2} \) were characterized with respect to sexual development in this unrelated homothallic ascomycete. Like \( \text{C. heterostrophus NPS2} \), \( \text{G. zeae NPS2} \) was weakly up-regulated under iron-depleted conditions (Fig. 2C) and \( \text{G. zeae NPS2} \) strains showed WT tolerance to iron depletion (data not shown). As observed for \( \Delta nps2 \) strains of \( \text{C. heterostrophus} \), \( \text{G. zeae NPS2} \) strains are defective in sexual development, in this case, in self-compatible sexual development. \( \text{G. zeae NPS2} \) strains form perithecia (Fig. 4D) as well as the selfed WT does (Fig. 4A), and there is no significant difference in the number of perithecia formed (Fig. 4C, left two bars). However, no mature asci or ascospores are found in these perithecia (Fig. 4E and F, middle bar), as for \( \Delta nps2 \) strains of \( \text{C. heterostrophus} \).

HPLC analyses of the \( \text{G. zeae WT} \) strain identified ferricrocin as an intracellular siderophore of this species. HPLC analyses of the \( \text{G. zeae NPS2} \) strain revealed that it completely lacks ferricrocin in its cells, demonstrating that the NRPS encoded by \( \text{G. zeae NPS2} \) is responsible for ferricrocin biosynthesis (Fig. 5, compare the top panel [arrow] to the panel second from the top). Biosynthesis of TAFC, the extracellular siderophore of \( \text{G. zeae} \), was observed in the broth extract of the \( \Delta nps2 \) strain (data not shown), indicating that the NRPS encoded by \( \text{G. zeae NPS2} \) does play a role in TAFC biosynthesis. Quantitative analyses of TAFC biosynthesis indicated that the amount of TAFC produced was increased in \( \Delta nps2 \) strains (Fig. 6A and B, second bar from the right) compared to that in WT strains (Fig. 6A and B, left bar).

**Deletion of \( \text{G. zeae NPS1} \), which is closely related to \( \text{G. zeae NPS2} \), does not affect sexual development.** \( \text{G. zeae NPS1} \) is up-regulated under iron-depleted conditions (Fig. 2C), as is \( \text{G. zeae NPS2} \), implying that the NRPS encoded by \( \text{G. zeae NPS1} \) is also involved in iron metabolism. Like \( \text{G. zeae NPS2} \) strains, \( \text{G. zeae NPS1} \) strains are as tolerant to iron depletion as the WT is (data not shown). In contrast to selfed strains of \( \text{G. zeae NPS2} \), however, selfed strains of \( \text{G. zeae NPS1} \) show WT fertility, producing perithecia (compare Fig. 4A and G) with mature asci and ascospores (compare Fig. 4B and H). No clear reduction in the number of perithecia (see Fig. S1A in the supplemental material) or in the number of asci per perithecium (see Fig. S1B in the supplemental material) was observed, compared to the WT.

Biosynthesis of both intracellular (arrow, Fig. 5, second row from the bottom) and extracellular (not shown) siderophores was observed in \( \text{G. zeae NPS1} \) strains. Quantitative HPLC analyses of WT and \( \Delta nps1 \) strains of \( \text{G. zeae} \), however, indicated that the amount of both extracellular (Fig. 6A and B, second bar from the left) and intracellular (Fig. 6C and D, right bar) siderophore made by the \( \Delta nps1 \) strain was reduced compared to that of the WT strain (Fig. 6A to D, left bar).
suggesting that the NRPS encoded by \(G. \text{zeae} \ \text{NPS1}\) is somehow involved in siderophore biosynthesis.

The sexual development phenotype of strains carrying deletions of both \(G. \text{zeae} \ \text{NPS1}\) and \(\text{NPS2}\) is identical to that of \(G. \text{zeae} \ \Delta \text{nps2}\) strains. Double deletion of \(G. \text{zeae} \ \text{NPS1}\) and \(\text{NPS2}\) does not affect tolerance to iron depletion (data not shown). The \(\Delta \text{nps1} \ \Delta \text{nps2}\) strain shows a defect in sexual development similar to that observed for \(\Delta \text{nps2}\) strains; perithecia form as in the WT and \(\Delta \text{nps2}\) strains (Fig. 4I and C, right bar; compare with panels A, D, and C, left two bars), but there are no mature ascis or ascospores (Fig. 4J and F, right bar [absence of]; compare with panels E and F, middle bar [absence of]).

As expected, \(G. \text{zeae} \ \Delta \text{nps1} \ \Delta \text{nps2}\) strains completely lacked the ability to biosynthesize ferricrocin (Fig. 5, bottom row), while they were still able to biosynthesize extracellular siderophores (not shown). Quantitative HPLC analyses showed that the amount of extracellular (Fig. 6A and B, right bar) siderophore made by the \(\Delta \text{nps1} \ \Delta \text{nps2}\) strain was increased.

**FIG. 4.** \(G. \text{zeae} \ \Delta \text{nps2}\) and \(\Delta \text{nps1} \ \Delta \text{nps2}\) strains form WT perithecia but fail to form mature ascis and ascospores. \(G. \text{zeae} \ \Delta \text{nps1}\) strains form perithecia with WT fertility. (A, D, G, I) Perithecia formed by WT, \(\Delta \text{nps2}\), \(\Delta \text{nps1}\), and \(\Delta \text{nps1} \ \Delta \text{nps2}\) selfed strains, taken at \(\times 6.4\) magnification, 14 days after cross setup; inserts, \(\times 40\) magnification. All strains form perithecia with WT morphology (compare panels D, G, and I with panel A).

(C) Average number of perithecia per plate. Ten replicates were set up for each strain. Error bars indicate 95% confidence intervals. No significant differences were observed in the number of perithecia among the WT, \(\Delta \text{nps2}\), and \(\Delta \text{nps1} \ \Delta \text{nps2}\) strains. For data on \(\Delta \text{nps1}\), see Fig. S1 in the supplemental material. (B, E, H, J) Microscope images of the contents of perithecia, taken at \(\times 500\) magnification. Crushed perithecia harvested from 14-day-old crosses were stained with lactophenol-cotton blue. Abnormal ascus-like structures were observed in perithecia of \(\Delta \text{nps2}\) and \(\Delta \text{nps1} \ \Delta \text{nps2}\) selfed strains (compare panels E and J with panel B). No ascospores were found in these ascus-like structures, in contrast to WT or \(\Delta \text{nps1}\) asci. (F) Average number of asci per perithecium. Thirty perithecia were opened for each strain. No mature asci were found in perithecia developed in \(\Delta \text{nps2}\) or \(\Delta \text{nps1} \ \Delta \text{nps2}\) selfed strains. Error bars indicate 95% confidence intervals. For data on \(\Delta \text{nps1}\) fertility, which is like that of the WT, see Fig. S1 in the supplemental material.
Exogenous application of iron partially restores fertility to *C. heterostrophus Δnps2* strains. The connection between the abnormal sexual development of *C. heterostrophus Δnps2* strains and their lack of intracellular siderophore biosynthesis was further examined by characterizing the sexual development of Δnps2 strains supplied with iron during mating. Application of 250 μM Fe(III)EDTA partially restored fertility to *C. heterostrophus Δnps2* strains (Fig. 7, compare panel B with panel D). *C. heterostrophus Δnps2* strains form pseudothecia with some mature asci and ascospores, although the number of asci per pseudothecium is less than that of a WT cross (Fig. 7E; also, compare panel D with panels A and C). No significant difference in viability was observed between the ascospores recovered from these crosses and those from WT crosses (data not shown). Note that a reduction in the number of asci per pseudothecium was observed in WT crosses when iron was applied, indicating a deleterious effect of iron overload on the sexual reproduction of *C. heterostrophus* WT strains.

Exogenous application of iron partially or completely restores fertility to *G. zeae Δnps2* and Δnps1 Δnps2 strains. Similarly, an exogenous supply of iron restores fertility to Δnps2 and Δnps1 Δnps2 strains of *G. zeae* (Fig. 8; see Fig. S2 in the supplemental material). On a plate supplied with 250 μM Fe(III)EDTA, both the *G. zeae Δnps2* and Δnps1 Δnps2 strains formed perithecia with mature asci and ascospores (see Fig. S2E and F in the supplemental material; compare to Fig. 8B and C, respectively), although the average number of asci per perithecium was less than that of a WT strain (Fig. 8). Application of 250 μM ferric citrate, however, restored WT levels of fertility to *G. zeae Δnps2* and Δnps1 Δnps2 strains (see Fig. S2H and I in the supplemental material; compare to Fig. 8B and C, respectively). No statistically significant difference (*P > 0.05*) was observed in the number of asci per perithecium among the WT, Δnps2, and Δnps1 Δnps2 strains when ferric citrate was applied (Fig. 8). Thus, the *G. zeae Δnps2* and Δnps1 Δnps2 strains show similar responses to an exogenous supply of iron, indicating that there is no significant difference between their defects in iron metabolism during sexual development.

Overall, these results obtained with *G. zeae* and the results obtained with *C. heterostrophus Δnps2* strains demonstrate that the abnormal sexual development seen is at least partly due to their deficiency in nutrient iron resulting from loss of intracellular siderophores.

Exogenous application of ferricrocin restores WT fertility to Δnps2 strains. Finally, we asked if application of ferricrocin, the intracellular siderophore of *C. heterostrophus* and *G. zeae*, is able to restore WT fertility to the Δnps2 strains. When ferricrocin was supplied, both the *C. heterostrophus* (Fig. 9) and *G. zeae Δnps2* (Fig. 10; see Fig. S3 in the supplemental material) strains were as fertile as WT strains were. No significant differences in the number of asci per *C. heterostrophus* pseudothecium (Fig. 9E) or *G. zeae* perithecium (Fig. 10) were observed between the Δnps2 and WT strains, demonstrating that

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**FIG. 5.** The NRPS encoded by *G. zeae NPS2* is responsible for intracellular siderophore biosynthesis. HPLC analyses of mycelial extracts of WT, Δnps2, Δnps1, and Δnps1 Δnps2 strains are shown. Arrows indicate the ferricrocin peak. The ferricrocin peak is missing in the Δnps2 and Δnps1 Δnps2 samples (second panel from the top, bottom panel), demonstrating that the NRPS encoded by *NPS2* is responsible for ferricrocin biosynthesis. Biosynthesis of ferricrocin (and TAFC [not shown]) is observed in the Δnps1 sample (arrow, second panel from the bottom).
the observed defect in the sexual development of Δnps2 strains is due to loss of the ability to biosynthesize ferricrocin.

**DISCUSSION**

By screening our collection of Δnps deletion strains, we have determined that a natural biological function of the intracellular siderophore ferricrocin, a metabolite biosynthesized by the NPS2 enzyme of the organisms that produce it, e.g., *C. heterostrophus* and *G. zeae*, is to participate in a fundamental fungal developmental process, i.e., production of sexual spores in meiosis. Thus, the observation that there is a close relationship between secondary-metabolite or small-molecule production and fungal development (7) is additionally documented. Furthermore, this observation provides support for the argument that products of multifunctional enzymes (e.g., NRPSs, best known as producers of secondary metabolites) can play fundamental roles in fungal cells themselves. The well-studied effects of these products on other organisms, either beneficial (e.g., as antibiotics) or harmful (e.g., as mycotoxins), may not address the primary “reason” why fungi and bacteria biosynthesize so-called secondary metabolites. The primary activities of these metabolites, one supposes, are those that benefit the fungal cell itself and not those that benefit pharmaceutical concerns.

**Ferricrocin, the intracellular siderophore of *C. heterostrophus*, plays a role in sexual development.** In this study, we characterized our collection of 12 Δnps strains of *C. heterostrophus* with respect to a possible role in sexual development and found that the Δnps2 strains fail to develop asci in homozygous Δnps2 crosses. Phylogenetic (18) and expression analyses of NPS2 indicated that the NRPS encoded by NPS2 is involved in siderophore biosynthesis. MS and HPLC analyses demonstrated that the product of the NRPS encoded by NPS2 is ferricrocin, the intracellular siderophore of *C. heterostrophus* (see Fig. 3 and 5 in reference 23). Exogenous application of iron restored the ability of *C. heterostrophus* Δnps2 strains to develop asci and ascospores, albeit at a reduced level. Exogenous application of ferricrocin itself fully restored WT fertility to the Δnps2 strains. To our knowledge, this is the first demonstration that iron and siderophores play a role in the sexual development of a heterothallic ascomycete fungus.

**Nutrient iron stored by intracellular siderophores is essential for ascus and ascospore development in *C. heterostrophus*.** Intracellular siderophores have been proposed to play a role in

![FIG. 6. Biosynthesis of intra- and extracellular siderophores is reduced in the *G. zeae* Δnps1 strain, compared to that in the WT strain.](image-url)
In this study, exogenous application of iron in the form of Fe(III)EDTA partially restored the ability of *C. heterostrophus* /H9004 nps2 strains to develop asci and ascospores, indicating that their defect in sexual development is at least partly due to loss of the ability to store iron and thus is due to starvation for nutrient iron. Whether a /H9004 nps2 strain acts male or female makes no difference in crosses between WT and /H9004 nps2 strains; these crosses are fully fertile, demonstrating that the stored iron supply in one of the two mating partners is sufficient for successful sexual development and that the mating type has no connection to the sexual developmental phenotype of /H9004 nps2 strains.

Iron storage in fungi (36). In this study, exogenous application of iron in the form of Fe(III)EDTA partially restored the ability of *C. heterostrophus* Δnps2 strains to develop asci and ascospores, indicating that their defect in sexual development is at least partly due to loss of the ability to store iron and thus is due to starvation for nutrient iron. Whether a Δnps2 strain acts male or female makes no difference in crosses between WT and Δnps2 strains; these crosses are fully fertile, demonstrating that the stored iron supply in one of the two mating partners is sufficient for successful sexual development and that the mating type has no connection to the sexual developmental phenotype of Δnps2 strains. In WT × Δnps2 crosses, iron bound to intracellular siderophores is accessible to the Δnps2 mating partner presumably only after cell-cell fusion with a WT mating partner; thus, nutrient iron stored by intracellular siderophores must be relatively unimportant for development prior to cell-cell fusion.

Furthermore, *C. heterostrophus* Δnps2 strains form WT pseudothecia even in homozygous Δnps2 crosses, demonstrating that absence of iron storage capability by siderophores does not affect pseudothecium development. The block in the sexual development of *C. heterostrophus* Δnps2 strains appears to be before initiation of ascus formation, although rare immature ascus-like structures can be found in pseudothecia of homozygous Δnps2 crosses. The block in the sexual development of *C. heterostrophus* Δnps2 strains appears to be before initiation of ascus formation, although rare immature ascus-like structures can be found in pseudothecia of homozygous Δnps2 crosses.
Iron stored by intracellular siderophores is essential for ascus/asco-spore development in *C. heterostrophus*, but dispensable for early sexual development, including pseudothecium formation. Extracellular and intracellular siderophores play fundamentally different roles in fungal developmental biology. Inhibition of extracellular siderophore biosynthesis through deletion of *NPS6* leads to a reduction of pigmentation and asexual sporulation in *C. heterostrophus* (23). By contrast, *nps2* strains show WT pigmentation and WT levels of asexual sporulation in planta, indicating that a major source of iron during asexual development of *C. heterostrophus* is iron influx through extracellular siderophores, rather than iron stored by intracellular siderophores. Extracellular siderophores are required for full virulence of *C. heterostrophus* to its host, while intracellular siderophores are dispensable for fungal infection, as evidenced by the WT virulence of the *nps2* strain to the host. Thus, iron acquired by extracellular siderophores is more important for iron metabolism of *C. heterostrophus* during sexual development. On the basis of the data presented here and our previous work on extracellular siderophores of *C. heterostrophus*, extracellular and intracellular siderophores play fundamentally different roles in fungal developmental biology.-application of ferricrocin restores WT fertility in homozygous *C. heterostrophus nps2* crosses (not shown). In summary, nutrient iron stored by intracellular siderophores is essential for ascus/asco-spore development in *C. heterostrophus* but dispensable for early sexual development, including pseudothecium formation.

**FIG. 9.** Application of ferricrocin restores WT fertility in homozygous *C. heterostrophus nps2* crosses. (A to D) Microscope images of the contents of crushed pseudothecia, taken at ×125 magnification, collected from 21-day-old cross plates stained with lactophenol-cotton blue. On plates containing 200 μM ferricrocin, *nps2* × *nps2* crosses developed pseudothecia with mature asci and ascospores (D). (E) Average number of asci per pseudothecium. Thirty pseudothecia were opened for each strain and for each condition. Error bars indicate 95% confidence intervals. When ferricrocin was supplied, no significant differences in the number of asci per pseudothecium were observed between *nps2* × *nps2* and WT × WT crosses (*P* > 0.05), demonstrating that ferricrocin restores WT fertility to the *nps2* strain. The standard was MMNOS.
heterostrophus (23), we propose that intra- and extracellular siderophores play fundamentally different roles in C. heterostrophus.

Intracellular siderophores, reactive oxygen species (ROS), and fungal sexual development. In addition to their role in iron storage, fungal intracellular siderophores are proposed to play a role also in sequestering iron to prevent Haber-Weiss/Fenton reactions, which generate highly cytotoxic hydroxyl radicals (22). The Haber-Weiss/Fenton reaction, summarized as \( \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_3^- + \text{OH}^- + \text{OH}^+ \), is catalyzed by intracellular free iron (14). To avoid this cytotoxic reaction, many organisms sequester intracellular iron in iron-binding proteins, such as transferrin and ferritin (16, 24). The antioxidant property of ferritins, through sequestration of iron, has been well characterized in bacteria, plants, and animals (3, 30). Ferritins, if present in fungi, are largely uncharacterized. A ferritin-like protein in a fungus was reported for the first time only recently (29). In fungal cells, the cytoprotective role that ferritin plays in other organisms (against hydroxy radicals produced by Haber-Weiss/Fenton reactions) is proposed for intracellular siderophores (22).

In *A. nidulans*, deletion of *sidC*, which encodes the NRPS responsible for intracellular siderophore biosynthesis, leads to an increase in intracellular free-iron levels and to hypersensitivity of mycelia to the redox-cycling agent paraquat (12). Asexual spores of an *A. nidulans* Δ*sidC* strain show hypersensitivity to ROS more clearly than mycelia (13). In our experiments, no significant difference in sensitivity to ROS was observed between WT and Δ*nps2* strains of *C. heterostrophus* during vegetative growth (see Fig. S4 in the supplemental material). Likewise, a mutant strain of *Schizosaccharomyces pombe* which lacks the ability to biosynthesize intracellular siderophores is as tolerant to paraquat as is the WT (28). The role of intracellular siderophores as an antioxidant may be more important in fungal spores than in mycelia, at least in some fungi.

Sensitivity to iron overload stress was also examined in *C. heterostrophus*, but no clear difference was observed between the WT and Δ*nps2* strains (data not shown). Nevertheless, exogenous application of iron led to reduced fertility in a homozygous WT cross of *C. heterostrophus*, suggesting that excess iron does have adverse effects on the sexual development of this fungus. It is noteworthy that exogenous application of iron bound to ferrirocin did not cause a reduction of fertility in a homozygous WT cross of *C. heterostrophus*, indirectly supporting the hypothesis of the cytoprotective role of intracellular siderophores. Exogenous application of iron only partially restored fertility to *C. heterostrophus* Δ*nps2* strains in a homozygous Δ*nps2* cross, while application of ferrirocin fully restored fertility. A simple interpretation of these results is that iron was not as efficiently taken up or distributed by fungal cells as was ferrirocin. Alternatively, the results could imply that there is an as-yet-unknown function of intracellular siderophores in fungal sexual development, in addition to their role as a source of nutrient iron. Recent studies imply a role for ROS in fungal development (reviewed in reference 1). For example, expression of *cpeA*, which encodes a catalase-peroxidase in *A. nidulans*, is induced during sexual development (26). Perhaps fungal intracellular siderophores play an antioxidant role in sexual development, in addition to their role as a source of nutrient iron.

The roles of *NPS2* in ferrirocin biosynthesis and in sexual development are conserved between the heterothallic ascomycete *C. heterostrophus* and the homothallic ascomycete *G. zeae*. To date, all of the known fungal siderophores are of the hydroxamate type (34), except for polycarboxylate siderophores produced by zygomycetes (31). Hydroxamate siderophores are largely divided into three groups, the ferrichrome type, the coprogen type, and the fusaragine type (25). Our previous study identified the gene (*NPS6*) that encodes an NRPS responsible for the biosynthesis of coprogen and its derivatives in *C. heterostrophus* (23). *NPS6* is one of only a few *NPS* genes conserved among diverse species of fungi (18). We demonstrated that the NRPSs encoded by *NPS6* orthologs are responsible for the biosynthesis of coprogen- and fusaragine-type extracellular siderophores in *A. brassicicola* and *G. zeae*, respectively, and proposed the functional conservation of *NPS6* in coprogen- or fusaragine-type siderophore biosynthesis among filamentous ascomycetes (23). In the present study, we revealed that the NRPS encoded by *C. heterostrophus* *NPS2* is responsible for biosynthesis of ferrirocin, a ferrichrome-type siderophore. *NPS2* is also conserved among diverse species of fungi, including ascomycetes and basidiomycetes (18). The present study and an independent study by Tobiasen et al. (32) identified ferrirocin as the product of the NRPS encoded by *G. zeae* *NPS2* Δ*nps2* and Δ*nps2*, to which *NPS2* genes show strong similarity, encode the NRPSs responsible for the biosynthesis of ferrirocin and ferrichrome in *A. nidulans* and *U. maydis*, respectively (12, 38). Schwecke et al. (28) demonstrated that the NRPS encoded by the *NPS2* ortholog of *S. pombe* (sib1) is responsible for ferrichrome biosynthesis. On the basis of in silico analyses, they also predicted that the NRPSs encoded by *NPS2* homologs are likely to produce similar products in different species of fungi. Taking the findings together, we propose that the function of the NRPS encoded by *NPS2* in ferrichrome-type siderophore biosynthesis is conserved among diverse species of fungi.

Our study on *NPS6* demonstrated that the role of extracellular siderophores in fungal virulence to plant hosts is conserved among filamentous ascomycetes (23). Here, we demonstrated that the role of intracellular siderophores in sexual development is conserved in the heterothallic ascomycete *C. heterostrophus* and the homothallic ascomycete *G. zeae*. Deletion of the *NPS2* ortholog in *G. zeae* resulted in concomitant defects in intracellular siderophore biosynthesis and ascus/ascospore development, as observed in *C. heterostrophus* Δ*nps2* strains. Application of ferrirocin fully restored WT fertility to the *G. zeae* Δ*nps2* strain. Recently, Eisendle et al. (13) reported that a Δ*sidC* strain is impaired in sexual development in the homothallic ascomycete *A. nidulans*. Application of ferrirocin restored the ability of the Δ*sidC* strain to undergo WT sexual development. Whether or not intracellular siderophores play a role in sexual development also in other species, such as the basidiomycete *U. maydis* and the fission yeast *S. pombe*, is the subject of future studies.

*GzNPS1* has a potential role in the siderophore biosynthesis of *G. zeae*. Like *G. zeae* *NPS2*, *G. zeae* *NPS1* shows high similarity to *A. nidulans* *sidC* and *U. maydis* *sid2* and groups with these genes and *C. heterostrophus* *NPS2* in phylogenetic anal-
yses, suggesting a role in ferrichrome-type siderophore biosynthesis. Tobiasen et al. (32) proposed also that G. zeae NPS1 is likely to encode the NRPS involved in siderophore biosynthesis, although no functional data were provided. Indeed, we found that G. zeae NPS1 is strongly up-regulated under iron-depleted conditions, further indicating its involvement in iron metabolism. In this study, however, we failed to obtain clear evidence that the NRPS encoded by G. zeae NPS1 has a role in siderophore biosynthesis. Deletion of NPS6 in G. zeae abolished extracellular siderophore biosynthesis (23), and deletion of G. zeae NPS2 led to complete loss of intracellular siderophore biosynthesis, demonstrating that the NRPSs encoded by NPS6 and NPS2 fully account for siderophore biosynthesis in G. zeae. Consistently, HPLC analyses of G. zeae Δnps1 strains confirmed the biosynthesis of ferricrocin and TAFc, the major intracellular and extracellular siderophores of G. zeae. Furthermore, any characteristic phenotype (sensitivity to low-iron and oxidative stress) associated with iron was not observed in G. zeae Δnps1 strains and the phenotype of G. zeae Δnps1 Δnps2 strains is identical to that of Δnps2 strains.

Nevertheless, quantitative HPLC analyses revealed an interesting difference in the levels of siderophore biosynthesis between WT and Δnps1 strains of G. zeae. When the amounts of siderophore biosynthesized per liter of culture broth, or per gram of mycelia, were compared, the amount of extracellular siderophore produced by G. zeae Δnps1 strains was approximately half of the amount produced by WT strains (Fig. 6A and B). Similarly, G. zeae Δnps1 strains produced only a quarter of the amount of intracellular siderophore produced by WT strains (Fig. 6C and D). Ferricrocin and TAFc are both trihydroxamate siderophores, and the proposed role of the NRPSs encoded by NPS2 and NPS6 is to conjugate 3 units of N\(^2\)-acyl-N\(^5\)-hydroxy-L-ornithine (AHO), the direct precursor of fungal trihydroxamate siderophores. Perhaps the NRPS encoded by NPS1 is partially functional and able to biosynthesize dihydroxamates but not trihydroxamates. These dihydroxamates may then be used by the NRPSs encoded by NPS2 and NPS6 to biosynthesize trihydroxamate siderophores via incorporation of a single AHO unit. Thus, perhaps the contribution of the NRPSs encoded by NPS1 may be in achieving efficient siderophore biosynthesis. This hypothesis can be tested by examining the biosynthesis of dihydroxamates in Δnps2 Δnps6 and Δnps1 Δnps2 Δnps6 strains of G. zeae. If the hypothesis is supported, production of dihydroxamates should be detected only in the former. Alternatively, the function of G. zeae NPS1 could be examined in the C. heterostrophus Δnps2 strain by separately expressing the heterologous G. zeae NPS1 and NPS2 genes to see if they both complement (i.e., restore the WT phenotype to the C. heterostrophus Δnps2 strain), which would indicate functional redundancy.

Quantitative HPLC analyses also pointed out that Δnps2 and Δnps1 Δnps2 strains produce more TAFc than WT G. zeae strains (Fig. 6). A similar observation was made in the qualitative analyses of siderophore biosynthesis in WT and Δnps6 strains of C. heterostrophus. In this case, C. heterostrophus Δnps6 strains produced more intracellular siderophores than WT strains did (23; see Table S3 in the supplemental material). These results are due, most likely, to a change in the flow of AHO, the common precursor of intra- and extracellular siderophores. In the G. zeae Δnps2 strain, inhibition of the pathway for intracellular siderophore production probably led to an increase in AHO flow to the NRPS encoded by NPS6, resulting in an increase in TAFc biosynthesis. These results indicate that deletion of an NPS gene affects the biosynthesis of a metabolite which is not the product of the NRPS encoded by the NPS gene. Thus, reduction of siderophore biosynthesis in G. zeae Δnps1 strains could be due to secondary effects of deletion of NPS1.

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