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

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The genome of the maize pathogen *Cochliobolus heterostrophus* encodes three unlinked monofunctional catalase-encoding (*CAT*) genes that singly or in combination could offer protection against the harmful effects of oxidative stress. Phylogenetic analysis placed the *CAT2* and *CAT3* proteins in a cluster with large subunit catalases (*CAT3* has a secretory signal sequence and was grouped with known secreted catalases), whereas *CAT1* clustered with small subunit catalases. Single, double, and triple *cat* mutants were created and screened for sensitivity to hydrogen peroxide and altered virulence on maize. All mutants deficient in *CAT3* had enhanced sensitivity to hydrogen peroxide, as compared with wild type or with mutants deficient in *CAT1*, *CAT2*, or both. All catalase-deficient mutants had normal virulence to maize. Thus, the secreted *CAT3* protein protects the fungus from oxidative stress during vegetative growth, but members of this enzyme family, alone or in combination, are not essential for virulence.

Additional keywords: fungi, genome sequence, pathogenicity.

When under attack, plant hosts of pathogenic fungi release reactive oxygen species (ROS), presumably as a defense mechanism (Shim and Dunkle 2002; Wang et al. 1996). Production of ROS involves reduction of molecular oxygen to form superoxide anions (O_2^-), followed by spontaneous dismutation of the superoxide anion to yield hydrogen peroxide and oxygen. Hydroxyl radicals ($OH^\cdot$) develop via metal reduction in a hydrogen peroxide-dependent manner. Hydroxyl radicals are the major cause of cellular damage, which involves lipid peroxidation, enzyme inactivation, and degradation of nucleic acids (Thompson et al. 1987). These activities can be effective against the plant cells that produce ROS and can also be di-

rected against invading pathogens (Keppler et al. 1989). Production of ROS is well documented in microbe-dicot interactions (Bestwick et al. 1998) and has also been observed after infection of monocots, such as barley and rice (Kumar et al. 2001; Mehdy 1994).

Many fungi produce catalases and other enzymes that help them survive stress caused by ROS, e.g., O_2^- , $OH^\cdot$, and hydrogen peroxide (Schouten et al. 2002). Although enzymes such as superoxide dismutases, peroxidases, and catalases are well-known ROS scavengers, their importance during infection and survival of a fungus in its host remains uncertain. There is evidence that hydrogen peroxide and catalase activity are associated with infection of maize by the fungus *Exserohilum turcicum* (Keissar et al. 2002).

In this study, the potential contribution of all monofunctional catalases to virulence of *Cochliobolus heterostrophus* on maize was investigated. *C. heterostrophus* belongs to the Pleosporales, a sister taxon of *E. turcicum*, and causes Southern Corn Leaf Blight (Drechsler 1925), whereas *E. turcicum* causes northern leaf blight. Our investigation was made possible by availability of the *C. heterostrophus* genome sequence, which was sequenced by Celera Genomics for Torrey Mesa Research Institute (TMRI)/Syngenta, and the fact that targeted mutations are generated efficiently in this organism (Catlett et al. 2003; Turgeon et al. 1987; Wirsal et al. 1996). Furthermore, it is easy to do crosses in the laboratory with this fungus. Three monofunctional catalase genes were identified in the genome, single, double, and triple deletion mutants were generated, and their phenotypes determined. We found that deletion of one of these genes enhanced oxidative stress sensitivity but did not affect virulence to maize.

RESULTS

Structures of catalase genes and deduced amino-acid sequences.

Three monofunctional catalase (*CAT*) genes, designated *CAT1*, *CAT2*, and *CAT3*, were identified in the *C. heterostrophus* genome, using a tblastn query with protein sequences of several known catalases. ReBLAST of the *C. heterostrophus* genome with the putative *C. heterostrophus* catalases did not reveal additional ones. The putative *CAT* coding sequences and corresponding predicted introns are: *CAT1*, 1,641 bp with one intron (72 bp); *CAT2*, 2,220 bp with one intron (54 bp); and *CAT3*, 2,190 bp with six introns (53, 48, 62, 55, 54, and 55 bp). Proteins deduced from the *CAT1*, *CAT2*, and *CAT3* open reading

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frames were 547, 740, and 730 amino acids long, with predicted molecular masses (and isoelectric points) of 63 (5.97), 83.2 (5.78), and 79.9 kDa (5.94), respectively. All three catalases have both the proximal heme-ligand and active site PROSITE signature motif sequences that distinguish true catalases.

To predict functions of the *C. heterostrophus* catalases, phylogenetic analyses were conducted (data not shown). Both maximum likelihood analysis of amino acid sequences based on Bayesian inference and maximum parsimony analysis showed two well-supported clades, corresponding to small (50 to 65 kDa) and large (approximately 80 kDa) subunit catalases (Klotz et al. 1997).

Distinct clades representing the three groups of catalases (Groups I, II, and III) defined in previous studies (Bussink and Oliver 2001; Klotz et al. 1997) were also evident, as was strong support for distinct secreted and nonsecreted subgroups of Group II. Fungal catalases fell in Groups II and III only, as found previously (Klotz et al. 1997). CAT2 (Group II) clustered with nonsecreted catalases and is closely related to CAT2 of *Cladosporium fulvum* (Bussink and Oliver 2001). CAT3 (Group II) clustered with the secreted catalases. Consistent with this, a signal peptide leader sequence with a cleavage site between amino acid positions 19 and 20 in the CAT3 protein is predicted (Nielsen et al. 1997). CAT1 is a putative small sub-

Fig. 1. Positions (indicated by letters) of primers used for the design of single and triple deletion constructs and analysis of catalase-encoding (*CAT*) gene deletions. Black arrows indicate primers used to construct deletions only, gray arrows indicate primers used for the design of deletion constructs and analysis of deletions, white arrows indicate primers used to analyze gene deletions only, and dotted vertical lines indicate borders of gene replacement. Sizes of flanks used for deletion ranged from 327 to 461 bp. On the diagram, 5 to 3' orientation of *CAT1*, *CAT3*, *hygB*, and *bar* is from right to left. *CAT2* 5 to 3' orientation is left to right. Note orientation of *hygB* is reversed in the *CAT2* deletion strain, as compared with the *CAT1* and *CAT3* mutants.

Table 1. Primer combinations (primer position in brackets) used to check integration events in single (a), double (b), and triple (c) catalase mutants

Gene deletion	Primer combinations	Size (kb) of product expected with DNA from mutants	Size (kb) of product expected with wild-type DNA
<i>CAT1</i> ab	GCAT1-P0 (A) and NCL38 (YG)	2.9	none
	GCAT1-P5 (F) and NCL37 (HY)	2.1	none
<i>CAT2</i> ab	GCAT2-P0 (G) and NCL38 (YG)	2.8	none
	GCAT2-P5 (L) and NCL37 (HY)	2.25	none
<i>CAT3</i> ab	GCAT3-P0 (M) and H2	1.4	none
	GCAT3-P5 (R) and H1	1.3	none
<i>CAT1</i> c	GCAT1-P0 (A) and GCAT1-P5 (F)	4.5	3.8
<i>CAT2</i> c	GCAT2-P0 (G) and GCAT2-P5 (L)	4.6	4.4
<i>CAT3</i> c	GCAT3-P0 (M) and GCAT3-P5 (R)	3.9	5.1

unit catalase that clustered in Group III, distinct from the large subunit catalases in Group II. All predicted and known peroxisomal catalases cluster in Group III. However, *C. heterostrophus* CAT1 does not contain signature motifs proposed for peroxisomal catalases, (Gould et al. 1989; Swinkels et al. 1991).

Fig. 2. Polymerase chain reaction confirmation of single catalase gene deletions. Template DNA: *cat1* = D.C5.cat1-1B, *cat2* = D.C5.cat2-3A, *cat3* = D.C5.cat3-1B. For *cat1* and *cat3*, left and right lanes correspond to checks of targeted deletion of the 3' and 5' ends of the genes, respectively; for *cat2*, left and right lanes correspond to checks of targeted deletion of the 5' and 3' ends of the gene, respectively. Outside lanes, 1 kb plus DNA ladder. Sizes are in kb.

Confirmation of single, double, and triple *cat* deletions.

Figure 1 is a diagram of the *CAT* gene-deletion constructs and the locations of primers used to make and confirm deletions. Polymerase chain reaction (PCR) product sizes expected when DNA from wild-type vs. deletion mutants is used as template are listed in Table 1. PCR analysis using DNA from transformants carrying putative single, double (Figs. 2 and 3), or triple *CAT* deletions (Fig. 4) confirmed homologous integration of the selectable marker at the target site in each case.

Sensitivity of single, double, and triple *cat* mutants to hydrogen peroxide.

Growth rates of wild-type strains (C5 and N21-R-24) and *cat1* and *cat2* mutants were not significantly different ($P > 0.05$) on hydrogen peroxide-amended vs. nonamended medium (Fig. 5). *Cat3* mutant strains, however, grew normally on nonamended plates but significantly ($P < 0.05$) slower on amended medium (Fig. 5). The hydrogen peroxide-sensitive phenotype cosegregated with resistance to hygromycin B (Fig. 5), confirming that sensitivity is due to deletion of *CAT3*. Growth rates of *cat1;cat3* and *cat2;cat3* double mutants or *cat1;cat2;cat3* triple mutant were also significantly reduced ($P < 0.001$) on hydrogen peroxide, compared with that of wild-type strains, whereas *cat1;cat2* double mutants were not ($P > 0.05$). Mutations in *CAT1* or *CAT2*, or both, did not enhance sensitivity of

Fig. 3. Polymerase chain reaction analysis of double catalase mutants to confirm deletion of pairs of *CAT* genes. **A**, *cat1;cat2* double mutants, **B**, *cat2;cat3* double mutants, and **C**, *cat1;cat3* double mutants. Strains used for template DNA are indicated at the top of each panel. Primer combinations are listed in Table 1. For *cat1* and *cat3*, left and right lanes correspond to checks of targeted deletion of the 3' and 5' ends of the gene, respectively; for *cat2*, left and right lanes correspond to checks of targeted deletion of the 5' and 3' ends of the gene, respectively. Expected product sizes are listed in Table 1. Outside lanes, 1 kb plus DNA ladder. Note that each panel represents three independent mutants. The left part of each panel is an assessment of deletion of one of the two *CAT* genes, the right side is the same template DNA assessed for the second *CAT* gene.

cat3 mutants (Fig. 5). Thus, CAT3 alone contributes to protection of *C. heterostrophus* against oxidative stress during vegetative growth.

Virulence of single, double, and triple *cat* mutants.

Symptoms caused by *cat1*, *cat2*, or *cat3* mutants or double or triple *cat* mutants were similar to those caused by wild-type strains of *C. heterostrophus* on maize (Fig. 6A and B). These results came from analysis of progeny of crosses between purified mutant transformants and wild-type testers. Primary *cat2* transformant D.C5.Cat2-3A, after purification by single sporing, was exceptional in that it caused smaller and fewer lesions on maize seedlings than did wild type. However, this transformant also had slow growth on nonamended minimal medium (MM) and the reduced virulence phenotype did not cosegregate with resistance to hygromycin B in progeny of crosses to wild type. Therefore, reduced virulence was not associated with *cat2* and was eliminated in progeny of a cross.

DISCUSSION

Results of this study indicate that *C. heterostrophus* monofunctional catalases are not required for normal virulence of the fungus to maize. This conclusion could be drawn because we were able to extract all putative monofunctional catalase genes from the *C. heterostrophus* genome sequence and delete them all by taking advantage of the highly efficient system for gene deletion via homologous recombination in this fungus. In addition, the ease of doing crosses in the laboratory allowed us to quickly construct double mutants. These resources positioned us to assay the virulence phenotype of single, double, and triple monofunctional catalase mutants on maize.

Several previous studies of catalase-deficient fungal mutants and their virulence to plants have been reported. In all of these studies, only single gene mutations were evaluated (Bussink and Oliver 2001; Calera et al. 1997; Garre et al. 1998; Schouten et al. 2002; Wysong et al. 1998). Among the CAT genes studied was that of *Candida albicans*, which, when mutated, caused reduced virulence in an intravenous infection model of disseminated candidiasis in mouse (Wysong et al. 1998). Three studies involving plant-fungal pathogen systems yielded no catalase mutants that caused reduced virulence. These included *C. fulvum* on tomato (two catalases reported, Bussink and Oliver 2001), *Claviceps purpurea* on rye (one catalase reported, Garre et al. 1998) and *Botrytis cinerea* on to-

mato and bean (two catalases reported, Schouten et al. 2002). One study with *Aspergillus fumigatus* (Calera et al. 1997) investigated the effect of deletion of one of two catalases on pathogenicity in a mouse model of aspergillosis. No differences were observed between the *cat1* mutant and its parental CAT1 strain.

Our observation of normal virulence in monofunctional catalase-deficient mutants does not imply that resistance to oxidative stress is unimportant for fungal virulence, since there are other enzymes known to be involved in detoxification of ROS. In fact, three different gene families that encode catalases able to detoxify hydrogen peroxide are known: i) manganese catalases (Barynin et al. 1986), ii) catalase peroxidases (Fraaije et al. 1996), and (iii) the true (monofunctional) catalases. This paper focused on true catalases, which are homotetrameric, heme-containing enzymes found in eukaryotes and prokaryotes (Klotz et al. 1997; Schonbaum and Chance 1976). Catalase peroxidases are also found in lower eukaryotes, but manganese catalases have been reported only in prokaryotes. Related to this, a reported fourth true catalase in *A. nidulans* was reannotated as a catalase-peroxidase (Kawasaki and Aguirre 2001; Scherer et al. 2002). A tblastn search of the *C. heterostrophus* genome, using the protein sequence of the *A. nidulans* catalase-peroxidase as a query, produced one strong hit. A catalase previously reported to be a virulence factor for tumorigenesis in the *Agrobacterium tumefaciens*-rye interaction also appears to be a catalase-peroxidase, not a true catalase. It lacks the two distinct PROSITE motifs found in monofunctional catalases and is homologous to the same *C. heterostrophus* hit identified in the query with the *A. nidulans* catalase-peroxidase. The *C. heterostrophus* catalase-peroxidase could have compensated for a possible virulence function of monofunctional catalases in *C. heterostrophus*. A tblastn search of the *C. heterostrophus* genome for the presence of manganese catalases did not yield any high hits, as would be predicted given their known prokaryote distribution.

The catalase triple mutant of *C. heterostrophus* had normal virulence, despite hyphae being clearly sensitive to hydrogen peroxide. This observation raises several questions to be addressed in the future. Were plant-produced hydrogen peroxide levels sufficiently high under our experimental conditions to cause inhibition of fungal growth during infection? If so, was the hydrogen peroxide in contact with the fungus? Was the

Fig. 4. Polymerase chain reaction confirmation of deletion of CAT3 in *cat1/cat2* double mutant BR23-R-10. The *cat1* (assayed with primer combination GCAT1-P0/ GCAT1-P5) and *cat2* (assayed with primer combination GCAT2-P0/ GCAT2-P5) controls (from left, lanes 2 to 5) confirm that this pair of CAT genes is deleted in BR23-R-10. Lanes 6 and 7, *cat3* (assayed with primer combination GCAT3-P0/GCAT3-P5) controls, C5 and BR23-R-10, which are wild type for CAT3 and thus yield the wild-type band. Lanes 8 to 10, triple catalase mutants D.Ch.59R, D.Ch.61R, and D.Ch.68R are deleted for *cat3* (assayed with primer combination GCAT3-P0/ GCAT3-P5). Outside lanes, 1 kb plus DNA ladder.

Fig. 5. Growth of wild-type and *cat1*, *cat2*, and *cat3* deletion progeny in 24 h, calculated as a change in colony diameter on 5 mM H₂O₂-amended minimal medium. Strains are wild-type strains (C5 and N21-R-24) and catalase deletion mutants, *cat1* (BR14-1-4 and BR14-R-1), *cat2* (BR12-2-2 and BR12-2-3), *cat3* (BR11-R-2 and BR11-R-6), *cat1/cat2* (BR23-R-10 and BR23-R-27), *cat1/cat3* (BR40-R-4 and BR40-R-11), *cat2/cat3* (BR30-R-4 and BR30-R-5), and *cat1/cat2/cat3* (D.Ch.59R, D.Ch.61R, and D.Ch.68R). Bar = standard deviation.

plant test sensitive enough to detect small changes in fungal virulence? Given these open questions, the role of fungal tolerance to ROS in virulence remains unresolved. Future studies should include deletion of all genes encoding enzymes that use ROS as substrates.

MATERIALS AND METHODS

Strains, media, crosses, and transformation.

C. heterostrophus culture media, growth conditions, crossing (Leach et al. 1982), and transformation procedures (Straubinger et al. 1990; Turgeon et al. 1987) were described previously. Strains used or constructed in this study are listed in Table 2. Transformations were conducted in strain C5, using the gene for resistance to hygromycin B (*hygB*) as a selectable marker (Turgeon et al. 1987) or, in strain BR23-R-10, using the gene for resistance to bialaphos (*bar*) as a se-

lectable marker (Straubinger et al. 1990). All transformants were purified by isolation of single conidia on complete medium (CM) without salts amended with 50 µg hygromycin B per ml (Invitrogen, San Diego, CA, U.S.A.). Strains were grown on CM with xylose (CMX) (Tzeng et al. 1992) at 22°C under black lights (Sylvania Inc., Danvers, MA, U.S.A.) for conidiation. Purified transformants were crossed to strain N21-R-24 (Table 2) for progeny tests.

Double mutants were created by crossing single mutants in all possible combinations (Table 2). Ascospores were transferred to CMX plates, after which mycelium from germinated spores was transferred to selective medium to determine hygromycin B sensitivity. Triple mutant strains D.Ch.59R, D.Ch.61R, and D.Ch.68R were created by deletion of *CAT3* in *cat1;cat2* double mutant BR23-R-10. Transformants were purified by isolation of single conidia on MM amended with 75 µg of bialaphos per ml (Meiji Seika Kaisha, Tokyo). Single-spored

Fig. 6. Virulence assays. Top, Lesions on corn leaves caused by wild-type strains (C5 and N21-R-24) and *cat1;cat2*, *cat1;cat3*, and *cat2;cat3* double mutants. No differences in quantity or quality of lesions were observed. Bottom, Lesions on W64-N-corn seedlings caused by wild type (C5 and N21-R-24), *cat1;cat2* double mutants, and *cat1;cat2;cat3* triple mutants. All are like wild type, indicating that none of these catalases alone or in combination is essential for virulence. BR23-R-10 is the *cat1;cat2* progenitor strain in which *cat3* was deleted. D.Ch.64S, D.Ch.57S, and D.Ch.67S are *cat1;cat2;CAT3* strains resulting from the purification of transgenic heterokaryons.

bialaphos-sensitive double mutant strains D.Ch.57S, D.Ch.64S, and D.Ch.67S were also obtained during single sporing of the primary bialaphos-resistant putative triple mutant, indicating that the original transformant was a heterokaryon, consisting of both bialaphos-resistant and sensitive nuclei. These were retained for comparison with bialaphos-resistant triple mutants in virulence assays (Table 2, Fig. 6B). Strains were stored at -80°C in 25% glycerol and were retrieved from storage prior to each experiment.

DNA isolation and PCR.

Fungal genomic DNA was isolated using a DNeasy Plant mini or 96-well kit (Qiagen, Valencia, CA, U.S.A.). PCR amplification was performed, using either Hotstart master mix (Qiagen) or Expand polymerase (Roche, Indianapolis, IN, U.S.A.). The PCR program used with Hotstart master mix was as follows: step 1, 95°C , 15 min; step 2, 96°C , 30 s; step 3, 54°C , 30 s; and step 4, 72°C , 1 min per kb. Steps 2 to 4 were repeated 36 times. The PCR program used with Expand was as follows: step 1, 94°C , 2 min; step 2, 94°C , 30 s; step 3, 54°C , 30 s; step 4, 68°C , 3.5 min, and step 5, 72°C for 10 min. Steps 2 to 4 were repeated 30 times.

Identification of catalases

using comparative molecular and phylogenetic analyses.

Amino acid sequences of known catalase genes were used to query (tblastn; Altschul et al. 1997) the *C. heterostrophus* genome sequence generated by Celera Genomics for TMRI/Syngenta to identify all monofunctional catalases. Open reading frames were annotated using Artemis (Rutherford et al. 2000). The *CAT1*, *CAT2*, and *CAT3* sequences were assigned GenBank accession numbers AY369262, AY369263, and AY369264, respectively. Protein sequences of selected catalases in GenBank were aligned with the *C. heterostrophus* sequences using T-Coffee (Notredame et al. 2000). Alignments were assessed manually and were improved, in certain cases, using Genedoc (Nicholas et al. 1997). For phylogenetic analysis, a maximum likelihood analysis using Bayesian inference was performed with the program MrBayes (Huelsenbeck and Ronquist 2001). MrBayes was run with the following parameters: a general model of DNA distribution (GTR) with gamma-distributed rate variation across sites. A Markov chain Monte Carlo analysis with four chains was run for 1,000,000 generations, with a sample frequency of 100. This yielded 10,000 trees, of which 200 were discarded. For additional comparison,

Table 2. *Cochliobolus heterostrophus* strains used in this study

Name	Genotype	Source
N21-R-24	WT; <i>MAT1-2</i>	N21-R-24 = progeny of N3-4-2 (<i>MAT1-2</i>) x N3-4-4 (<i>MAT1-1</i>) cross N3-4-2 = progeny of N2-2-6 (<i>MAT1-2</i>) x C5 cross N2-2-6 = progeny of C4 x C5 cross
C5	WT; <i>MAT1-1</i>	ATCC number 48331 (Leach et al. 1982)
C4	WT; <i>MAT1-2</i>	ATCC number 48332 (Leach et al. 1982)
D.C5.cat1-1B	<i>cat1</i> ; <i>MAT1-1</i> ; <i>hygB</i>	C5 carrying a <i>CAT1</i> deletion
D.C5.cat2-3A	<i>cat2</i> ; <i>MAT1-1</i> ; <i>hygB</i>	C5 carrying a <i>CAT2</i> deletion
D.C5.cat3-1B	<i>cat3</i> ; <i>MAT1-1</i> ; <i>hygB</i>	C5 carrying a <i>CAT3</i> deletion
BR14-1-4	<i>cat1</i> ; <i>hygB</i> ; <i>MAT1-1</i>	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-R-1	<i>cat1</i> ; <i>hygB</i> ; <i>MAT1-1</i>	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-R-4	<i>cat1</i> ; <i>hygB</i> ; <i>MAT1-2</i>	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-1-1	<i>cat1</i> ; <i>hygB</i> ; <i>MAT1-2</i>	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-R-8	WT	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-R-11	WT	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-R-12	WT	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR14-R-14	WT	Progeny of a N21-R-24 x D.C5.cat1-1B cross
BR12-2-1	<i>cat2</i> ; <i>hygB</i>	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-2-2	<i>cat2</i> ; <i>hygB</i> ; <i>MAT1-2</i>	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-2-3	<i>cat2</i> ; <i>hygB</i> ; <i>MAT1-1</i>	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-R-3	<i>cat2</i> ; <i>hygB</i>	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-1-1	WT	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-1-4	WT	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-R-2	WT	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR12-R-4	WT	Progeny of a N21-R-24 x D.C5.cat2-3A cross
BR11-R-2	<i>cat3</i> ; <i>hygB</i> ; <i>MAT1-2</i>	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-R-6	<i>cat3</i> ; <i>hygB</i> ; <i>MAT1-1</i>	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-R-7	<i>cat3</i> ; <i>hygB</i> ; <i>MAT1-1</i>	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-3-1	<i>cat3</i> ; <i>hygB</i> ; <i>MAT1-1</i>	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-R-1	WT	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-R-4	WT	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-R-5	WT	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR11-2-3	WT	Progeny of a N21-R-24 x D.C5.cat3-1B cross
BR23-R-10	<i>cat1</i> ; <i>cat2</i> ; <i>hygB</i>	Progeny of a BR14-R-1 x BR12-2-2 cross
BR23-R-14	<i>cat1</i> ; <i>cat2</i> ; <i>hygB</i>	Progeny of a BR14-R-1 x BR12-2-2 cross
BR23-R-27	<i>cat1</i> ; <i>cat2</i> ; <i>hygB</i>	Progeny of a BR14-R-1 x BR12-2-2 cross
BR20-R-6	<i>cat1</i> ; <i>cat3</i> ; <i>hygB</i>	Progeny of a BR14-R-4 x BR11-R-6 cross
BR30-R-4	<i>cat1</i> ; <i>cat3</i> ; <i>hygB</i>	Progeny of a BR14-R-4 x BR11-R-6 cross
BR30-R-5	<i>cat1</i> ; <i>cat3</i> ; <i>hygB</i>	Progeny of a BR14-R-4 x BR11-R-6 cross
BR40-R-4	<i>cat2</i> ; <i>cat3</i> ; <i>hygB</i>	Progeny of a BR12-2-2 x BR11-3-1 cross
BR40-R-9	<i>cat2</i> ; <i>cat3</i> ; <i>hygB</i>	Progeny of a BR12-2-2 x BR11-3-1 cross
BR40-R-11	<i>cat2</i> ; <i>cat3</i> ; <i>hygB</i>	Progeny of a BR12-2-2 x BR11-3-1 cross
D.Ch.59R	<i>cat1</i> ; <i>cat2</i> ; <i>cat3</i> ; <i>hygB</i> ; <i>bar</i>	BR23-R-10 carrying a deletion of <i>CAT3</i>
D.Ch.61R	<i>cat1</i> ; <i>cat2</i> ; <i>cat3</i> ; <i>hygB</i> ; <i>bar</i>	BR23-R-10 carrying a deletion of <i>CAT3</i>
D.Ch.68R	<i>cat1</i> ; <i>cat2</i> ; <i>cat3</i> ; <i>hygB</i> ; <i>bar</i>	BR23-R-10 carrying a deletion of <i>CAT3</i>
D.Ch.57S	<i>cat1</i> ; <i>cat2</i> ; <i>hygB</i>	Transformation of <i>BR23-R-10</i>
D.Ch.64S	<i>cat1</i> ; <i>cat2</i> ; <i>hygB</i>	Transformation of <i>BR23-R-10</i>
D.Ch.67S	<i>cat1</i> ; <i>cat2</i> ; <i>hygB</i>	Transformation of <i>BR23-R-10</i>

a maximum parsimony analysis with 200 random addition sequences and 200 bootstrap replicates was performed using PAUP*4.0 (Swofford 2002).

Catalase gene disruptions.

Each *CAT* gene (*CAT1*, *CAT2*, or *CAT3*) gene was entirely deleted, using the split marker method described by Catlett and associates (2003), based on a modification of Fairhead and associates (1998). In the first round of PCR (Fig. 1, Tables 3 and 4), the *hygB* cassette (2.5 kb) was amplified from pUCATPH (Lu et al. 1994), using M13F and M13R primers (Amberg et al. 1995). The flanking regions of *CAT1*, *CAT2*, and *CAT3* were amplified using primer combinations CAT-P1/CAT-P2 and CAT-P3/CAT-P4 (Fig. 1, Tables 3 and 4). A second round of PCR amplified one or the other flank and about two-thirds of *hygB*, as indicated in Table 3. The final PCR products (5' flank-HY and YG-3' flank for *cat1* and *cat3*, 5' flank-YG and HY-3' flank for *cat2*) were purified using Qiagen columns (Qiagen) and were used for transformation.

Double mutants were created by crossing single mutants. PCR analysis was used to screen for deletion of pairs of catalase genes in the progeny (Fig. 1, Table 1). Triple mutant (*cat1;cat2;cat3*) strains D.Ch.59R, D.Ch.61R, and DCh.68R were created by deletion of *CAT3* in a *cat1;cat2* double mutant (BR23-R-10), using a construct carrying the gene for resistance to bialaphos (*bar*, 1.5 kb) as the selectable marker (Straubinger et al. 1990) (Table 2). For this, three rounds of PCR were performed. In the first round, the *bar* gene was amplified from pBG (Straubinger et al. 1990) using M13F/M13R. The 'primary' 5 and 3' flanks were amplified with primer combinations GCAT3-P0/CAT3-P2 and CAT3-P3b/GCAT3-P5, respectively (Fig. 1, Tables 3 and 5). In the second round, GCAT3-P0/M13F and M13R/GCAT3-P5 were used to amplify the primary 5' flank-*bar* and *bar*-primary 3' flank pieces, respectively (Fig. 1). In the third round, nested primers CAT3-P1 and CAT3-P4 were used to amplify the final product (5' flank-*bar*-3' flank) that was used for trans-

formation (Fig 1, Tables 3 and 5). The product used for transformation was purified using Qiagen columns (Qiagen).

Oxidative stress assay.

Sensitivity to oxidative stress was tested on solid MM, with or without hydrogen peroxide in petri dishes (90-mm diameter). Autoclaved MM was cooled to 50°C, and then hydrogen peroxide (30%) (Sigma) was added to a final concentration of 5 mM. Mycelial disks (3-mm diameter) were cut with a cork borer 1 cm from the edge of an actively growing culture. Disks were inverted and placed in the centers of plates. Single, double, and triple catalase deletion mutants were represented by three to four strains in each assay, and assays were repeated. Colony diameters on four replicate plates per strain per concentration were measured after 2 and 3 days incubation at 22°C in the dark. Subsequently, colony diameter increase per 24 h was calculated, and the data analyzed. One-way ANOVA with Bonferroni's posttest was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software, San Diego, CA, U.S.A.).

Table 4. Primer combinations (primer positions in brackets) used to produce constructs for individual *CAT* gene deletions

Primer combinations	PCR product
First-round PCR	
CAT1-P3 and CAT1-P4 (D/E)	5' flank
CAT2-P1 and CAT2-P2 (H/I)	5' flank
CAT3-P1 and CAT3-P2 (N/O)	5' flank
CAT1-P1 and CAT1-P2 (B/C)	3' flank
CAT2-P3 and CAT2-P4 (J/K)	3' flank
CAT3-P3a and CAT3-P4 (Pa/Q)	3' flank
M13F and M13R	<i>hygB</i> cassette
Second-round PCR	
CAT1-P4 (E) and NLC 37 (HY)	5' flank-YG or -HY
CAT2-P1 (H) and NLC 38 (YG)	5' flank-YG or -HY
CAT3-P1 (N) and NLC 37 (HY)	5' flank-YG or -HY
NLC38 (YG) and CAT1-P1 (B)	YG- or HY-3' flank
NLC37 (HY) and CAT2-P4 (K)	YG- or HY-3' flank
NLC38 (YG) and CAT3-P4 (Q)	YG- or HY-3' flank

Table 3. Primers used in this study

Gene	Primer location	Primer name	Sequence 5 to 3'
CAT1	A	GCAT1-P0	CAGAACGACGCTACACTTGA
	B	CAT1-P1	TCTATGCGCCAGTAGCATGA
	C	CAT1-P2	GTCGTGACTGGGAAAACCCCTGGCG-TCGTTTCGCACCTAGCTTTAC
	D	CAT1-P3	TCCTGTGTGAAATTGTTATCCGCT-CGCCAGAAGCAGCTCCCTTA
	E	CAT1-P4	GGCATCTGCTGGTTGGATAA
CAT2	F	GCAT1-P5	GGTGAGATGGCGGAACGATT
	G	GCAT2-P0	CCAGCGACAGAAGAGGTCAA
	H	CAT2-P1	GAGTCTCGCGTGGTACATCG
	I	CAT2-P2	GTCGTGACTGGGAAAACCCCTGGCG-AAGTCGAGTCGGCTGGCAAT
	J	CAT2-P3	TCCTGTGTGAAATTGTTATCCGCT-GAACGCCATGGTTGCATACT
CAT3	K	CAT2-P4	AGAGATGTGACGCCGACGAG
	L	GCAT2-P5	ATTGCGTCTTGGTCGACGTT
	M	GCAT3-P0	ATCTATGCCACGCCGTACGA
	N	CAT3-P1	TGCGTGGCAAGCACAGTGTA
	O	CAT3-P2	GTCGTGACTGGGAAAACCCCTGGCG-CGACGTAAGATGGCCTGGAA
	Pa	CAT3-P3a	TCCTGTGTGAAATTGTTATCCGCT-CGGTCCGTTGGTGATGAATG
	Pb	CAT3-P3b	TCCTGTGTGAAATTGTTATCCGCT-ATCGGTCCGTTGGTGATGAA
	Q	CAT3-P4	TTCGCCGTGACGTCGTGAG
	R	GCAT3-P5	TGCGACGCATTGCTCAGATT
<i>hygB</i>	M13F	M13F	CGCCAGGGTTTTCCAGTCACGAC
	M13R	M13R	AGCGGATAACAATTTACACAGGA
	HY	NLC37 ^a	GGATGCCTCCGCTCGAAGTA
	YG	NLC38 ^a	CGTTGCAAGACCTGCCTGAA
	H1	H1	TCTCCTTGATGCACCATTCCTT
<i>bar</i>	H2	H2	TTGTGATCCGCCTGGACGACT
	M13F	M13F	Discussed above
	M13R	M13R	Discussed above

^a Catlett et al. 2003.

Table 5. Primer combinations (primer positions in brackets) used to produce a construct for deletion of *CAT3* from double mutant BR23-R-10 (*cat1;cat2*)

Primer combinations	PCR product
First-round PCR	
(M/O) GCAT3-P0 and CAT3-P2	Primary 5' flank
(Pb/R) CAT3-P3b and GCAT3-P5	Primary 3' flank
M13F and M13R	<i>bar</i> cassette
Second-round PCR	
M13F and (M) GCAT3-P0	<i>Bar</i> -primary 5' flank
GCAT3-P5 (R) and M13R	Primary 3' flank- <i>bar</i>
Third-round PCR	
(N/Q) CAT3-P1 and CAT3-P4	5' flank- <i>bar</i> -3' flank

Virulence assays.

Maize (*Zea mays* L.) seedlings (inbred W64A-N, 14 days old) grown in a Conviron (Winnipeg, Canada) growth chamber were inoculated by spraying with conidial suspensions in 0.05% Tween 20 harvested from 10-day-old cultures. Spore suspensions (1,000 spores per ml, 15 ml per strain) were sprayed on five or six seedlings, using a pressurized thin layer chromatography sprayer (Alltech Associates, Deerfield, IL, U.S.A.). Inoculated seedlings were placed in a dark mist chamber for 24 h (22°C, 100% relative humidity), after which plants were transferred to a Conviron growth chamber (23°C, 12-h light, and 90% relative humidity). Symptoms were evaluated five days after inoculation, and lesion sizes on the fourth oldest leaves were compared with those of wild-type strains C5 and N21-R-24. Single, double, and triple catalase deletion mutations were represented by three to four strains in each plant test. All plant tests were duplicated.

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