Iron, Oxidative Stress, and Virulence: Roles of Iron-Sensitive Transcription Factor Sre1 and the Redox Sensor ChA1 in the Maize Pathogen *Cochliobolus heterostrophus*

Ning Zhang,^{1,2} Mohd Zainudin NurAinIzzati,^{1,3} Keren Scher,⁴ Bradford J. Condon,¹ Benjamin A. Horwitz,⁴ and B. Gillian Turgeon¹

¹Plant Pathology & Plant-Microbe Biology, Cornell University, Ithaca, NY 14853, U.S.A.; ²Department of Plant Biology & Pathology, Rutgers University, New Brunswick, NJ 08901, U.S.A.; ³Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 Serdang Selangor, Malaysia; ⁴Department of Biology, Technion, Haifa 32000, Israel

Submitted 23 February 2013. Accepted 17 August 2013.

The gene *SRE1*, encoding the GATA transcription factor siderophore biosynthesis repressor (Sre1), was identified in the genome of the maize pathogen *Cochliobolus heterostrophus* and deleted. Mutants were altered in sensitivity to iron, oxidative stress, and virulence to the host. To gain insight into mechanisms of this combined regulation, genetic interactions among *SRE1* (the nonribosomal peptide synthetase encoding gene *NPS6*, which is responsible for extracellular siderophore biosynthesis) and *ChA1* (encoding a transcription factor regulating redox homeostasis) were studied. To identify members of the Sre1 regulon, expression of candidate iron and oxidative stress-related genes was assessed in wild-type (WT) and *sre1* mutants using quantitative reverse-transcription polymerase chain reaction. In *sre1* mutants, *NPS6* and *NPS2* genes, responsible for siderophore biosynthesis, were derepressed under iron replete conditions, whereas the high-affinity reductive iron uptake pathway associated gene, *FTR1*, was not, in contrast to outcomes with other well-studied fungal models. *C. heterostrophus* L-ornithine-N⁵-monooxygenase (*SIDA2*), ATP-binding cassette (*ABC6*), catalase (*CAT1*), and superoxide dismutase (*SOD1*) genes were also derepressed under iron-replete conditions in *sre1* mutants. *Chap1nps6* double mutants were more sensitive to oxidative stress than either *Chap1* or *nps6* single mutants, while *Chap1sre1* double mutants showed a modest increase in resistance compared with single *Chap1* mutants but were much more sensitive than *sre1* mutants. These findings suggest that the *NPS6* siderophore indirectly contributes to redox homeostasis via iron sequestration, while Sre1 misregulation may render cells more sensitive to oxidative stress. The double-mutant phenotypes are consistent with a model in which iron sequestration by *NPS6* defends the pathogen against oxidative stress. *C. heterostrophus sre1*,

nps6, *Chap1*, *Chap1nps6*, and *Chap1sre1* mutants are all reduced in virulence toward the host, compared with the WT.

NPS6 and iron acquisition.

Extracellular siderophores have been documented as virulence factors for some (Greenshields et al. 2007; Hwang et al. 2008; Oide et al. 2006) but not all (Eichhorn et al. 2006; Mei et al. 1993) fungal pathogens. In the maize pathogen *Cochliobolus heterostrophus*, the extracellular siderophore coprogen requires nonribosomal peptide synthetase (Nps)6 for biosynthesis. Mutants deleted for the corresponding gene (*NPS6*), as might be expected, have decreased growth when iron is limited. They are also more sensitive to oxidative stress, and are reduced in virulence to the host compared with the wild type (WT) (Oide et al. 2006). Iron and oxidative stresses are interrelated. Fe²⁺ and Fe³⁺ catalyze, by nonenzymatic pathways collectively referred to as Fenton reactions, interconversion of reactive oxygen species (ROS). Fe²⁺ is oxidized to Fe³⁺ by hydrogen peroxide (H₂O₂), and Fe³⁺ is reduced by superoxide to Fe²⁺, regenerating dioxygen (Barbusinski 2009; Pierre and Fontecave 1999).

The association between iron and ROS is pivotal in the interface between a pathogen and its host. For some biotrophic fungus–cereal interactions, it has been demonstrated that Fe³⁺ accumulates at infection sites (Liu et al. 2007). This “Fe burst,” reminiscent of the well-studied oxidative burst, is apparently plant-host derived. Where it has been studied, Fe has been shown to accrue in epidermal cells at the infection site, while mesophyll cells of the host become depleted of iron, as inferred from the expression of two reporter genes 24 h postinoculation of wheat with *Blumeria graminis* f. sp. *tritici* (Liu et al. 2007). The co-presence of ROS and Fe³⁺ could be very effective against an invading pathogen (Liu et al. 2007). In a diametrically opposed mechanism, pathogen and host may compete for iron, and sequestering of Fe by the host makes it unavailable for the pathogen. This mechanism is well established in the medical literature; transferrin sequesters iron in the plasma and ferritin sequesters iron within host cells (Weinberg 2009). To extract host-bound iron and thus compete, pathogens secrete high-affinity siderophores or use membrane-bound reductive iron assimilation (RIA) mechanisms. Although

N. Zhang and M. Z. NurAinIzzati contributed equally to this work. B. A. Horwitz and B. G. Turgeon co-directed the research.

Corresponding author: B. G. Turgeon; E-mail: bgt1@cornell.edu

*The e-Xtra logo stands for “electronic extra” and indicates that three supplementary tables and two supplementary figures are published online and that Figures 2 through 6 appear in color online.

the *C. heterostrophus* genome carries genes encoding the proteins required for both mechanisms, virulence depends on siderophores, as noted above, as it does in the mammalian pathogen *Aspergillus fumigatus* (Schrettl et al. 2010). The bZIP regulator HapX, expressed under Fe starvation, is essential for *A. fumigatus* to attain full virulence to mice, suggesting that the pathogen needs to overcome Fe limitation in the host (Schrettl et al. 2010). Fe deficiency in *Arabidopsis thaliana* makes it more difficult for both *Dickeya dadantii* and *Botrytis cinerea* to attack, apparently due to Fe limitation for the pathogen rather than an enhanced plant defense response. Ferritin expression is increased during infection, suggesting competition for iron in the plant-pathogen interaction (Kieu et al. 2012).

Sre and iron homeostasis.

The siderophore biosynthesis repressor (Sre), a GATA transcription factor, is known, in some fungi, to detect available iron levels and regulate iron acquisition (An et al. 1997; Chao et al. 2008; Labbe et al. 2007; Pelletier et al. 2002; Schrettl et al. 2008). In *Aspergillus nidulans*, genes involved in siderophore biosynthesis and uptake are repressed under iron-sufficient conditions in the WT and derepressed in an *sreA* mutant (Haas et al. 1999). In the *sreA* mutant of the human pathogen *A. fumigatus*, genes for iron uptake are derepressed in the presence of iron, and some genes in antioxidative pathways are upregulated (Schrettl et al. 2008).

ChAP1 and redox homeostasis.

The *C. heterostrophus* ortholog (ChAp1) of the yeast redox-sensitive transcription factor Yap1 responds to H₂O₂ levels (Lev et al. 2005; Shanmugam et al. 2010). *ChAP1/YAP1* orthologs have been studied in several other fungal pathogens of plants (Cho et al. 2009; Molina and Kahmann 2007; Temme and Tudzynski 2009). According to the yeast paradigm, oxidation of vicinal cysteine residues in the C-terminal domain of ChAp1p masks a nuclear export signal, resulting in nuclear retention. In *Saccharomyces cerevisiae*, a peroxiredoxin acts as an intermediate, oxidized first and, in turn, oxidizing ChAP1. The corresponding glutathione-dependent phospholipid peroxidase, Hyr1 is a virulence factor for *Magnaporthe oryzae* (Huang et al. 2011). In *C. heterostrophus*, the return to redox homeostasis is delayed by lack of ChAp1, as measured by expression of a genetically encoded redox reporter in the WT and a *Chap1* mutant (Ronen et al. 2013).

Interrelationships among iron, oxidative stress, and virulence.

To address the issue of why *C. heterostrophus nps6* mutants are hypersensitive to oxidants, an understanding of the signaling pathways and associated transcription factors by which fungal cells respond to and contend with Fe and ROS stresses is needed. In this article, we describe manipulation of *SRE1*, the ortholog of *A. nidulans SreA*, in *C. heterostrophus*. We report quantitative reverse-transcription polymerase chain reaction (qRT-PCR) studies of 31 genes associated with iron and oxidative stress and compare their expression in WT and the *sre1* mutant. We show that loss of *SRE1* deregulates a subset of iron and oxidative stress-responsive genes, including *NPS6*, but not the high-affinity iron permease encoding gene, *FTR1*, associated with the RIA mechanism of iron acquisition.

If an increased level of oxidants inside the cell (compared with the WT under the same conditions) is the explanation for hypersensitivity of the *C. heterostrophus* extracellular siderophore *nps6* mutant to oxidative stress, we would predict that *Chap1nps6* double mutants would be more sensitive than either single *Chap1* or *nps6* mutants. If excess free Fe⁺⁺⁺ is responsible for oxidant sensitivity of *nps6* mutants, overexpres-

sion of the iron regulon in *sre1* and *Chap1sre1* double mutants should make the fungus more resistant to oxidative stress than *Chap1* mutants. We used a genetic approach to address and corroborate both predictions.

RESULTS

SRE1 bioinformatic and phylogenetic analyses.

A BLASTP query using the *A. nidulans* SreA amino acid sequence against the protein catalog of *C. heterostrophus* reference strain C5 identified a homologous sequence (protein ID 109473, E value = 1.91E-41, score = 796). The corresponding gene, *SRE1*, was used to identify the ortholog (Joint Genome Institute [JGI] ID number 135674) in isogenic *C. heterostrophus* C4, which was used to construct (below) the *sre1* deletion strain used in this work. The Sre1 predicted protein sequences of *C. heterostrophus* race T strain C4 and race O strain C5 are 100% identical.

The phylogenetic relationship of the predicted Sre1 protein to other similar proteins is shown in Figure 1. The predicted Sre1 protein groups with 80% bootstrap support with other Sre-like proteins such as *A. nidulans* SreA (*Emericella nidulans*), which supports orthology. The *SRE1* open reading frame (ORF) is predicted to be 1,563 bp, with two introns (101 and 65 bp). A Pfam sequence search detected two GATA Zn finger domains, starting at residues 131 and 287, with E values of 9.2 E-15 and 2.7E-17, respectively.

Deletion of *SRE1* and complementation of the *sre1* mutant.

Candidate independently isolated hygromycin B (HygB)-resistant *sre1* mutant strains (*sre1-1*, *sre1-2*, *sre1-3*, and *sre1-4*) (Table 1) were chosen following the targeted gene deletion experiment and each was purified by subculturing from a single conidium. Deletion of *SRE1* in transformants was confirmed by diagnostic PCR (discussed below; Supplementary Table S1) with the following primer pairs: i) ChSREAF1/ChSREAR1, ii) NLC37/NLC38, iii) ChpSREAU1/NLC37, and iv) ChSREAD1/NLC38. The first primer pair confirmed the absence of *SRE1* in candidate mutants and the second confirmed the presence of the selectable marker *hygB*. The third and fourth primer pairs confirmed correct insertion of the deletion cassette carrying *hygB* into the 5' and 3' flanks, respectively, of the native *SRE1* locus.

The *sre1* mutants showed an approximately 34% decrease in radial growth rates compared with WT strains ($P < 0.002$). Ability of conidia to germinate was comparable in mutant and WT strains; by 3 h, each showed 100% germination on glass slides.

For complementation, *sre1* mutant strain *sre1-2-1* was transformed following the scheme shown in Supplementary Fig. S1A. Candidate HygB-sensitive, G418-resistant strains were identified. Two such candidates, *sre1-2[SRE1]-1* and *sre1-2[SRE1]-4*, were restored to WT growth characteristics and confirmed by diagnostic PCR, as described below, to be complemented strains.

Sre1 regulation of gene expression.

Expression of 29 genes hypothesized to be involved in iron or oxidative stress regulation was measured and compared in the WT and an *sre1* mutant strain, grown under iron-depleted and replete conditions (Fig. 2; Table 2). Four genes—*NPS6*, L-ornithine-N⁵-monooxygenase (*SIDA2*), ATP-binding cassette gene *ABC6*, and *FTR1*—showed clear repression by iron in the WT. *NPS6*, *SIDA2*, and *ABC6* are adjacent to each other in the genome and our data (N. Zhang, B. J. Condon, and B. G. Turgeon, unpublished) indicate that the latter is involved in transport of coprogen out of the cell. All three genes are derepressed under iron-sufficient conditions in the *sre1* mutant

(Fig. 2). Expression of the *FTR1* gene involved in RIA iron uptake was not altered in the *sre1* mutant, which indicates that not all repression by iron is Sre1 dependent (Fig. 2). Expression of *NPS2*, the gene for intracellular siderophore biosynthesis, was upregulated under iron-sufficient conditions in the *sre1* mutant but not in the WT (Fig. 2). *CAT1*, encoding one of three catalases (CAT) in the genome (Robbertse et al. 2003), was significantly upregulated in the *sre1* mutant in +Fe conditions (Fig. 2). Genes that were modestly influenced by iron or

lack of Sre1 include *ABC39*, *PKS1*, *NOX1*, *NOX2*, *NOX4*, *NOXR*, *VEL3*, *FET1*, *SOD1*, and *SOD2* (Fig. 2; Table 2).

Overexpression of *CAT1* in the *sre1* mutant in Fe-replete conditions could be due to oxidative stress. If so, one would expect other genes with antioxidant functions to be overexpressed as well. In a second set of experiments, we compared expression of two previously studied targets of the redox homeostasis regulator ChApl: a glutathione reductase gene (*GLR1*) and a thioredoxin reductase gene (*TRR1*). *GLR1* and

Fig. 1. *Cochliobolus heterostrophus* Sre1 groups with orthologs in other fungal species. The most parsimonious tree based on the predicted amino acid sequences of *C. heterostrophus* C5 Sre1 (SRE1) and similar iron regulators in other fungal species. Bootstrap values equal or higher than 50% are labeled on the branches. *Cochliobolus* spp. protein ID numbers are from the Joint Genome Institute; all others are from GenBank.

TRR1 were not affected by *SRE1* loss (data not shown). The expression patterns of *NPS6*, *SRE1*, and *CAT1* in the second experiment confirmed those shown in Figure 2. The expression of *SRE1* increased by 4.5-fold in Fe-replete compared with Fe-depleted conditions (Fig. 2), and this was confirmed in the second data set (6.6-fold; data not shown).

***Chap1* and *nps6* mutations contribute additively to oxidative stress sensitivity.**

Double *Chap1nps6* mutants, created by crossing *Chap1* and *nps6* single mutants (as described below), were more sensitive to H_2O_2 than *Chap1* mutants, and *Chap1* mutants were more sensitive than *nps6* mutants (Fig. 3; Supplementary Fig. S2). Thus, double *Chap1nps6* mutant strains are more sensitive than either single mutant parent, with approximately twofold less growth than *Chap1* at 1 mM and no growth at 2 mM H_2O_2 (Fig. 3). WT, *Chap1*, and *nps6* progeny from the cross are similar to the corresponding parents. This relationship indicates that the ChAp1 and Nps6 proteins have additive contributions to oxidative stress resistance. Their growth phenotypes on complete medium with xylose (CMX) are illustrated in Figure 4; the *Chap1nps6* mutant grows as well as either single mutant or WT.

Absence of *Sre1* partially complements *Chap1* mutant oxidative stress sensitivity.

Loss of *Sre1* releases expression of *NPS6* and *NPS2* in the *sre1* mutant in Fe-replete conditions (Fig. 2; Table 2). Assuming that there is a corresponding increase in extracellular and intracellular siderophore levels, this should promote sequestering of iron. Improved sequestering of iron should make the fungus less sensitive to extracellular H_2O_2 (including that deployed by the host), in a model where free iron promotes oxidative damage via the Fenton reactions. Therefore, we tested whether loss of *Sre1* might compensate to some extent for loss of ChAp1. At a concentration of 2 mM, *Chap1* progeny from a cross (number 1756) between *Chap1* and *sre1* mutant parents showed extreme sensitivity to oxidative stress, while double

Chap1sre1 mutant progeny were slightly but significantly less sensitive (Fig. 5) and *sre1* mutant progeny were considerably less sensitive than *Chap1* or the double mutant (Fig. 5). Although the radial growth rates of both *sre1* and *Chap1sre1* strains were less than the WT (Fig. 4), resistance to H_2O_2 at 2.0 mM was higher in *Chap1sre1* than in the single *Chap1* mutant, as shown by approximately twofold increased growth at 2 mM H_2O_2 in two double-mutant progeny compared with the *Chap1* strains (Fig. 5). Thus, the *sre1* mutation is partially epistatic to the *Chap1* mutation. This is not a large effect but it does support a model in which increased expression of *NPS6* in the *sre1* mutant compensates for *Chap1* sensitivity to oxidative stress. Observation of enhanced resistance in the double mutant at only a moderate level of H_2O_2 but not at higher or lower levels suggests that opposing contributions of the loss of *SRE1* add to give the observed result.

Virulence of single and double mutants is less than the WT.

The *nps6*, *Chap1*, and *Chap1nps6* mutant progeny of cross 1756 produced lesions approximately twofold smaller than those of WT progeny. We reported previously that a *Chap1* mutant produced lesions the same size as those produced by the WT (Lev et al. 2005). We speculate that the discrepancy between the two experiments results from our use of different host lines. We know that, for *nps6* mutants, lesion size, as well as the ratio of mutant lesion size over WT lesion size, depends on the maize host genotype (B. J. Condon, B. A. Horwitz, and B. G. Turgeon, unpublished).

The *nps6* virulence data are in agreement with previous data (Oide et al. 2006) and serve as an internal control (Fig. 6). Thus, in contrast to in vitro oxidant sensitivity (Fig. 3), loss of *NPS6* does not exacerbate the *Chap1* virulence phenotype. This implies that, in planta, the phenotypes depend on additional factors.

Loss of *Sre1*, on the other hand, caused a modest but significant decrease in lesion size that corresponds nicely (approximately 35%) to the decrease in radial colony growth rate

Table 1. Fungal strains^z

Strain	Genotype	Genetic background or reference
C4	<i>MAT1-2</i>	ATCC 48331 (Leach et al. 1982)
C5	<i>MAT1-1</i>	ATCC 48332 (Leach et al. 1982)
CB7	<i>MAT1-1, alb1</i>	WT, B30-A3-R-20, albino (Wu et al. 2012)
CB11	<i>MAT1-2, alb1</i>	WT, B30-A3-R-11 (Oide et al. 2010)
<i>sre1-1</i>	<i>sre1, MAT1-2, hygB</i>	C4
<i>sre1-2</i>	<i>sre1, MAT1-2, hygB</i>	C4
<i>sre1-3</i>	<i>sre1, MAT1-2, hygB</i>	C4
<i>sre1-4</i>	<i>sre1, MAT1-2, hygB</i>	C4
<i>sre1-2[SRE1]-1</i>	<i>SRE1, MAT1-2, NPTII</i>	<i>sre1-2-1</i> , a single conidiated strain of <i>sre1-2</i> , above
<i>sre1-2[SRE1]-4</i>	<i>SRE1, MAT1-2, NPTII</i>	<i>sre1-2-1</i> , a single conidiated strain of <i>sre1-2</i> , above
<i>Chnps6-3</i>	<i>nps6, MAT1-2, hygB</i>	C4 (Oide et al. 2006)
1689-R10	<i>Chap1, MAT1-1, hygB</i>	<i>Chap1</i> (Lev et al. 2005)
1756-1-1	<i>Chap1, MAT1-2, hygB</i>	This study
1756-1-2	WT, <i>MAT1-1</i>	This study
1756-1-3	<i>Chap1nps6, MAT1-1, hygB</i>	This study
1756-1-4	<i>Chap1nps6, MAT1-1, hygB</i>	This study
1756-1-5	<i>nps6, MAT1-2, hygB</i>	This study
1756-1-6	<i>nps6, MAT1-2, hygB</i>	This study
1756-1-7	<i>Chap1, MAT1-2, hygB</i>	This study
1756-1-8	WT, <i>MAT1-1</i>	This study
1757-1-1	WT, <i>MAT1-1</i>	This study
1757-1-2	<i>Chap1, MAT1-1, hygB^r</i>	This study
1757-1-3	<i>Chap1sre1 MAT1-2, hygB^r</i>	This study
1757-1-4	<i>sre1, MAT1-2, hygB^r</i>	This study
1757-1-5	<i>Chap1, MAT1-1, hygB^r</i>	This study
1757-1-6	WT, <i>MAT1-1</i>	This study
1757-1-7	<i>Chap1sre1, MAT1-2, hygB^r</i>	This study
1757-1-8	<i>sre1, MAT1-2, hygB^r</i>	This study

^z *hygB^r* = hygromycin B resistant, ATCC = American Type Culture Collection, and WT = wild type.

Fig. 2. Expression of genes regulated by Sre1 under conditions of high and low iron. Each bar represents at least three biological and two technical replicates. WT-Fe: wild type under iron-depleted condition, WT+Fe: WT under iron-replete condition, *sre1*-Fe: *sre1* deletion mutant under iron-depleted condition, *sre1*+Fe: *sre1* deletion mutant under iron-replete condition. Expression levels are relative to WT+Fe (set to 1 for each gene), and comparisons are made across treatments for a single gene at a time. Note differences in scale; in cases in which expression is very low in the WT+Fe condition, the corresponding bar is not visible in the graph. Treatments sharing the same letter are not significantly different according to analysis of variance at $P < 0.05$. *HISH3* was used as the reference gene. *SRE1* is not expressed in the *sre1*-deletion strain, as expected, and is upregulated in the WT under +Fe conditions. Note that *ABC6*, *FTR1*, *NPS6*, and *SIDA2* are upregulated in low Fe conditions and that *ABC6*, *CAT1*, *NPS2*, *NPS6*, *SIDA2*, and *SOD1* are derepressed in the *sre1* mutant compared with the WT under Fe-replete conditions.

in vitro. Cross 1757 progeny showed that *Chap1* mutants were more reduced in virulence than *sre1* mutants and that *Chap1sre1* double mutants were slightly more reduced in virulence than *Chap1* single mutants. None of the lesion measurements was statistically significant one from the other but all

were different from the WT. Because *Chap1* mutants are in both the 1756 and 1757 crosses, they serve as a robust internal control (Fig. 6).

Lesion type and characteristics vary for the WT and all the mutants. The lesions on leaves inoculated with the WT have

Table 2. Comparison of gene expression in the wild type (WT) and the *sre1* mutant under iron-depleted and replete conditions

Gene	Putative function	Strain and Fe condition ^x	Expression ^y	Standard error	Fe regulated?	Sre1 regulated?
<i>NPS2</i>	Nonribosomal peptide synthetase, intracellular siderophore biosynthesis	WT-Fe	1.2 a	0.48	No, in WT	Yes, up- in <i>sre1</i> in +Fe
		WT+Fe	1.0 a	0.14	Yes, in <i>sre1</i>	
		<i>sre1</i> -Fe	2.2 a	0.29		
		<i>sre1</i> +Fe	9.4 b	0.56		
<i>NPS6</i>	Nonribosomal peptide synthetase, extracellular siderophore biosynthesis	WT-Fe	58.1 bc	32.33	Yes, in WT and <i>sre1</i>	Yes, derepressed in <i>sre1</i> in +Fe
		WT+Fe	1.0 a	0.14		
		<i>sre1</i> -Fe	72.8 b	14.02		
		<i>sre1</i> +Fe	18.1 c	4.28		
<i>NPS8^z</i>	Nonribosomal peptide synthetase	WT-Fe	0.1	NA	ND	ND
		WT+Fe	1.0	2.97		
		<i>sre1</i> -Fe	5.3	NA		
		<i>sre1</i> +Fe	2.8	NA		
<i>NPS10</i>	Nonribosomal peptide synthetase	WT-Fe	1.0 a	0.32	No	No
		WT+Fe	1.0 a	0.06		
		<i>sre1</i> -Fe	1.5 a	0.09		
		<i>sre1</i> +Fe	1.2 a	0.09		
<i>SIDA1</i>	Flavin-containing monooxygenase	WT-Fe	4.5 ab	1.69	No	No
		WT+Fe	1.0 a	0.15		
		<i>sre1</i> -Fe	5.6 b	1.22		
		<i>sre1</i> +Fe	2.5 ab	0.66		
<i>SIDA2</i>	Flavin-containing monooxygenase	WT-Fe	22.7 b	9.41	Yes, in WT and <i>sre1</i>	Yes, derepressed in <i>sre1</i> in +Fe
		WT+Fe	1.0 a	0.23		
		<i>sre1</i> -Fe	19.8 b	4.70		
		<i>sre1</i> +Fe	8.1 b	2.60		
<i>ABC6</i>	ATP-binding cassette	WT-Fe	11.0 b	3.98	Yes, in WT and <i>sre1</i>	Yes, derepressed in <i>sre1</i> in +Fe.
		WT+Fe	1.0 a	0.29		
		<i>sre1</i> -Fe	32.4 c	8.44		
		<i>sre1</i> +Fe	11.0 b	4.50		
<i>ABC39</i>	ATP-binding cassette	WT-Fe	2.3 a	0.73	No, in WT Yes, in <i>sre1</i>	Yes, up- in <i>sre1</i> in -Fe
		WT+Fe	1.0 a	0.15		
		<i>sre1</i> -Fe	3.7 b	0.29		
		<i>sre1</i> +Fe	1.7 a	0.31		
<i>FTR1</i>	High affinity iron permease	WT-Fe	21.4 b	8.64	Yes in WT and <i>sre1</i>	No, repressed in both WT and <i>sre1</i> in +Fe
		WT+Fe	1.0 a	0.22		
		<i>sre1</i> -Fe	24.2 b	7.43		
		<i>sre1</i> +Fe	2.2 a	0.61		
<i>OPT1</i>	Oligopeptide transporter	WT-Fe	1.5 a	0.47	No	No
		WT+Fe	1.0 a	0.15		
		<i>sre1</i> -Fe	1.6 a	0.22		
		<i>sre1</i> +Fe	1.2 a	0.39		
<i>LAE1</i>	S-adenosylmethionine-dependent methyltransferase activity	WT-Fe	0.8 a	0.06	No	No
		WT+Fe	1.0 a	0.08		
		<i>sre1</i> -Fe	0.8 a	0.09		
		<i>sre1</i> +Fe	0.7 a	0.20		
<i>PKS1</i>	Polyketide synthase	WT-Fe	3.8 a	1.16	Yes, in WT Yes, in <i>sre1</i>	Yes, up- in <i>sre1</i> mutant in -Fe
		WT+Fe	1.0 a	0.59		
		<i>sre1</i> -Fe	9.8 b	3.39		
		<i>sre1</i> +Fe	0.3 a	0.26		
<i>NOX1</i>	Ferric reductase, NADH/NADPH oxidase	WT-Fe	0.9 ab	0.08	No, in WT Yes, in <i>sre1</i>	Yes, up- in <i>sre1</i> mutant in -Fe
		WT+Fe	1.0 ab	0.21		
		<i>sre1</i> -Fe	1.2 a	0.20		
		<i>sre1</i> +Fe	0.6 b	0.14		
<i>NOX2</i>	Ferric reductase, NADH/NADPH oxidase	WT-Fe	0.4 b	0.08	Yes, in WT No, in <i>sre1</i>	Yes, up in WT in +Fe
		WT+Fe	1.0 a	0.14		

(continued on next page)

^x WT-Fe = WT under iron-depleted condition, WT+Fe = WT under iron-replete condition, *sre1*-Fe = *sre1* deletion mutant under iron-depleted condition, and *sre1*+Fe = *sre1* deletion mutant under iron-replete condition.

^y Average gene expression levels (in fold) with no letter in common are significantly different ($P < 0.05$). *HISH3* was used as the control gene in the expression analysis.

^z Fewer than three replicates data were obtained for the *NPS8* gene and statistical analyses were not conducted.

dark-brown cores surrounded by large, beige necrotic areas (Fig. 6) and the collection of these leaves is more necrotic overall compared with the mutants (not shown). The *nps6* mutant necrotic lesions are smaller, with pinpoint dark cores, as we have reported earlier (Oide et al. 2006). *Chap1* mutants are similar to *nps6* in size; however, dark cores tend to be larger

(Fig. 6A and B). Lesion length of *sre1* mutants is longer, on average, than *Chap1* mutants but without statistical significance and cores are less dark than those of the WT or *Chap1* mutants. Statistically, neither double mutant was significantly different in size from the single mutants although, on average, the lesions were smaller (Fig. 6C).

Table 2. (continued from preceding page)

Gene	Putative function	Strain and Fe condition ^x	Expression ^y	Standard error	Fe regulated?	Sre1 regulated?
<i>NOX3</i>	Ferric reductase, NADH/NADPH oxidase	<i>sre1</i> -Fe	0.7 ab	0.14	No	No
		<i>sre1</i> +Fe	0.4 b	0.22		
		WT-Fe	1.3 a	0.50		
		WT+Fe	1.0 a	0.17		
<i>NOX4</i>	Ferric reductase, NADH/NADPH oxidase	<i>sre1</i> -Fe	0.8 a	0.59	Yes in WT No in <i>sre1</i>	Yes, up in WT in +Fe
		<i>sre1</i> +Fe	1.6 a	0.63		
		WT-Fe	0.3 b	0.07		
		WT+Fe	1.0 a	0.21		
<i>NOXR</i>	Regulatory, signal transduction mechanisms	<i>sre1</i> -Fe	0.5 ab	0.24	Yes in WT, No in <i>sre1</i>	Yes, up in WT in +Fe
		<i>sre1</i> +Fe	0.3 b	0.01		
		WT-Fe	0.6 b	0.06		
		WT+Fe	1.0 a	0.11		
<i>VEL1</i>	SWI-SNF chromatin-remodeling complex protein	<i>sre1</i> -Fe	0.8 ab	0.06	No	No
		<i>sre1</i> +Fe	0.8 ab	0.02		
		WT-Fe	0.9 a	0.05		
		WT+Fe	1.0 a	0.06		
<i>VEL3</i>	Serine/threonine protein kinase	<i>sre1</i> -Fe	0.9 a	0.08	No in WT Yes in <i>sre1</i>	Yes, up in <i>sre1</i> in -Fe
		<i>sre1</i> +Fe	0.8 a	0.15		
		WT-Fe	0.7 b	0.27		
		WT+Fe	1.0 ab	0.15		
<i>VEL2</i>	SWI-SNF chromatin-remodeling complex protein	<i>sre1</i> -Fe	1.9 a	0.30	No	No
		<i>sre1</i> +Fe	0.6 b	0.17		
		WT-Fe	0.8 a	0.17		
		WT+Fe	1.0 a	0.09		
<i>CAT1</i>	Catalase	<i>sre1</i> -Fe	1.0 a	0.17	No in WT Yes in <i>sre1</i>	Yes, up in <i>sre1</i> in +Fe condition
		<i>sre1</i> +Fe	0.9 a	0.13		
		WT-Fe	0.6 a	2.18		
		WT+Fe	1.0 a	0.64		
<i>CAT2</i>	Catalase	<i>sre1</i> -Fe	0.0 c	0.00	No	No
		<i>sre1</i> +Fe	32.7 b	31.89		
		WT-Fe	5.5 a	6.67		
		WT+Fe	1.0 a	0.30		
<i>CAT3</i>	Catalase	<i>sre1</i> -Fe	0.9 a	0.17	Yes in WT and <i>sre1</i>	Yes, up in <i>sre1</i> in +Fe
		<i>sre1</i> +Fe	0.7 a	0.13		
		WT-Fe	0.1 b	0.03		
		WT+Fe	1.0 a	0.38		
<i>FET1</i>	Low-affinity iron permease	<i>sre1</i> -Fe	0.1 b	0.01	No	Yes, up in <i>sre1</i> in +Fe
		<i>sre1</i> +Fe	3.3 a	1.27		
		WT-Fe	0.5 a	0.38		
		WT+Fe	1.0 ab	0.20		
<i>PPT1</i>	4'-Phosphopantetheinyl transferase	<i>sre1</i> -Fe	1.4 ab	0.14	No	No
		<i>sre1</i> +Fe	4.0 b	2.27		
		WT-Fe	1.7 a	0.34		
		WT+Fe	1.0 a	0.09		
<i>SOD1</i>	Copper/zinc superoxide dismutase	<i>sre1</i> -Fe	1.8 a	0.07	No	Yes, up in <i>sre1</i> under + and -Fe
		<i>sre1</i> +Fe	0.8 a	0.11		
		WT-Fe	1.6 ac	0.10		
		WT+Fe	1.0 a	0.03		
<i>SOD2</i>	Manganese and iron superoxide dismutase	<i>sre1</i> -Fe	2.5 b	0.49	No in WT Yes in <i>sre1</i>	Yes, up in <i>sre1</i> in +Fe
		<i>sre1</i> +Fe	1.9 bc	0.19		
		WT-Fe	0.5 a	0.17		
		WT+Fe	1.0 ab	0.25		
<i>SOD3</i>	Manganese and iron superoxide dismutase	<i>sre1</i> -Fe	0.6 a	0.07	No	No
		<i>sre1</i> +Fe	1.5 b	0.38		
		WT-Fe	1.2 a	0.20		
		WT+Fe	1.0 a	0.28		
<i>SRE1</i>	GATA-type transcription factor	<i>sre1</i> -Fe	1.0 a	0.23	Yes in WT	Up in WT in +Fe
		<i>sre1</i> +Fe	1.2 a	0.09		
		WT-Fe	0.2 b	0.04		
		WT+Fe	1.0 a	0.12		
		<i>sre1</i> -Fe	0.0 c	0.00		
		<i>sre1</i> +Fe	0.0 c	0.00		

DISCUSSION

The role of Sre1 in the regulation of gene expression by Fe.

The *C. heterostrophus* ortholog (Sre1) of the *A. nidulans* and *Ustilago maydis* iron regulatory transcription factors SreA/URBS1 (An et al. 1997; Haas et al. 1999) is responsible for repression of a group of iron-regulated genes under iron-replete conditions. The *NPS6* gene encoding the Nps6 protein responsible for biosynthesis of the extracellular siderophore, coprogen, belongs to this group, as does *ABC6*, an adjacent ABC transporter gene, involved in coprogen transport out of the cell (N. Zhang, B. J. Condon, and B. G. Turgeon, *unpublished*). Expression of *NPS6* was decreased in Fe-replete conditions in the WT and derepressed in the *sre1* mutant (Table 2; Fig. 2). Therefore, Sre1 mediates repression of *NPS6* expression by iron. In the experiment described in Table 2 and Figure 2, *NPS6* expression was decreased in Fe-replete conditions in the *sre1* mutant, suggesting a level of regulation in addition to that mediated by Sre1. In a second experiment, the latter was not statistically significant from expression in the *sre1* mutant grown in Fe-depleted conditions, though the average value decreased. However, repression of *FTR1*, encoding the high-affinity iron permease essential for the RIA pathway, was robust in the *sre1* mutant (Fig. 2), strongly suggesting that Sre1 alone does not account for all regulation by iron. In *A. nidulans*, loss of the transcription factor HapX resulted in partial

derepression of *SreA* (Hortschansky et al. 2007). We found that, in *C. heterostrophus*, *SRE1* expression increased under high Fe; HapX could be the factor responsible for this.

It may not be a coincidence that *NPS6* but not *FTR1* is regulated by iron in an *SRE1*-dependent manner. *C. heterostrophus* *ftr1* mutants have no observable altered developmental phenotype compared with the WT and are not reduced in virulence (B. J. Condon and B. G. Turgeon, *unpublished*), indicating that the *NPS6* high-affinity iron acquisition pathway is favored over the RIA pathway in terms of both Sre1 regulation and function in necrotrophic *C. heterostrophus*. The preference for the siderophore versus the RIA mechanism has, for plant pathogens examined thus far, aligned with pathogenic lifestyle. Necrotrophs (*Alternaria brassicicola*, *C. heterostrophus*, *C. miyabeanus*, and *Fusarium graminearum*) (Oide et al. 2006) require siderophores but not RIA (*F. graminearum* [Park et al. 2006] and *C. heterostrophus* [B. J. Condon and B. G. Turgeon, *unpublished*]) for successful interactions with their host. Biotrophs, on the other hand, tend to rely on RIA (*U. maydis* [Albarouki and Deising 2013; Eichhorn et al. 2006] but not siderophores (*U. maydis* [Mei et al. 1993] and *Microbotryum violaceum* [Birch and Ruddat 2005])). It could be that the two mechanisms have different efficacies in the unique environments of necrotrophic versus biotrophic host defense responses. For example, siderophores could be recognized as a pathogen-associated molecular pattern, discouraging their use by bio-

Fig. 3. Sensitivity to H_2O_2 of progeny of a tetrad from a *Chap1* by *nps6* cross. Bars reflecting concentration of H_2O_2 are color-coded. The x axis is the strain examined and the y axis is growth ratio (colony diameter at a given concentration of H_2O_2 per colony diameter on control without H_2O_2). Nomenclature: cross number is 1756, a cross between *Chap1* mutant 1689-R10 and *nps6* mutant *Chnp6*-3. The three strains on the left are wild type (WT) and the two parents of the cross. The remaining eight strains are progeny (1756-1-1 through 1756-1-8) collected from a tetrad (tetrad 1756-1) that have the following genotypes: 1756-1-2, 1-8 = WT; 1756-1-1, 1-7 = *Chap1*; 1756-1-5, 1-6 = *nps6*; 1756-1-3, 1-4 = *Chap1nps6*. Note that *Chap1nps6* double mutant progeny are more sensitive to H_2O_2 than either single *Chap1* or *nps6* mutant progeny.

trophs. Differential regulation of *NPS6* and *FTR1* by Sre1 could explain their relative importance in virulence.

The connection between Sre1 or ChApl to the oxidant-sensitive phenotype of *nps6* mutants.

Since the discovery that *nps6* mutants are sensitive to oxidative stress (Oide et al. 2006), the biochemical reason for this has been an open question. One hypothesis is that, in Fe-replete conditions, the Fenton reactions intensify oxidative damage in the absence of the secreted iron chelator. This is relevant in infection, because both the pathogen and the plant can produce H_2O_2 and superoxide. The ionic nature of superoxide makes it difficult for it to cross cellular membranes (Halliwell 2001), except perhaps through anion channels. Superoxide, however, is dismutated to peroxide, the most abundant and stable ROS, which can more easily cross the plasma membrane to raise the oxidant level and act as a signal in the cytoplasm (Paulsen and Carroll 2010). The redox-sensitive transcription factor ChApl is a major player in resistance of *C. heterostrophus* to H_2O_2 . Two histidine kinase two-component pathways, one including the response regulator, Ssk1, and the other including the response regulator, Skn7, are also necessary for full resistance to oxidative stress (Heller and Tudzynski 2011; Oide et al. 2010; Vargas-Perez et al. 2007). Loss of *C. heterostrophus* ChApl prevents expression of *TRR1* and *GLR1* (Lev et al. 2005). Lack of the *B. cinerea* YAP1 ortholog BAP1 rendered the mutant unable to bring the intracellular glutathione pool back to a reduced state, as detected with a genetically encoded sensor of the glutathione state (Heller and Tudzynski 2011). In *C. heterostrophus*, we have recently found evidence, using a genetically encoded indicator of H_2O_2 levels, that the return to homeostasis following H_2O_2 treatment is delayed in *Chap1* mutants (Ronen et al. 2013).

To examine the connection among *nps6*, *sre1*, and *Chap1* mutant phenotypes, we made and tested *Chap1nps6* and *Chap1sre1* double mutants for response to in vitro oxidative stress. We predicted that, if higher levels of oxidants inside the cell make the *C. heterostrophus* extracellular siderophore mutant *nps6* sensitive to oxidative stress, *Chap1nps6* double mutants should be more sensitive than either single *Chap1* or *nps6* mutants, based on the following model. Assuming that an extracellular oxidant makes the intracellular redox state more oxidized in the *nps6* mutant than the same extracellular concentration would in WT, *nps6* will be hypersensitive to oxidative stress. ChApl provides a major mechanism to overcome the hyperoxidized state and recover homeostasis. This mechanism is lost in *Chap1*; therefore, the double mutant is more sensitive than *nps6*. *Chap1* mutants are sensitive to oxidative stress because they cannot upregulate a subset of antioxidant genes in response to exposure to oxidants. If the *NPS6* gene is also lacking, as in the *nps6* mutant, the oxidative stress “seen” inside the cell is higher than when *NPS6* is present; thus, again, the double mutant is more sensitive than the single *Chap1* mutant. In the absence of ChApl, return to homeostasis is delayed or seriously deficient, because the cell must rely entirely on additional mechanisms (e.g., the Hog1 stress-response pathway). Our results demonstrate that the *Chap1nps6* mutant is, indeed, more sensitive than the *Chap1* mutant which, in turn, is more sensitive than the *nps6* mutant. This result rules out the possibility that ChApl relies primarily on coprogen production to deal with oxidative stress.

Chap1sre1 double mutants, on the other hand, are less sensitive than *Chap1* single mutants. The *sre1* mutants lose repression of many iron- and redox-related genes under iron-replete conditions. *Chap1sre1* double mutants are partially rescued for the *Chap1* oxidant-sensitive phenotype at high H_2O_2 levels.

Fig. 4. Growth on complete medium (CM) with xylose of wild type (WT), *Chap1*, *nps6*, and *sre1* single mutants and *Chap1nps6* and *Chap1sre1* double mutants. Mutant *sre1* has decreased growth on CM, as does the *Chap1sre1* mutant, indicating epistasis of the *sre1* mutation in the double mutant. Phenotypes of the single *Chap1* and *nps6* mutants and the *Chap1nps6* double are like the WT.

Sequestering of Fe by increased siderophore production could provide this protective effect. On the other hand, increased siderophore production could increase intracellular Fe levels and, thus, oxidative stress, as proposed for *Aspergillus fumigatus* (Schrettl et al. 2008). This could explain increased *CAT1* levels in the *sre1* mutant (Fig. 2). However, the strongly increased *CAT1* transcript level in the *sre1* mutant was not associated with enhanced resistance to H₂O₂ under standard Fe-replete conditions (Fig. 5). Sequestering of Fe by extra- and intracellular siderophores would protect against oxidant damage. Protection by sequestering Fe outside the cell might not be very efficient if only extracellular siderophore production is increased in the *sre1* mutant; measurement of siderophore levels inside and outside the cell could answer this question. Our expression analyses demonstrate that *NPS2*, encoding Nps2, responsible for synthesis of the intracellular siderophore, is up-regulated together with *NPS6* in response to high Fe levels in *sre1* mutants.

Alternative models also need to be considered in view of the opposing roles of iron, which can promote oxidative damage yet is also required to detoxify oxidants (Haas 2012). Enzymes with heme cofactors (CAT and peroxidases) depend on Fe for their activity. Therefore, iron limitation could increase sensitivity to oxidative stress. Loss of coprogen in the *nps6* mutant might limit Fe uptake, lowering with the activity of mutant might limit Fe uptake, lowering the activity of oxidant-detoxifying enzymes. In the *sre1* mutant, increased coprogen levels could facilitate Fe uptake. Construction of mutants in siderophore transport might support or exclude this explanation; however, it seems less likely than the first, because the loss of

NPS6 results in sensitivity to oxidants even under sufficient Fe where growth does not appear limited by iron. Nevertheless, direct measurement of local Fe levels may be necessary to support or exclude either mechanism.

The direct relationships found in vitro do not always correspond to virulence on the host. For example, it is shown in Figure 6 that the double *Chap1nps6* mutant elicited symptoms similar to the *Chap1* mutant whereas, in vitro, the double mutant was more sensitive to H₂O₂ (Fig. 3). In the infection court, the situation is more complex than in vitro. In the initial interaction with the host plant, the pathogen could encounter oxidative stress from the plant's oxidative burst or perhaps iron starvation resulting from sequestering of iron by the host. Studies of the interactions of the biotroph *Blumeria graminis* with its cereal host show that the plant produces an Fe burst at the infection site, intensifying the Fenton reactions (Liu et al. 2007). Thus, at least three mechanisms—ROS from the plant oxidative burst, sequestering of iron by the host, or, in contrast, a burst of Fe exacerbating oxidative stress—may be at work in the *C. heterostrophus*–maize interaction (Fig. 6). One of the first questions to answer will be whether the invading fungus encounters increased iron or, rather, must compete for iron, as recently studied in the interaction of *F. oxysporum* with tomato roots (Lopez-Berges et al. 2012). We found that expression of *SRE1* is repressed under Fe-depleted conditions. Therefore, observation of an *sre1* mutant low-virulence phenotype would suggest that the pathogen encounters sufficient or high Fe in the infection court. High-resolution imaging of ROS and Fe levels in fungus and pathogen on the plant, together with the mutant lines studied here, may help resolve some of these questions.

Fig. 5. Sensitivity to H₂O₂ of progeny of a tetrad from a *Chap1* × *sre1* cross. Cross 1757 is a cross between *Chap1* mutant 1689-R10 and *sre1* mutant *sre1*-1-1, a single conidiated strain of *sre1*-1. The eight progeny (1757-1-1 through 1757-1-8) collected from a tetrad (tetrad 1757-1) have the following genotypes: 1757-1-1, 1-6 = WT; 1757-1-2, 1-5 = *Chap1*; 1757-1-4, 1-8 = *sre1*; 1757-1-3, 1-7 = *Chap1sre1*. Note that *Chap1sre1* double-mutant progeny are less sensitive than *Chap1* and more sensitive than *sre1* mutant progeny.

MATERIALS AND METHODS

Strains, growth conditions, and crosses.

Fungal strains used in this study are listed in Table 1. The *sre1* mutants were constructed in *C. heterostrophus* C4 (*MAT1-2*; American Type Culture Collection 48331). To obtain the *Chap1* mutant used here, a *Chap1* strain available from previous work (Lev et al. 2005) was crossed to WT strain C5. An H_2O_2 -sensitive, HygB-resistant, *MAT1-1 Chap1* progeny (1689-R10) was isolated from this cross. Strains were grown on CMX (Tzeng 1990) at 24°C under a fluorescent light cycle of 12 h of light and 12 h of darkness unless mentioned otherwise. For iron-depleted conditions, fungi were grown in minimal medium (MM) (Leach et al. 1982) with iron omitted (MM-Fe). For iron-replete conditions, 10 μ M $FeCl_3$ was added to MM as a supplement (MM+Fe). *Zea mays* ‘W64A-N’ was grown in a growth chamber at 24°C with a cycle of 16 h of light and 8 h of darkness and used for virulence assays when plants were 21 days old.

To generate *Chap1nps6* double mutants, *Chap1* mutant 1689-R10 (*MAT1-1*) was crossed with *nps6* mutant *Chnps6-3* (*MAT1-2*). For *Chap1sre1* double mutants, *Chap1* mutant 1689-R10 was crossed with *sre1* mutant *sre1-1* (*MAT1-2*). Crosses were set up on Sach’s medium in triplicate and incubated for 3 weeks at 23°C in the dark (Leach et al. 1982). Tetrads were collected from each cross and tetrad member phenotypes examined on CMX, and CMX without salts plus HygB to screen for resistance to the drug, and tetrads with hygB-sensitive members were selected. Their genotype was determined by diagnostic

PCR amplification. Absence of *NPS6*, *SRE1*, and *ChAP1* was confirmed with primer pairs ChNPS6qF1/ChNPS6qR1, ChSREAF1/ChSREAR1, and DW301/DW302, respectively.

Bioinformatic and phylogenetic analyses.

BLASTp analysis using the *A. nidulans* SreA amino acid sequence against the genomic sequence of *C. heterostrophus* reference strain C5 identified a homologous sequence (E value = $1.91E-41$, score = 796). This gene, *SRE1* (JGI protein ID number 109473), was used to identify the corresponding gene (JGI ID number 193544) in *C. heterostrophus* C4. The Sre1-predicted protein sequences of C4 and C5 are 100% identical. Twenty-six representative top hits (E value < $4E-34$) with the *C. heterostrophus* *SRE1* sequence were downloaded from GenBank and orthologs in other *Cochliobolus* spp. were downloaded and aligned with ClustalX (Thompson et al. 1997). The most parsimonious tree was generated with PAUP*4b10 (Swofford 1993) for the aligned protein sequence dataset. Bootstrap analysis was carried out with 1,000 replicates.

DNA manipulations and fungal transformation.

Fungal genomic DNA was isolated using the MoBio microbial DNA isolation kit (MoBio Laboratories Inc., Carlsbad, CA, U.S.A.). PCR amplification was conducted with GoTaq (Promega, Madison, WI, U.S.A.) or iProof high-fidelity DNA polymerase (Bio-Rad, Hercules, CA, U.S.A.), following the manufacturer’s instructions. Split-marker constructs were prepared for targeted deletion of *SRE1* (Catlett et al. 2003). A

Fig. 6. Virulence phenotypes of single- and double-mutant progeny on maize. **A**, Examples of leaves inoculated with progeny of crosses and the wild type (WT), photographed 5 days postinoculation. **B** and **C**, Quantification of lesion sizes (y axes) on leaves inoculated with the indicated progeny. Error bars are standard deviation. Different letters indicate significant differences at $P < 0.05$ when analyzed using one-way analysis of variance (SPSS version 17.0). Note that the lesions on leaves inoculated with the WT are larger than those of any of the mutants or mutant progeny. The *nps6* extracellular siderophore mutant, used as a control, made smaller lesions than WT (A), whereas *sre1* progeny produced slightly larger lesions (but with no significant difference) than *Chap1* and double-mutant progeny, which were approximately the same size as those of *nps6* (C).

1.8-kb DNA sequence including the *SRE1* ORF was deleted from strain C4 and replaced with the *hygB* gene that functions as a drug resistance marker (Oide et al. 2006). Transformation of *C. heterostrophus* was carried out as previously described (Turgeon et al. 2010). Deletion of *SRE1* in transformants was confirmed by PCR with the following primer pairs: i) ChSREAF1/ChSREAR1, ii) NLC37/NLC38, iii) ChSREAU1/NLC37, and iv) ChSREAD1/NLC38. The first primer pair confirmed the absence of *SRE1* in candidate mutants, the second confirmed the presence of the selectable marker *hygB*, and the third and fourth confirmed correct insertion of the deletion cassette carrying *hygB* into the 5' and 3' flanks, respectively, of the native *SRE1* locus.

Complementation of the *sre1* mutant strain *sre1-2-1* (a single conidiated isolate of *sre1-2*) was carried out by transformation with the *SRE1* ORF placed adjacent to the *NPTII* gene for resistance to G418. To do this, *SRE1* with upstream and downstream flanking regions (totaling 1,800 bp) was amplified (3,500 bp) using the primer pair SRE1FP2npt/SRE1RP2npt. An additional 800 bp of downstream sequence was amplified using the primer pair SRE14F/SRE14R. This downstream region was fused to the *NPTII* gene, driven by the TrpC promoter and terminator (generated using primer pair DW69/DW70 from plasmid pII99) (Oide et al. 2006; Sevim et al. 2012), by means of overlapping sequence adaptors on DW69 and Sre14F. The upstream sequence of DW70 overlaps with the adaptor sequence on Sre1RP2. The net result is a construct which replaces *HygB* with the native *SRE1* gene and promoter, immediately tandem to the *NPTII* gene under the control of the TrpC promoter.

Transformation was carried out as above for the *SRE1* gene deletion, except protoplasts were harvested from cultures that were grown for 2 days in liquid CM, blended, then grown in fresh liquid CM for 12 h overnight. Transformants were confirmed to be G418 resistant and HygB sensitive, then single conidiated and retested for antibiotic resistance.

Complementation was confirmed by PCR using the following primer combinations: NPTII-F/NPTII-R to confirm presence of the *NPTII* gene, ChSREAF1/ChREAR1 to confirm presence of the *SRE1* gene, and NLC37/NLC38 to confirm absence of the *HygB* gene.

Growth rate and conidial germination.

To assess growth rate, 4-mm-diameter plugs cut from the edge of each 1-week-old fungal colony were placed on CMX and cultured at 22°C with a cycle of 12 h of light and 12 h of darkness for 5 days. Diameter of the fungal colonies was then measured. To measure asexual spore germination rate, conidia were suspended in a drop of sterile distilled water on glass slides, then incubated at 30°C in a humid chamber. At 2 and 3 h, 50 conidia from the *sre1* mutant and WT were examined under a microscope for percent germination. The whole experiment was replicated three times.

Oxidative stress sensitivity.

To evaluate the sensitivity to oxidative stress (Oide et al. 2006), an agar plug of each strain was grown at 22°C in the dark for 5 days on MM and MM supplemented with 0.5, 1.0, 2.0, and 4 mM H₂O₂ (Sigma, St. Louis), and colony radius was recorded. The growth ratio was calculated by measuring colony diameter at a given concentration of H₂O₂ per colony diameter on the MM control plate. Experiments were repeated three times, with at least two independent mutant strains of each type.

Virulence assays.

To evaluate the effect of deletion of *SRE1* and double mutants on virulence to the host, 3-week-old maize plants (W64A-N)

were inoculated by spraying plants with conidial suspensions prepared at a concentration of 2×10^4 conidia per milliliter in 0.02% Tween 20 (2 ml per plant). Three plants were used for each fungal strain. Plants were incubated in a mist chamber for 24 h after inoculation, then moved to a growth chamber for an additional 4 days at 24°C under a cycle of 16 h of light and 8 h of darkness. The fourth true leaves were photographed at 5 days postinoculation and the experiment was repeated three times. The data were analyzed using SPSS analysis version 17.0.

Gene expression analysis with qRT-PCR.

For gene expression analysis of the WT and *sre1-1* mutant strains described in Figure 2, 10^5 conidia were transferred to 50 ml of MM-Fe or MM+Fe (10 μ M FeCl₃) broth in a 200-ml Erlenmeyer flask (HCl washed) and cultured in the dark at 22°C with an 150 rpm shake speed. After 96 h, fungal mycelium was harvested and total RNA was isolated using a MoBio microbial RNA isolation kit (MoBio Laboratories Inc.). RNA was treated with Turbo DNase (Ambion, Austin, TX, U.S.A.). An iScript cDNA synthesis kit (BioRad) was used to prepare cDNA from 1 μ g of DNase-treated RNA for qPCR assays. qPCR was conducted with SYBR ER supermix (Invitrogen, Carlsbad, CA, U.S.A.) and run on an ABI PRISM 7000 real-time thermocycler. In total, 28 genes were included in the qPCR, with *HISH3* as a reference gene. Primers used in the qPCR assays were synthesized by Integrated DNA Technologies and the sequences are listed in Supplementary Table S2. Specificity of the assays was tested with the melt-curve analysis. qPCR data were analyzed following the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Gene expression of the 29 target genes was normalized relative to the expression of *HISH3* gene (Table 2; Fig. 2) and calculated relative to the WT+Fe treatment. Three biological and two technical replicates were analyzed. In an additional experiment, data for *NPS6*, *SRE1*, *CAT1*, *CAT2*, *CAT3*, *GLR1*, and *TRR1* were obtained, and calculated relative to the WT+Fe treatment. Procedures were essentially the same as for Figure 2. A γ actin gene (JGI v1.0 ID 31040/v2.0 ID 1197727) was used as the reference; there were six biological and two technical replicates, and primers for this experiment (synthesized by Sigma) are listed in Supplementary Table S3.

ACKNOWLEDGMENTS

The research was supported by United States Department of Agriculture National Institute of Food and Agriculture grant number 2010-65108-20500 to B. G. Turgeon. K. Scher's postdoctoral fellowship was supported by BARD grant IS 4167-08C to B. A. Horwitz and B. G. Turgeon. M. Z. NurAinIzzati was sponsored by the Universiti Putra Malaysia (UPM) and The Ministry of Higher Education (MOHE). We thank K. Loeffler, Department of Plant Pathology and Plant-Microbe Biology photographer, for superb photographic assistance.

LITERATURE CITED

- Albarouki, E., and Deising, H. B. 2013. Infection structure-specific reductive iron assimilation is required for cell wall integrity and full virulence of the maize pathogen *Colletotrichum graminicola*. Mol. Plant-Microbe Interact. 26:695-708.
- An, Z. Q., Zhao, Q., McEvoy, J., Yuan, W. M., Markley, J. L., and Leong, S. A. 1997. The second finger of Urbs1 is required for iron-mediated repression of sid1 in *Ustilago maydis*. Proc. Natl. Acad. Sci. U.S.A. 94:5882-5887.
- Barbusinski, K. 2009. Fenton reaction—Controversy concerning the chemistry. Ecol. Chem. Eng. 16:347-358.
- Birch, L. E., and Ruddat, M. 2005. Siderophore accumulation and phytopathogenicity in *Microbotryum violaceum*. Fungal Genet. Biol. 42:579-589.
- Catlett, N., Lee, B.-N., Yoder, O., and Turgeon, B. 2003. Split-marker recombination for efficient targeted deletion of fungal genes. Fungal

- Genet. Newsl. 50:9-11.
- Chao, H. M., Chen, Y. H., Liu, J. H., Lee, S. M., Lee, F. L., Chang, Y., Yeh, P. H., Pan, W. H. T., Chi, C. W., Liu, T. Y., Lui, W. Y., Ho, L. T., Kuo, C. D., Lin, D. E., Chan, C. C., Yang, D. M., Lin, A. M. Y., and Chao, F. P. 2008. Iron-generated hydroxyl radicals kill retinal cells in vivo: Effect of ferulic acid. *Hum. Exp. Toxicol.* 27:327-339.
- Cho, Y., Kim, K. H., La Rota, M., Scott, D., Santopietro, G., Callihan, M., Mitchell, T. K., and Lawrence, C. B. 2009. Identification of novel virulence factors associated with signal transduction pathways in *Alternaria brassicicola*. *Mol. Microbiol.* 72:1316-1333.
- Eichhorn, H., Lessing, F., Winterberg, B., Schirawski, J., Kamper, J., Muller, P., and Kahmann, R. 2006. A ferrooxidation/permeation iron uptake system is required for virulence in *Ustilago maydis*. *Plant Cell* 18:3332-3345.
- Greenshields, D. L., Liu, G. S., Feng, J., Selvaraj, G., and Wei, Y. D. 2007. The siderophore biosynthetic gene *SID1*, but not the ferroxidase gene *FET3*, is required for full *Fusarium graminearum* virulence. *Mol. Plant Pathol.* 8:411-421.
- Haas, H. 2012. Iron—A key nexus in the virulence of *Aspergillus fumigatus*. *Front. Microbiol.* 3 28. doi: 10.3389/fmicb.2012.00028. Published online.
- Haas, H., Zadra, I., Stoffer, G., and Angermayr, K. 1999. The *Aspergillus nidulans* GATA factor SREA is involved in regulation of siderophore biosynthesis and control of iron uptake. *J. Biol. Chem.* 274:4613-4619.
- Halliwell, B. 2001. Role of free radicals in the neurodegenerative diseases: Therapeutic implications for antioxidant treatment. *Drugs Aging* 18:685-716.
- Heller, J., and Tudzynski, P. 2011. Reactive oxygen species in phytopathogenic fungi: Signaling, development, and disease. *Annu. Rev. Phytopathol.* 49:369-390.
- Hortschansky, P., Eisendle, M., Al-Abdallah, Q., Schmidt, A. D., Bergmann, S., Thon, M., Kniemeyer, O., Abt, B., Seeber, B., Werner, E. R., Kato, M., Brakhage, A. A., and Haas, H. 2007. Interaction of HapX with the CCAAT-binding complex—A novel mechanism of gene regulation by iron. *EMBO (Eur. Mol. Biol. Organ.) J.* 26:3157-3168.
- Huang, K., Czymmek, K. J., Caplan, J. L., Sweigard, J. A., Donofrio, N. M. 2011. *HYR1*-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus *PLoS Pathol.* 7:e1001335. doi:10.1371 Published online.
- Hwang, L. H., Mayfield, J. A., Rine, J., and Sil, A. 2008. Histoplasma requires *SID1*, a member of an iron-regulated siderophore gene cluster, for host colonization. *PLoS Pathog.* 4:e1000044. Published online.
- Kieu, N. P., Aznar, A., Segond, D., Rigault, M., Simond-Cote, E., Kunz, C., Soulie, M. C., Expert, D., and Dellagi, A. 2012. Iron deficiency affects plant defence responses and confers resistance to *Dickeya dadantii* and *Botrytis cinerea*. *Mol. Plant Pathol.* 13:816-827.
- Labbe, S., Pelletier, B., and Mercier, A. 2007. Iron homeostasis in the fission yeast *Schizosaccharomyces pombe*. *Biometals* 20:523-537.
- Leach, J., Lang, B. R., and Yoder, O.C. 1982. Methods for selection of mutants and in vitro culture of *Cochliobolus heterostrophus*. *J. Gen. Microbiol.* 128:1719-1729.
- Lev, S., Hadar, R., Amedeo, P., Baker, S. E., Yoder, O. C., and Horwitz, B. A. 2005. Activation of an AP1-like transcription factor of the maize pathogen *Cochliobolus heterostrophus* in response to oxidative stress and plant signals. *Eukaryot. Cell* 4:443-454.
- Liu, G. S., Greenshields, D. L., Sammynaiken, R., Hirji, R. N., Selvaraj, G., and Wei, Y. D. 2007. Targeted alterations in iron homeostasis underlie plant defense responses. *J. Cell Sci.* 120:596-605.
- Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. *Methods* 25:402-408.
- Lopez-Berges, M. S., Capilla, J., Turra, D., Schafferer, L., Matthijs, S., Jochl, C., Cornelis, P., Guarro, J., Haas, H., and Di Pietro, A. 2012. HapX-mediated iron homeostasis is essential for rhizosphere competence and virulence of the soilborne pathogen *Fusarium oxysporum*. *Plant Cell* 24:3805-3822.
- Mei, B. G., Budde, A. D., and Leong, S. A. 1993. *Sid1*, a gene initiating siderophore biosynthesis in *Ustilago maydis*—molecular characterization, regulation by iron, and role in phytopathogenicity. *Proc. Natl. Acad. Sci. U.S.A.* 90:903-907.
- Molina, L., and Kahmann, R. 2007. An *Ustilago maydis* gene involved in H₂O₂ detoxification is required for virulence. *Plant Cell* 19:2293-2309.
- Oide, S., Moeder, W., Krasnoff, S., Gibson, D., Haas, H., Yoshioka, K., and Turgeon, B. G. 2006. NPS6, encoding a nonribosomal peptide synthetase involved in siderophore-mediated iron metabolism, is a conserved virulence determinant of plant pathogenic ascomycetes. *Plant Cell* 18:2836-2853.
- Oide, S., Liu, J., Yun, S. H., Wu, D., Michev, A., Choi, M. Y., Horwitz, B. A., and Turgeon, B. G. 2010. Histidine kinase two-component response regulator proteins regulate reproductive development, virulence, and stress responses of the fungal cereal pathogens *Cochliobolus heterostrophus* and *Gibberella zeae*. *Eukaryot. Cell* 9:1867-1880.
- Park, Y. S., Choi, I. D., Kang, C. M., Ham, M. S., Kim, J. H., Kim, T. H., Yun, S. H., Lee, Y. W., Chang, H. I., Sung, H. C., and Yun, C. W. 2006. Functional identification of high-affinity iron permeases from *Fusarium graminearum*. *Fungal Genet. Biol.* 43:273-282.
- Paulsen, C. E., and Carroll, K. S. 2010. Orchestrating redox signaling networks through regulatory cysteine switches. *ACS Chem. Biol.* 5:47-62.
- Pelletier, B., Beaudoin, J., Mukai, Y., and Labbe, S. 2002. Fep1, an iron sensor regulating iron transporter gene expression in *Schizosaccharomyces pombe*. *J. Biol. Chem.* 277:22950-22958.
- Pierre, J. L., and Fontecave, M. 1999. Iron and activated oxygen species in biology: The basic chemistry. *Biometals* 12:195-199.
- Robbertse, B., Yoder, O. C., Nguyen, A., Schoch, C. L., and Turgeon, B. G. 2003. Deletion of all *Cochliobolus heterostrophus* monofunctional catalase-encoding genes reveals a role for one in sensitivity to oxidative stress but none with a role in virulence. *Mol. Plant-Microbe Interact.* 16:1013-1021.
- Ronen, M., Shalaby, S., and Horwitz, B. A. 2013. Role of the transcription factor ChAP1 in cytoplasmic redoxhomeostasis: Imaging with a genetically encoded sensor in the maize pathogen *Cochliobolus heterostrophus*. *Mol. Plant Pathol.* doi:10.1111/mpp.12047. Published online.
- Schrettl, M., Kim, H. S., Eisendle, M., Kragl, C., Nierman, W. C., Heinekamp, T., Werner, E. R., Jacobsen, I., Illmer, P., Yi, H., Brakhage, A. A., and Haas, H. 2008. SreA-mediated iron regulation in *Aspergillus fumigatus*. *Mol. Microbiol.* 70:27-43.
- Schrettl, M., Ibrahim-Granet, O., Droin, S., Huerre, M., Latge, J. P., and Haas, H. 2010. The crucial role of the *Aspergillus fumigatus* siderophore system in interaction with alveolar macrophages. *Microbes Infect.* 12:1035-1041.
- Sevim, A., Donzelli, B. G. G., Wu, D. L., Demirbag, Z., Gibson, D. M., and Turgeon, B. G. 2012. Hydrophobin genes of the entomopathogenic fungus, *Metarhizium brunneum*, are differentially expressed and corresponding mutants are decreased in virulence. *Curr. Genet.* 58:79-92.
- Shanmugam, V., Ronen, M., Shalaby, S., Larkov, O., Rachamim, Y., Hadar, R., Rose, M. S., Carmeli, S., Horwitz, B.A., and Lev, S. 2010. The fungal pathogen *Cochliobolus heterostrophus* responds to maize phenolics: Novel small molecule signals in a plant-fungal interaction. *Cell. Microbiol.* 12:1421-1434.
- Swofford, D. L. 1993. PAUP (Phylogenetic Analysis Using Parsimony). Illinois Natural History Survey, Chicago.
- Temme, N., and Tudzynski, P. 2009. Does *Botrytis cinerea* ignore H₂O₂-induced oxidative stress during infection? Characterization of *Botrytis* activator protein 1. *Mol. Plant-Microbe Interact.* 22:987-998.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876-4882.
- Turgeon, B. G., Condon, B., Liu, J., and Zhang, N. 2010. Protoplast transformation of filamentous fungi. *Methods Mol. Biol.* 638:3-19.
- Tzeng, T. H. 1990. A restriction fragment length polymorphism map of *Cochliobolus heterostrophus*. Ph.D. thesis, Iowa State University, Ames, IA, U.S.A.
- Vargas-Perez, I., Sanchez, O., Kawasaki, L., Georgellis, D., and Aguirre, J. 2007. Response regulators SrrA and SskA are central components of a phosphorelay system involved in stress signal transduction and asexual sporulation in *Aspergillus nidulans*. *Eukaryot. Cell* 6:1570-1583.
- Weinberg, E. D. 2009. Iron availability and infection. *Biochim. Biophys. Acta* 1790:600-605.
- Wu, D. L., Oide, S., Zhang, N., Choi, M. Y., and Turgeon, B. G. 2012. ChLae1 and ChVel1 regulate T-toxin production, virulence, oxidative stress response, and development of the maize pathogen *Cochliobolus heterostrophus*. *PLoS Pathog.* 8. Published online.

AUTHOR-RECOMMENDED INTERNET RESOURCES

United States Department of Energy (DOE) Joint Genome Institute (JGI)
Fungal Genomics Program database:
genome.jgi.doe.gov/programs/fungi/index.jsf
DOE JGI Genome Portal: genome.jgi.doe.gov
Wellcome Trust Sanger Institute Pfam database: pfam.sanger.ac.uk