

# Catalase overexpression reduces the germination time and increases the pathogenicity of the fungus *Metarhizium anisopliae*

Claudia Erika Morales Hernandez · Israel Enrique Padilla Guerrero ·  
Gloria Angelica Gonzalez Hernandez · Eduardo Salazar Solis ·  
Juan Carlos Torres Guzman

Received: 18 December 2009 / Revised: 20 February 2010 / Accepted: 22 February 2010 / Published online: 2 April 2010  
© Springer-Verlag 2010

**Abstract** Catalases and peroxidases are the most important enzymes that degrade hydrogen peroxide into water and oxygen. These enzymes and superoxide dismutase are the first lines of cell defense against reactive oxygen species. *Metarhizium anisopliae* displays an increase in catalase–peroxidase activity during germination and growth. To determine the importance of catalase during the invasion process of *M. anisopliae*, we isolated the *cat1* gene. *cat1* cDNA expression in *Escherichia coli* and the subsequent purification of the protein confirmed that the *cat1* gene codes for a monofunctional catalase. Expression analysis of this gene by RT-PCR from RNA isolated from fungus grown in liquid cultures showed a decrease in the expression level of the *cat1* gene during germination and an increase during mycelium growth. The expression of this gene in the fungus during the infection process of the larvae of *Plutella xylostella* also showed a significant increase during invasive growth. Transgenic strains overexpressing the *cat1* gene had twice the catalase activity of the wild-type strain. This increase in catalase activity was accompanied by a higher level of resistance to exogenous hydrogen peroxide and a reduction in the germination time. This

improvement was also observed during the infection of *P. xylostella* larvae. *M. anisopliae* transgenic strains overexpressing the *cat1* gene grew and spread faster in the soft tissue of the insect, reducing the time to death of the insect by 25% and the dose required to kill 50% of the population 14-fold.

**Keywords** Catalase · *Metarhizium anisopliae* · Entomopathogenic fungi · Biocontrol

## Introduction

*Metarhizium anisopliae* is an entomopathogenic fungus used for the biological control of a wide range of insect pests. The interaction between the entomopathogenic fungus *M. anisopliae* and the host insect is a multifactorial process that culminates with the death of the insect. Germination, adhesion of conidia to the surface of the insect, penetration, invasive growth, and conidiation are the different stages of the pathogenic process. In each of these stages, the fungus faces environmental and nutritional challenges in addition to the defense mechanisms deployed by the host (for a review, see Roberts and St Leger 2004). *M. anisopliae* germination is marked by a significant increase in oxygen consumption which starts a few hours before germ tube emergence and reaches its highest level during exponential growth (Braga et al. 1999). Therefore, it is possible that the amount of hydrogen peroxide increases during germination and growth. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is one of the most frequently occurring reactive oxygen species (ROS), and it is produced by aerobic metabolism and enzymatic activity (Zamocky et al. 2008). Hydrogen peroxide formed during aerobic metabolism is capable of

**Electronic supplementary material** The online version of this article (doi:10.1007/s00253-010-2517-3) contains supplementary material, which is available to authorized users.

C. E. Morales Hernandez · I. E. Padilla Guerrero ·  
G. A. Gonzalez Hernandez · J. C. Torres Guzman (✉)  
Department of Biology, University of Guanajuato,  
Noria Alta s/n, Col. Noria Alta,  
Guanajuato, Guanajuato CP. 36050, Mexico  
e-mail: torguz@quijote.ugto.mx

E. Salazar Solis  
Department of Agronomy, University of Guanajuato,  
Guanajuato, Mexico

producing other reactive oxygen species which can damage many cellular components (Kawasaki and Aguirre 2001). Catalases and peroxidases are the most important enzymatic systems used to degrade  $H_2O_2$  into water and oxygen, thereby lowering intracellular hydrogen peroxide levels (Wang et al. 2007). These enzymes and superoxide dismutase are the cell's primary defense mechanisms against ROS. In fact, *M. anisopliae* displays increased catalase–peroxidase activity during germination and growth (Miller et al. 2004), and this may be necessary to counteract the hyperoxidant state produced by oxidative metabolism. Interestingly, genetic and biochemical studies indicate that reactive oxygen species play crucial roles in various aspects of cell physiology, cellular differentiation, signaling, and pathogen defense (Kawasaki and Aguirre 2001). In *Neurospora crassa*, *CAT-3* is involved in growth and differentiation (Michan et al. 2003), while *CAT-1* is involved in the oxidative stress response (Wang et al. 2007). In the phytopathogenic fungus *Claviceps purpurea*, it has been observed that this fungus secretes catalases in response to host plant defense mechanisms (Garre et al. 1998). In *Aspergillus oryzae*, *CATB*'s main function seems to be to reduce the toxic effects of oxidative stress that are induced by hydrogen peroxide (Hisada et al. 2005). In *Beauveria bassiana*, the specific activity of peroxisomal catalases is increased when glucose is replaced by an insect-like hydrocarbon. It has been suggested that in this fungus, the peroxisomal catalase could be involved in insect hydrocarbon catabolism (Pedrini et al. 2006) which proceeds through complete  $\beta$ -oxidation (Crespo et al. 2008). Thus, the catalase genes in fungi participate in the processes of cellular differentiation, detoxification, and catabolism.

In this work, we isolated the genomic copy of the *M. anisopliae cat1* gene and investigated its function in the invasion process that targets *Plutella xylostella* larvae by overexpressing the gene. Increased *cat1* expression was accompanied by improved  $H_2O_2$  tolerance and an increased germination rate, all of which improved the dosage–mortality data.

## Materials and methods

### Strains and growth conditions

*M. anisopliae* strain *Ma10* (CNRCB MaPL10) was obtained from the Centro Nacional de Referencia de Control Biológico and was originally isolated from *Geraeus senilis* (Coleoptera: Curculionidae) in the state of Colima (Mexico). The fungus was grown in minimal media (MM) consisting of 0.2%  $NH_4NO_3$ , 2% dextrose, 0.3%  $KH_2PO_4$ , and 2% 50 $\times$  salt stock solution (25 g  $MgSO_4$ , 0.09 g  $ZnSO_4$ , 0.05 g  $FeSO_4$ , 0.015 g  $MnSO_4$ , and 0.02 g  $CuSO_4$ ). Cultures were

grown in solid MM containing 2% agar, and conidia were collected by scraping the colony. The hyphae were removed by filtration through a sheet of sterile cheesecloth, and the conidia were washed three times by centrifugation and suspended in sterile 0.1% Triton X-100 solution. To obtain mycelia, conidia ( $1 \times 10^6$  ml $^{-1}$ ) were grown in minimal media containing either 2% glucose or 1% *P. xylostella* cuticle or in Sabouraud dextrose medium (SDM) and collected by filtration. The cultures were incubated in flasks at 28°C with shaking at 160 rpm. For developmental studies in solid medium plates, we proceeded as described (Staats et al. 2007) using a cellophane disc overlaid onto the appropriate solid medium to facilitate cell treatment or harvest.

*Escherichia coli* The DH5 $\alpha$  strain (Invitrogen, Carlsbad, CA, USA) was employed for DNA manipulations and transformations and was maintained in Luria–Bertani (LB) medium (Sambrook and Russell 2001) at 37°C. Ampicillin (100  $\mu$ g ml $^{-1}$ ) was added as required.

### Catalase activity

*M. anisopliae* homogenized cell-free extracts were prepared accordingly (Miller et al. 2004) by resuspending the material in 50 mM potassium phosphate buffer (pH 7.5) containing 10  $\mu$ M PMSF, 11.2  $\mu$ M E64, 2.5  $\mu$ g ml $^{-1}$  leupeptin, and 2  $\mu$ g ml $^{-1}$  pepstatin. The suspensions were centrifuged at 13,000 $\times$ g for 15 min at 4°C to remove cell debris, and the supernatants were assayed immediately for total protein and catalase activity. Protein concentration was quantified by the Lowry method (Lowry et al. 1951) using bovine serum albumin as the standard. Catalase activity was measured spectrophotometrically as described (Hisada et al. 2005) using a UV/vis spectrophotometer (Ultrospec 4300 Pro, Amersham Biosciences). One unit of catalase activity is defined as the amount of enzyme that decomposes 1  $\mu$ mol of  $H_2O_2$  per minute at 25°C. Consumption of  $H_2O_2$  was measured as the decrease in absorbance at 240 nm. Catalase activity in non-denaturing 8% polyacrylamide gels was determined as described (Lledias et al. 1998) with minor modifications, incubating the gel after electrophoresis 5 min in 5% methanol and then after rinsing three times with distilled water, 5 min in 10 mM  $H_2O_2$ . The gel, rinsed with distilled water, was incubated in a 1:1 mixture of freshly prepared 1% potassium ferric cyanide and 1% ferric chloride. Blue color developed in the gel except at zones where  $H_2O_2$  was decomposed by catalase. Staining was stopped by soaking the gel in a 10% acetic acid solution.

### Double-staining method

Visualization of catalase and catalase peroxidase activity on non-denaturing polyacrylamide gels was performed

using the ferric chloride–potassium ferric cyanide, double-staining method (Wayne and Diaz 1986; Yamashita et al. 2007) with minor modifications. The samples were separated by 8% native polyacrylamide gel electrophoresis. The gels were washed with a 50 mM phosphate buffer (pH 7.0) and treated with 50 mM phosphate buffer (pH 7.0) containing 0.02% 3,3'-diaminobenzidine and 0.006% hydrogen peroxide for 30 min at room temperature. The gels were washed twice with distilled water and soaked in 0.006% hydrogen peroxide for 10 min with gentle shaking. The gels were then stained with a potassium ferricyanide (1%, w/v) and ferric chloride solution (1%, w/v) until bands were visible. The gels were destained in distilled water overnight and photographed. Catalases with peroxidase activity appeared as dark bands within a zone of clearing in the green background. Catalases appeared only as a clear zone.

#### Nucleic acid isolation and *cat1* gene cloning

Fungal genomic DNA was isolated from mycelia grown for 24 h in SDM. DNA was extracted following standard protocols (Sambrook and Russell 2001). Total RNA was extracted from mycelia grown in different culture media or from larvae previously inoculated with conidia using Trizol® Reagent (Invitrogen) according to the manufacturer's instructions. All samples were DNase-treated using RNase-Free DNase (Promega Co., Madison, WI, USA) according to the manufacturer's instructions. The concentration and purity of both total RNA and DNA were determined by the ratio of absorbance's at 260 and 280 nm. A pair of specific primers, *catfd1* and *catr2* (Electronic supplementary materials (ESM), Table S1), was designed according to the cDNA sequence of the *cat1* gene (accession number DQ393583) and used to amplify a fragment containing the complete open reading frame (ORF) from *M. anisopliae*. The reaction mixture (50 µl) contained total DNA, each primer, and PCR Super Mix High Fidelity (Invitrogen). The amplification product was cloned into the pCR®2.1-TOPO® vector (Invitrogen) and sequenced. The nucleotide sequence of the clone was generated on both strands using universal primers and the specific primers *catfd3*, *catr4*, *catr5*, and *catfd6*, which were based on the *cat1* genomic sequence (sequence deposited in EMBL database, accession number FN641683). The DNA sequence was analyzed using the Lasergene 8 program (DNASTAR Inc., Madison WI, USA), and its identity was confirmed by comparison to published catalase sequences in the database ([www.ebi.ac.uk](http://www.ebi.ac.uk)).

#### Copy number of *cat1* by Southern blot analysis or semi-quantitative PCR

For Southern blot analysis, restricted chromosomal DNA was prepared, and Southern hybridization was performed

following standard procedures (Sambrook and Russell 2001). A radioactively labeled DNA probe of *M. anisopliae cat1* was used as a homologous probe. Two pairs of primers were used simultaneously in semi-quantitative PCR reactions: One pair was used to amplify the gene of interest (*cat1*), and at the same time in the same reaction tube, a second pair of primers was used to amplify a mono-copy gene used as control. Genomic DNA (100 ng) was added as the template in addition to PCR Super Mix High fidelity (Invitrogen) according to the manufacturer's recommendations and the specific primers *catfd7* and *catr8* for the *cat1* gene and the pair TEF1-(α)221 SEN and TEF1(α)221 ANTI for the *tef-1α* gene, which was used as a mono-copy control (ESM Table S1; Nakazato et al. 2006). All samples were subjected to PCR in a GeneAmp System 9700 (Applied Biosystems, Foster City, CA, USA). Aliquots of the PCR products were analyzed on 2% agarose gels and were quantified by densitometric analysis (Quantity One version 4.1.1, Bio-Rad, Hercules, CA, USA).

#### In vitro expression of the *cat1* gene from *M. anisopliae* grown in *P. xylostella* cuticle by semi-quantitative RT-PCR analysis

To obtain mycelia, conidia ( $1 \times 10^6 \text{ ml}^{-1}$ ) were grown in liquid minimal media containing 1% *P. xylostella* cuticle. The cultures were incubated in Erlenmeyer flasks at 28°C with shaking at 160 rpm. At specific times (0, 4, 8, 12, 16, 24, 36, and 48 h), the fungal material was collected by filtration and frozen immediately in liquid nitrogen and stored at  $-70^\circ\text{C}$  until use. Half of the material was used for catalase isozyme migration pattern detection in non-denaturing 8% polyacrylamide gels as previously described (Lledias et al. 1998). The other half was employed for RT-PCR analysis of the *cat1* gene and AJ274118 (loading control) to compare transcript abundances. RT-PCR was performed using the Superscript™ III One-step RT-PCR System with Platinum® taq DNA Polymerase kit (Invitrogen) according to the manufacturer's recommendations. The reaction employed specific primers: *catintsen* and *catintanti* for the *cat1* gene and *conest100-sen* and *conest100-anti* for the AJ274118 gene (ESM Table S1), which was used as a constitutive expression control (Freimoser et al. 2005) and which does not show a significant match with any other reported sequence in the relevant databases. Both oligomer pairs were used simultaneously in the same reaction tube. All samples were subjected to One-Step RT-PCR under the following conditions. The reverse transcription reactions were carried out at 58°C for 30 min and DNA amplifications were then carried out in a GeneAmp System 9700 (Applied Biosystems). Aliquots of the RT-PCR products were analyzed on 3% agarose gels (Ultrapure™ Agarose-1000, Invitro-

gen). Expression results were quantified by densitometric analysis (Quantity One version 4.1.1, Bio-Rad).

*In vivo* expression of the *M. anisopliae cat1* gene from infected larvae (*P. xylostella*) by semi-quantitative RT-PCR analysis

Petri dishes containing 25 third-instar *P. xylostella* larvae were placed on the stage of a Potter spray tower (Potter-Precision Laboratory Spray Tower, Burkard Scientific, Uxbridge, Middx, UK) and sprayed with a conidial suspension (500 all of  $1.5 \times 10^9$  conidia per milliliter) at 10 lb plg<sup>-2</sup> pressure. One hour after inoculation, the larvae were fed with a leaf disk of *Brassica oleracea*. Every 48 h, the leaf disk was changed. Petri dishes were incubated at 25°C and maintained in a humid environment with sterilized moist paper. At death, conidia from infected insect cadavers were collected with sterile 0.1% Triton X-100 solution. As a control, Petri dishes containing 25 third-instar *P. xylostella* larvae were sprayed with 500 µl of 0.1% Triton X-100 solution. Different stages of infection were monitored over an 8-day period. The stages of infection were categorized as follows: (1) 0 (conidia) to 24 h post-infection; (2) invasion of the infected insect (24–48 h); (3) death (48–72 h); (4) growth of mycelia on the surface of the cadaver (72–144 h); and (5) conidial growth on the surface of the cadaver (+144 h). At each time point in the observed infection, 10–40 larvae were collected and kept safe at -70°C until RNA extraction. From a parallel control experiment, larvae treated only with 0.1% Triton X-100 solution were collected. RNA was extracted from these larvae, and RT-PCR analysis of the *cat1* gene and AJ274118 (loading control) was performed to compare transcript abundance. RT-PCR was performed using the Superscript TM III One-step RT-PCR System with Platinum® taq DNA Polymerase kit (Invitrogen) according to the manufacturer's recommendations and as described in the previous paragraph.

Vector construction for overexpression of the *cat1* gene in *M. anisopliae*

An *XbaI*–*XbaI* fragment containing the *bar* gene of *Streptomyces hygrosopicus*, encoding resistance to ammonium glufosinate, fused to the *Aspergillus nidulans trpC* promoter from plasmid pBARGPE1 (Fungal Stock Center, [www.fgsc.net](http://www.fgsc.net)), was inserted into the *XbaI* site in plasmid pGPDAsGFP (Fernandez-Abalos et al. 1998). The resultant plasmid was designated pGG269. The *cat1* open reading frame of 2,480 bp was amplified by PCR using genomic DNA from *M. anisopliae* strain *Ma10* and the primers *catfd1PciI* and *catr2BamHI* (ESM Table S1). The resultant PCR product was digested with *BamHI* and *PciI* and inserted into the corresponding sites (*BamHI* and *NcoI*

sites) of the pGG269 plasmid. The resultant vector was designated pGG345 and contained the *cat1* ORF, which was up-regulated by the strong promoter *gpdA* from *A. nidulans*.

Transformation of *M. anisopliae*

Protoplasts of *M. anisopliae* were produced and transformed by the polyethylene glycol method as described previously (Goettel et al. 1990). Ammonium glufosinate-resistant colonies were selected on solid minimal medium supplemented with 500 µM ammonium glufosinate (Sigma-Aldrich, St. Louis, MO, USA). Colonies of transformants were collected for 5 to 7 days after the start of growth at 28°C in selective medium, and ten colonies were selected at random. Monospore isolates of each selected transformant were obtained for analysis in non-selective medium. Putative transformants were confirmed by PCR.

Expression of the *cat1* cDNA in *E. coli*

Expression of the *M. anisopliae cat1* cDNA in *E. coli* BL21 (DE3)pLysS (Invitrogen) was accomplished by sub-cloning the *cat1* cDNA into a pRSETB expression vector (Invitrogen). The *cat1* cDNA from *M. anisopliae Ma10* was amplified with primers *catBamHI* and *catSacI* (ESM Table S1) in a reaction mixture (50 µl) with PCR Super Mix High Fidelity (Invitrogen). The amplification product was digested with both *BamHI* and *SacI* restriction enzymes and ligated to *BamHI/SacI* digested pRSETB DNA plasmid, resulting in the recombinant plasmid pGG461. *E. coli* BL21(DE3)pLysS cells were transformed with plasmid pGG461, and colony selection occurred in LB liquid medium supplemented with ampicillin and chloramphenicol. For CAT1 expression and purification, bacterial cells were grown until O.D. of 0.4 at 37°C in LB liquid medium supplemented with ampicillin at 100 µg ml<sup>-1</sup>, chloramphenicol at 35 µg ml<sup>-1</sup>, and 1 mM IPTG for 3 h at 37°C. Induced cells were collected by centrifugation (8,000×g for 5 min at 4°C) and resuspended in binding buffer (20 mM phosphate, 500 mM NaCl, and 20 mM imidazole with 0.2 mg ml<sup>-1</sup> lysozyme, 20 µg ml<sup>-1</sup> DNase). Collected cells were broken by three to four alternating periods of freezing in liquid N<sub>2</sub> followed by thawing at 42°C. Cell debris was separated by centrifugation (40,000×g, for 30 min at 4°C), and the supernatant was subsequently filtered through a 0.22-µm syringe filter. The crude extracts were passed through a 1-ml HisTrap FF column (GE Healthcare, Piscataway, NJ, USA) equilibrated with 50 mM phosphate buffer, pH 7.5, and 20 mM imidazole according to the directions of the supplier. The proteins bound to the column were eluted with a gradient of 20 to 300 mM imidazole. The CAT1 product was detected between 100 and



300 mM imidazole. Fractions were saved at  $-70^{\circ}\text{C}$  until their use in catalase assays. The catalase activity was measured spectrophotometrically (Lledias et al. 1998; Hisada et al. 2005).

#### Assay of sensitivity to $\text{H}_2\text{O}_2$

The sensitivity of conidia to  $\text{H}_2\text{O}_2$  was estimated using a previously described protocol (Paris et al. 2003). Conidia ( $1 \times 10^6 \text{ ml}^{-1}$ ) were incubated at  $28^{\circ}\text{C}$  for 30 min with various  $\text{H}_2\text{O}_2$  concentrations (5, 10, 15, 25, and 50 mM). The  $\text{H}_2\text{O}_2$  was discarded by centrifugation, and conidia were suspended in the original water volume. A total of 500 conidia from each treatment were plated on Sabouraud dextrose (SD) agar medium (in triplicate) and incubated at  $28^{\circ}\text{C}$  for 72 h. The numbers of viable colonies was counted and their survival rates determined. The results depicted are the mean  $\pm$  standard deviation of three independent experiments. The data were analyzed by probit analysis using PoloPlus software (LeOra Software Company, Petaluma, CA, USA).

#### Conidia germination

To determine the rate of conidia germination,  $6 \times 10^6$  conidia per milliliter were gently streaked over SD agar medium, which was prepared on sterile microscope slides, and incubated at  $28^{\circ}\text{C}$  for 12 h. Conidia germination was measured at 2-h intervals. Populations of at least 200 cells were counted under the microscope, and the percentage of germlings and ungerminated conidia was determined. A germling was defined as a cell with a germ tube equal in length to the width of the conidia. The values are presented as the mean  $\pm$  standard deviation of three independent experiments.

#### Insect bioassay

Fresh conidia of *M. anisopliae* strains obtained from cultures on MM were used in bioassays against laboratory-reared third-instars *P. xylostella* larvae. Ten larvae per dose were sprayed with 0.5 ml conidial suspension using a Potter spray tower (Potter-Precision Laboratory Spray Tower, Burkard Scientific) at  $10 \text{ lb plg}^{-2}$ . Each treatment consisted of four replicates. The nine doses ranged from  $1 \times 10^5$  to  $5 \times 10^8$  conidia per milliliter. Each conidia dose was tested in four independent assays. One-hour post-inoculation larvae were fed with a leaf disk of *B. oleracea*. Every 48 h, the leaf disk was changed. Petri dishes were incubated at  $25^{\circ}\text{C}$ , and a humid environment was maintained with sterilized moist paper. Mortality was recorded at 8-h intervals, and the data were analyzed by probit analysis using PoloPlus software (LeOra Software Company).  $\text{LC}_{50}$  values were considered significantly different if their 95% confidence limits did not overlap.

## Results

### Cloning of the *cat1* gene and sequence analysis

Using the representational differences analysis approach to identify genes that are differentially expressed during the invasion of *M. anisopliae* into its host, the diamondback moth (*P. xylostella*), we isolated approximately 1,500 independent clones. One of them contained a 450-bp fragment that has high similarity to catalase genes. This sequence corresponded to a clone of 2,315-bp with accession number DQ393583 (<http://www.ebi.ac.uk>) containing a complete *cat1* mRNA from *M. anisopliae* strain ARSEF2575. We isolated the genomic copy of this gene and its respective cDNA from *M. anisopliae* strain Ma10 using the primers catfd1 and catr2 designed according to the catalase mRNA sequence (ESM Table S1). The results showed an amplification product of 2,480 bp corresponding to the genomic copy and a cDNA product of 2,145 bp. Both fragments were cloned and sequenced. Analysis of the *cat1* gene revealed an open reading frame interrupted by five introns that was indicative of a 715-amino acid polypeptide. The introns were corroborated by sequence alignments between the genomic and cDNA sequences from the Ma10 strain. The predicted protein sequence was compared with those of other catalases from filamentous fungi: monofunctional catalases and catalase–peroxidases. This sequence is homologous to: *C. purpurea* CAT1 (78.2% identity, accession number O60038); *N. crassa* CAT-3 (65.6% identity, accession number Q9C169); *A. nidulans* CATB (62% identity, accession number P78619); and *A. oryzae* CATB (64% identity, accession number Q877A8; ESM Fig. S1). A search of the PROSITE database identified the canonical catalase motifs. The predicted protein sequence showed the conserved catalytic site FDHERVPERAV HARGAG in the amino acid chain at positions 91–107. This site contains the conserved H-102 residue which is present in all heme catalases and is required for proper binding and reduction of the  $\text{H}_2\text{O}_2$  molecule. The heme binding site is composed of amino acid residues RIF SYLDTQL at positions 385–394. A possible secretion signal peptide (MVQARLV AISLALASLASS) at the amino terminal end is 57.9% identical (84.2% similarity) to the putative secretion signal of CAT1 from *C. purpurea* (Garre et al. 1998). The *cat1* gene exists as a unique copy in the *M. anisopliae* genome, as revealed by Southern blot analysis and semi-quantitative PCR analysis (data not shown).

### Expression of the *cat1* cDNA in *E. coli*

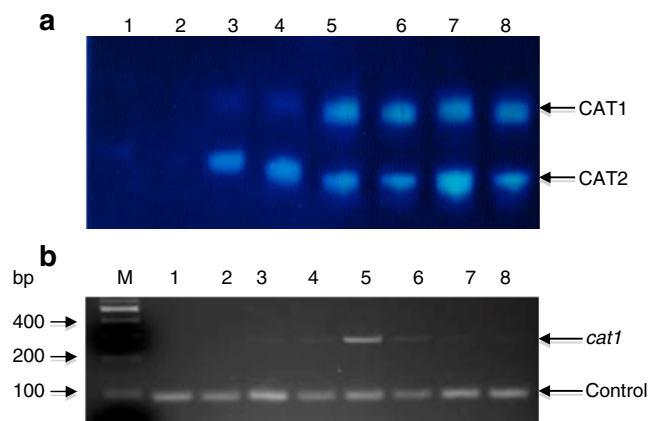
To determine whether the *cat1* gene codes for a monofunctional catalase or a catalase–peroxidase protein, we expressed the *M. anisopliae cat1* cDNA in *E. coli* cells using the

pRSETB vector. After recombinant CAT1 purification with a Ni<sup>+</sup> agarose column, CAT1 showed only catalase activity with in-gel enzyme assays (ESM Fig. S2a, b). This result confirms the amino acid sequence analysis and indicates that *cat1* codes for a monofunctional catalase.

#### RT-PCR analysis of the *M. anisopliae cat1* gene in vitro

*M. anisopliae* displays different responses to different growth conditions and undergoes both pathogenic and saprophytic growth (St Leger et al. 1994; St Leger et al. 1997). EST and cDNA microarray analyses have demonstrated that *M. anisopliae* can rapidly adjust its genomic expression patterns to adapt to insect cuticle, producing specific responses to different cuticle conditions and undertaking saprophytic growth (Freimoser et al. 2003; Dutra et al. 2004; Freimoser et al. 2005; Wang and St Leger 2005). To investigate the *cat1* expression profile during the invasion of *M. anisopliae* into its host *P. xylostella*, we carried out an expression analysis of the *cat1* mRNA by semi-quantitative RT-PCR analysis. To perform this kind of assay, we looked for a constitutively expressed control gene. From an analysis of the variation in gene expression patterns in *M. anisopliae* performed with microarrays (Freimoser et al. 2005), house-keeping genes for cell metabolism and certain other genes that had no significant match with any well-known genes showed stable expression levels. One of these is the EST AJ274118 which does not show significant changes in its expression under any of the tested conditions. Therefore, we designed pairs of primers to amplify DNA fragments of 100–300 bp in length for each gene studied by RT-PCR analysis. The same concentration of total RNA from cells grown on various carbon sources (glucose, chitin, or *P. xylostella* cuticle) was used. Fragments of cDNA from AJ274118 and *chit1* were amplified simultaneously in the same reaction tube. Amplification of the constitutively expressed control AJ274118 showed a similar amount of cDNA in each sample, with a constant amount of transcript throughout, using either glucose, chitin, or *P. xylostella* cuticle as the carbon source (ESM Fig. S3a), consistent with the microarray data (Freimoser et al. 2005). In contrast, high levels of *chit1* expression were observed in cells grown on chitin, along with low expression levels on glucose or with *P. xylostella* cuticle, consistent with the induction of chitinase activity previously described (St Leger et al. 1986). Therefore, AJ274118 was used as a constitutively expressed control in semi-quantitative RT-PCR assays. In these experiments, *cat1* expression was highest with glucose as the carbon source, followed by chitin and then *P. xylostella* cuticle (ESM Fig. S3b). Because these experiments represent only single points in the fungal life cycle, we performed the analysis during *M. anisopliae* growth in minimal medium with *P. xylostella* cuticle as the carbon source, inoculating

conidia and taking samples at different time points (0, 4, 8, 12, 16, 24, 36, and 48 h) post-inoculation. Half of the sample was used for RT-PCR analysis and the other half used to detect catalase activity in non-denaturing electrophoresis gels. As shown in Fig. 1a, *M. anisopliae* Ma10 conidia extracts displayed two catalase activity bands. These bands declined in activity level in extracts from 4-h samples and appeared again in extracts from 8-h samples. The intensity of the fastest moving isozyme band increased drastically after 8 h (conidia germination starts between 6 and 8 h), with the maximum activity level at 36 h. The slower moving isozyme band was detected in extracts from 8-h samples, with maximal intensity in extracts from 16-h samples (mycelial growth). This behavior resembles the expression level of *CAT-1* and *CAT-3* from *N. crassa*. The RT-PCR analysis showed *cat1* expression from 8 h until the end of the experiment (48 h), with the maximum at 16 h (Fig. 1b). These analyses suggest a correlation between *cat1* and the slower moving isozyme band. All catalases that have been previously detected in two different isolates from *M. anisopliae*, ARSEF2575 and ARSEF 324, showed peroxidase activity (Miller et al. 2004). To identify the monofunctional catalase in *M. anisopliae*, we took samples from the growth of *M. anisopliae* Ma10 in minimal medium with *P. xylostella* cuticle during a 48-h period and tested these samples for catalase and peroxidase activity by in-gel analysis. The fastest moving band showed catalase and



**Fig. 1** In vitro expression of *cat1* in *M. anisopliae* grown on *P. xylostella* cuticle. Conidia from *M. anisopliae* Ma10 were grown in liquid minimal media containing 1% *P. xylostella* cuticle. The cultures were incubated in Erlenmeyer flasks at 28°C as described in “Materials and methods.” The fungal material was collected by filtration and cellular extracts were obtained; half of the material was used to determine the catalase isozyme migration pattern in non-denaturing 8% polyacrylamide gels while the other half was used to compare transcript abundance by RT-PCR analysis. The lanes correspond to samples harvested at specific time points: lane 1 (0 h), lane 2 (4 h), lane 3 (8 h), lane 4 (12 h), lane 5 (16 h), lane 6 (24 h), lane 7 (36 h), and lane 8 (48 h) post-inoculation. **a** Non-denaturing 8% polyacrylamide gel (50 µg protein/lane). **b** Relative RT-PCR analysis of *cat1* and AJ274118 (loading control) using total RNA

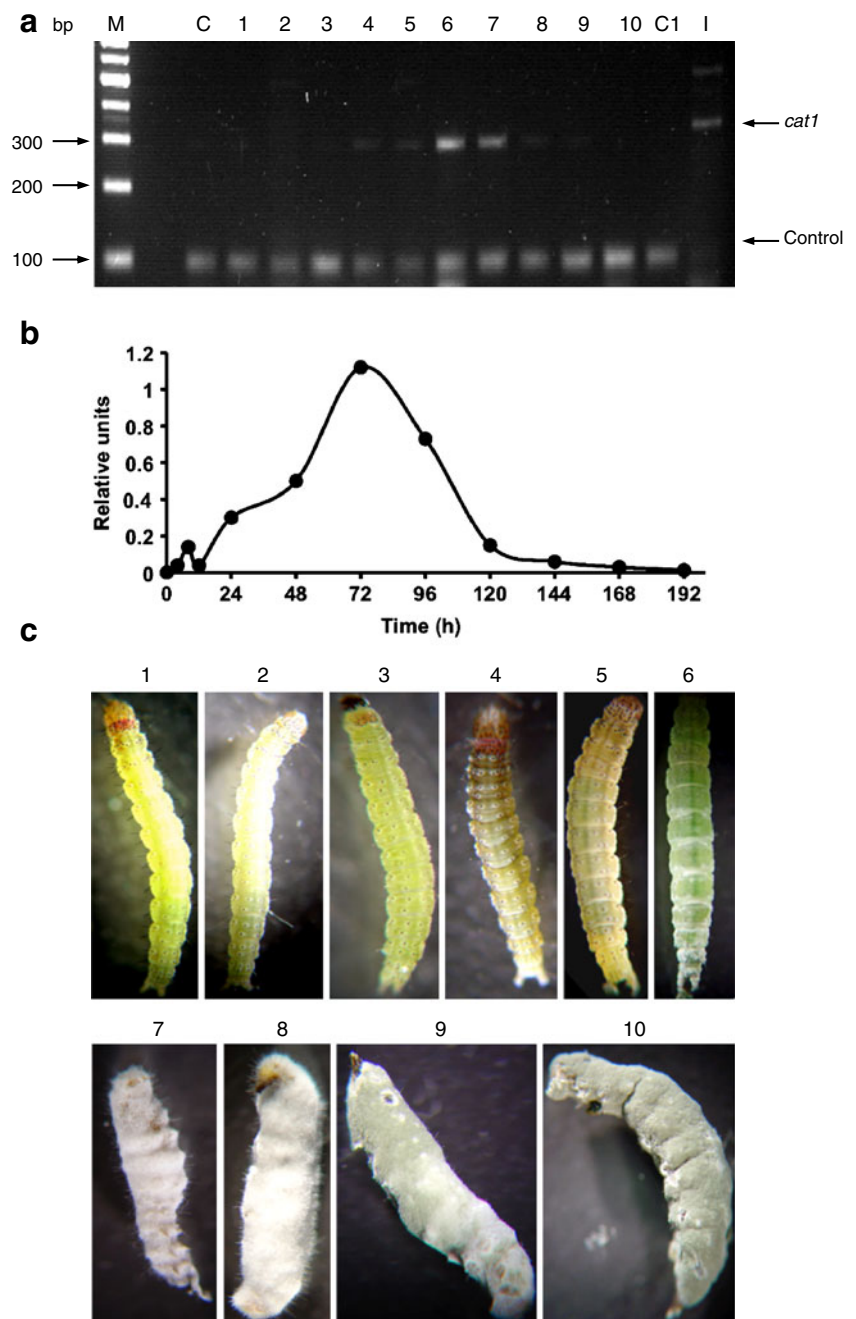
peroxidase activity, while the slower moving band showed only catalase activity (ESM Fig. S2c).

#### RT-PCR analysis of the *M. anisopliae cat1* gene during insect infection

The above experiments were performed *in vitro*. In order to analyze *cat1* expression *in vivo*, *P. xylostella* was infected with *M. anisopliae* conidia, different stages of infection in the larvae were monitored, and observations were made over an 8-day period. Eighty-seven percent of infected insects died by day 3, while the other 13% died the next

day. The times at which the insects reached the various stages of infection were similar. The infected larvae were collected at different times post-inoculation, and RNA was extracted and used to synthesize cDNA. The samples were used as templates for semi-quantitative PCR analysis, amplifying simultaneously in the same reaction tube *cat1* and AJ274118 (loading control; Fig. 2a). A *cat1* product of 293 bp was observed during the first 24 h post-infection, during invasion of the infected insect (24–48 h), and at larvae death (48–72 h). In contrast, an AJ274118 product of 100 bp was observed throughout the entire course of insect infection by *M. anisopliae*. The densitometric analysis

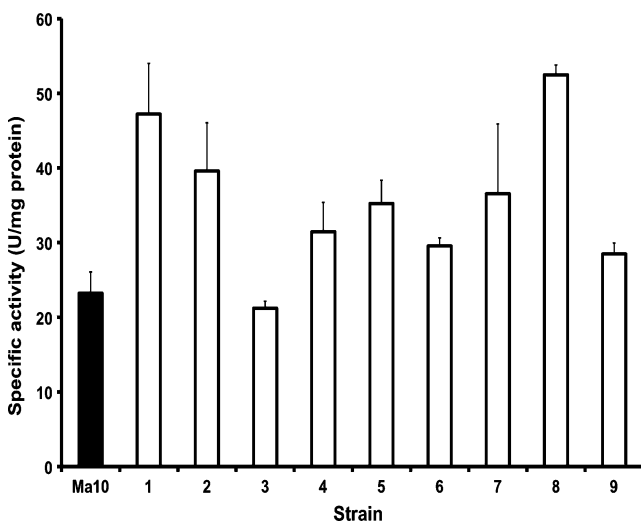
**Fig. 2** Expression of *M. anisopliae cat1* during insect infection. **a** RT-PCR analysis of *cat1* and AJ274118 (loading control) gene expression during the progressive infection of third-instars diamondback moth (*P. xylostella*) larvae with *M. anisopliae* Ma10. Lane M corresponds to DNA marker. Lane C corresponds to amplification products from the total RNA of *M. anisopliae* Ma10 conidia. Lanes 1–10 correspond to RT-PCR amplification products from the total RNA of third-instars diamondback moth larvae infected with *M. anisopliae* Ma10 conidia at different time points post-infection: lane 1 (4 h), lane 2 (8 h), lane 3 (12 h), lane 4 (24 h), lane 5 (48 h), lane 6 (72 h), lane 7 (96 h), lane 8 (120 h), lane 9 (144 h), lane 10 (168 h). Lane C1 corresponds to RT-PCR amplification products from the total RNA of *M. anisopliae* Ma10 conidia recollected from dead insects; lane I correspond to RT-PCR amplification products from the total RNA of uninfected third-instars diamondback moth (*P. xylostella*) larvae. **b** Relative expression of *cat1* during invasion measured by densitometric analysis. **c** Morphology of the progression of infection in third-instars diamondback moth (*P. xylostella*) samples; numbers correspond to the same time points post-infection as described in **a**. Results are representative from two separate RNA extractions and RT-PCR analysis, from independent infections assays



shows the major *cat1* expression during the invasion process, taking place inside the insect (Fig. 2b). The two RT-PCR products amplified from uninfected insects using the *cat1* and AJ274118 primers correspond to unspecific amplification. Figure 2c illustrates normal larval morphology during the course of infection.

#### Overexpression of the *M. anisopliae cat1* gene

To investigate the potential function of *cat1* during the *M. anisopliae* infection process, we performed overexpression experiments where the *cat1* gene was placed under the control of the constitutive promoter *gpdA* from *A. nidulans* (which was previously reported to be a functional promoter in *M. anisopliae*; St Leger et al. 1996) in a vector containing the *bar* gene as a selective marker. The resulting vector (named pGG345) was introduced by transformation into *M. anisopliae* protoplasts. Ten independent colonies were selected randomly for analysis. The transformant strains were grown in SD medium for 48 h, and the specific activity of catalase was quantified in the crude extract supernatant in recombinant and wild-type strains as reported (Hisada et al. 2005) using hydrogen peroxide as the substrate. The results for the specific activity indicate that most of the recombinant strains showed an increase in catalase activity in contrast to the wild-type strain (Fig. 3).



**Fig. 3** Specific catalase activity in *M. anisopliae* strain *Ma10* and *cat1* overexpression strains. *M. anisopliae* strain *Ma10* and the *cat1* overexpression strains were grown in Sabouraud dextrose (SD) medium for 48 h, and the specific activity of catalase was determined spectrophotometrically in the crude extract supernatant using hydrogen peroxide as the substrate. Specific activity was calculated by dividing the units of catalase activity by the total protein. Data represent the mean  $\pm$  standard error of three independent experiments. *Ma10* is the wild-type strain; 1 to 9 correspond to eight different transformants: 1 *C19cat1*-1, 2 *C19cat1*-2, 3 *C19cat1*-3, 4 *C19cat1*-4, 5 *C19cat1*-5, 6 *C19cat1*-6, 7 *C19cat1*-7, and 8 *C19cat1*-8

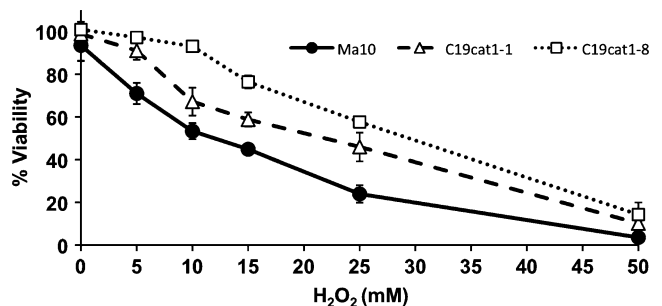
The strains *C19cat1*-1 and *C19cat1*-8 presented 1.8 and 2 times higher catalase activity, respectively, than the wild-type strain and were used in the next experiments.

#### Sensitivity of conidia to exogenous $H_2O_2$

We analyzed the sensitivity of conidia to  $H_2O_2$  exposure in strains overexpressing *cat1* by examining the ability of the conidia to germinate and form colonies. For this purpose, conidia from the *cat1* overexpressing strains *C19cat1*-1 and *C19cat1*-8 were exposed to increasing concentrations of hydrogen peroxide. The surviving conidia were evaluated by quantifying their ability to germinate and produce colonies on SD medium. The results indicated that the viability of the transformed strains was superior to that of the wild-type strain (Fig. 4). The hydrogen peroxide lethal dose 50 ( $LD_{50}$ ) was 60% higher in *C19cat1*-1 ( $LD_{50}$ =18.5) and 240% higher in *C19cat1*-8 ( $LD_{50}$ =28) than in the wild-type strain *Ma10* ( $LD_{50}$ =11.5). Thus, *cat1* overexpression dramatically improved the viability of conidia against exogenous hydrogen peroxide.

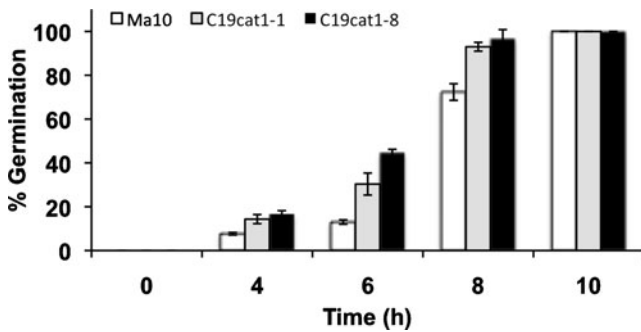
#### Velocity of germination

If the amount of ROS during germination in *M. anisopliae* increases because of the rise in oxidative metabolism (Braga et al. 1999) and if the observations about decreases in catalase–peroxidase activity during conidial germination are related to oxidative stress (Miller et al. 2004), it is possible that the concentration of ROS exceeds the antioxidant capacity of the cell during conidia germination. Our interest was to determine the efficiency of germination in *cat1*-overexpressing strains. Conidia from the *C19cat1*-1 and *C19cat1*-8 strains were streaked on SD medium and germination was quantified. The results showed that the *cat1*-overexpressing strains germinated faster than the wild-type strain (Fig. 5), with median germination times of 5.9,



**Fig. 4** Effect of hydrogen peroxide on the survival of *M. anisopliae* conidia. Conidia ( $1 \times 10^6$  ml<sup>-1</sup>) were incubated at 28°C for 30 min with various concentrations of  $H_2O_2$ . The number of surviving conidia was determined by plating on Sabouraud dextrose (SD) agar medium. Results depicted are the mean  $\pm$  standard deviation of three independent experiments





**Fig. 5** *cat1* overexpression reduces the germination time. Conidia germination was measured at 2-h intervals. Populations of at least 200 cells were counted under the microscope, and the percentage of germlings and ungerminated conidia was determined. A germling was defined as a cell with a germ tube equal in length to the width of the conidia. The values were depicted as the mean  $\pm$  standard deviation of three independent experiments

5.5, and 6.8 h for the C19cat1-1, C19cat1-8, and *Ma10* strains, respectively. This result suggests that overexpression of catalase diminishes oxidative stress and accelerates the germination process.

#### Evaluation of fungal virulence

We assessed the biological activity of the transgenic strain C19cat1-8 compared to the wild-type strain *Ma10* by inoculating third-instar diamondback moth (*P. xylostella*) larvae with conidia at concentrations ranging from  $1 \times 10^5$  to  $5 \times 10^8$  conidia per milliliter. Constitutive expression of *cat1* improved insecticidal activity (Table 1). The representative  $LD_{50}$  value for strain *Ma10* was  $4.4 \times 10^5$ . Meanwhile, in transgenic strain C19cat1-8, the  $LD_{50}$  value was  $3 \times 10^4$ . This represents a 14-fold reduction in the number of conidia necessary to kill 50% of the population. At the same time, *cat1* overexpression reduced the median time-to-mortality by 22% in C19cat1-8 ( $MT_{50}=72$  h) compared with *Ma10* ( $MT_{50}=93$  h). There were no differences in the appearance of larvae infected with the transgenic strain as opposed to wild type. Thus, catalase overexpression significantly increased the virulence of *M. anisopliae*.

#### Discussion

In this work, we have described the isolation of genomic and cDNA copies of the *cat1* gene from *M. anisopliae* strain *Ma10*. Based on sequence identity, this gene codes for a protein related to monofunctional catalases from the phytopathogenic fungi *C. purpurea* (CAT1, AJ001386) and *Magnaporthe grisea* (CATB, MG06442), to CAT-3 from *N. crassa*, and to CATB from *A. nidulans*, among others. Expression of *M. anisopliae cat1* cDNA in *E. coli* and subsequent in-gel enzyme assays confirmed that this protein is a monofunctional catalase because it is not stained with 3,3'-diaminobenzidine, which has been used as a chromogenic substrate for peroxidases (Yamashita et al. 2007). Additionally, the catalase activity is inhibited with 20 mM 3-amino-1,2,4 triazole (data not shown). This compound is an inhibitor for typical catalases without having any effect on peroxidase activity (Brown-Peterson and Salin 1993). The UV/vis spectrum of the purified protein indicated the presence of a heme prosthetic group (ESM Fig. S4). The  $A_{407}/A_{280}$  is 0.65; typical monofunctional heme catalases exhibit a ratio of approximately 1 (Shima et al. 1999).

A previous phylogenetic analysis of fungal catalases (Giles et al. 2006) revealed four different classes of these proteins: P (peroxisomal catalases), C (cytoplasmic catalases), A (spore-specific catalases), and B (secreted catalases). The amino acid sequence of the *cat1* gene shows an amino terminal signal peptide, and by sequence identity, *M. anisopliae* CAT1 can be categorized as a member of class B. However, to this point, we have been unable to detect extracellular catalase activity. Like other secreted catalases, the majority of the protein is cell wall-associated (Garre et al. 1998; Schliebs et al. 2006), and this may interfere with the detection of activity. The aforementioned phylogenetic analysis (Giles et al. 2006) shows multiple catalases for most fungal species. Through a database search ([www.ebi.ac.uk](http://www.ebi.ac.uk)), we found four predicted products of ESTs related to catalases in *M. anisopliae*: Translated sequence of EST BQ143257 exhibits 91.7% identity with *M. anisopliae* CAT1; predicted product of EST BQ143305 exhibits 33% identity with *M. anisopliae* CAT1 and 75% identity with *N.*

**Table 1** Dose–mortality response of third-instar larvae of *P. xylostella* infected with *M. anisopliae Ma10* or *cat1* recombinant strains

| Strain      | $LC_{50}$ (conidia $ml^{-1}$ , 95% confidence limit) | Slope             | $MT_{50}$ ( $h^{-1}$ , 95% confidence limit) | Slope             |
|-------------|--|-------------------|--|-------------------|
| Control     | –  | –                 | –  | –                 |
| <i>Ma10</i> | $4.4 \times 10^5$ ( $3.6$ – $7.2 \times 10^5$ )      | $0.376 \pm 0.053$ | 93 (86–102)                                  | $4.297 \pm 0.208$ |
| C19cat1-8   | $3 \times 10^4$ ( $1.5$ – $6 \times 10^4$ )          | $0.447 \pm 0.052$ | 72 (67–76)                                   | $3.637 \pm 0.156$ |

Ten larvae per dose were sprayed with 0.5 ml of a conidial suspension using a Potter spray tower at  $10 \text{ lb plg}^{-2}$ . Each treatment consisted of four replicates. The nine doses ranged from  $1 \times 10^5$  to  $5 \times 10^8$ . Each conidia dose was tested in four independent assays. Mortality was recorded at 8 h, and the data were analyzed by probit analysis using PoloPlus software.  $LC_{50}$  values were considered significantly different if their 95% confidence limits did not overlap. Control samples consisted of larvae sprayed with 0.1% Triton X-100

*crassa* CAT-1, representing two putative monofunctional catalases. The other two translated sequences of ESTs, BQ143330 and BQ142683, exhibit 65–80% identity to catalase–peroxidase proteins. The catalases previously detected in *M. anisopliae* showed peroxidase activity. However, important differences exist between strains ARSEF2575 and ARSEF324 (Miller et al. 2004). In addition to *M. anisopliae* Ma10 used in this work, we used *P. xylostella* cuticle as carbon source. This is the likely reason (besides the use of this particular strain) why we were able to identify, in addition to the catalase–peroxidase activity, the monofunctional catalase CAT1.

In *M. anisopliae*, germination is accompanied by a decrease in catalase activity (Miller et al. 2004). In our analysis, *M. anisopliae* Ma10 conidia extracts displayed two catalase activity bands. These bands declined at 4 h in extracts from Ma10 grown in *P. xylostella* cuticle, with catalase activity increasing at 8 h. The putative catalase peroxidase (the fastest moving band) increased earlier than the monofunctional catalase (the slower moving band). During mycelial growth, both enzymes should be important for controlling the level of oxidative stress. The expression pattern of the slower moving band correlates with the expression pattern of the *cat1* gene. This protein is also induced with hydrogen peroxide (data not shown), but until a deletion mutant is constructed, we can only suggest that the catalase activity observed in our cellular extracts corresponds to this particular gene.

The *cat1* expression pattern in vitro resembles the expression pattern of *catB* from *A. nidulans*: It was barely detectable in spores, disappeared after 4 h during germination, and started to accumulate after roughly 10 h of mycelial growth (Kawasaki et al. 1997). These results suggest that *cat1* is regulated at the level of transcription, but they do not rule out other possible regulatory mechanisms. In vivo, the *cat1* expression pattern resembles the expression pattern of the *CATB* gene from the plant pathogen fungus *M. grisea*, which is up-regulated in vivo coincident with the penetration process (Skamnioti et al. 2007). It is likely that *M. anisopliae* *cat1* is not exclusively associated with mycelial growth. Our hypothesis is that it is also necessary for the invasion process.

To understand the role of the *M. anisopliae* catalase genes during the pathogenic and saprophytic life cycle, it is important to identify and delete all the possible catalase genes (probably four genes)—in particular the *cat1* gene isolated in this work. Until then, we can only evaluate the participation of *cat1* during the invasion process of *P. xylostella* using overexpression protocols. The transgenic strains overexpressing the *cat1* gene have twice the catalase activity of the wild-type strain, in accord with the extra copy of the gene which is detected in both transgenic samples tested (data not shown). This higher catalase

activity produced a higher level of resistance to exogenous hydrogen peroxide and a reduction in the germination time. Excessive amounts of H<sub>2</sub>O<sub>2</sub> is harmful to the cells, so the quick and efficient removal of hydrogen peroxide is essential for an aerobic living organism (Zamocky et al. 2008). With twice the amount of catalase activity, this removal process may be more efficient, allowing the cells to grow in higher concentrations of H<sub>2</sub>O<sub>2</sub>. To our knowledge, there are few reports regarding catalase overexpression in fungi. In *Schizosaccharomyces pombe*, a catalase gene mutant that completely lacks catalase activity is more sensitive to hydrogen peroxide, whereas transformed cells that overexpress catalase activity are more resistant to hydrogen peroxide when compared to the wild type (Mutoh et al. 1999). In *Saccharomyces cerevisiae*, catalase overexpression reduces the levels of ROS and increases specific growth rates in the presence of high lactic acid concentrations (Abbott et al. 2009).

A hyperoxidant state is defined as an unstable, transient state in which ROS surpass the antioxidant capacity of the cell (Michan et al. 2003). A hyperoxidant state develops when morphogenetic transitions start and during the germination of conidia (Michan et al. 2003). According to this hypothesis, during the germination of *M. anisopliae* conidia, a high level of oxidative metabolism occurs and a hyperoxidant state ensues. Overexpression of *cat1* could be responsible for removing the excessive amount of H<sub>2</sub>O<sub>2</sub> generated, allowing the cells to germinate 13–19% faster than the wild type. This improvement was also observed during in vivo assays with *P. xylostella* where *M. anisopliae* grew and spread faster in the soft tissue of the insect, resulting in a significant decrease (25%) in the time of death for the insect and requiring 14 times less than the usual dose to kill 50% of the population. This result is important because overexpression of the protease Pr1 reduces (by the same level) the time of death (St Leger et al. 1996). The improvement for *M. anisopliae* as a biopesticide is even greater if we consider that insects got sick and stopped eating much earlier than those who were infected with the wild-type fungus. We conclude that overexpression of the *cat1* gene makes the *M. anisopliae* Ma10 strain genetically robust in its ability to control foliage pests such as *P. xylostella*, which is a major pest in cruciferous crops. We conclude that the *M. anisopliae* *cat1* gene encodes a protein with catalase activity that could be important during germination and growth for maintaining control over the ROS produced by germination, mycelial growth, and possibly during the invasion process. Deletion studies focused on the *cat1* gene would be expected to support the results observed from its overexpression. Determination of the kinetic parameters for the protein and the effect of catalase inhibitors would be important to corroborate that CAT1 is a monofunctional catalase. The

isolation and study of each catalase and catalase–peroxidase gene will help us understand the role of each of these enzymes in the physiology of *M. anisopliae*.

**Acknowledgments** This work was supported by the Consejo Nacional de Ciencia y Tecnología (CONACyT), Consejo Estatal de Ciencia y Tecnología del Estado de Guanajuato (CONCyTEG), Secretaría de Educación Pública (SEP), and the University of Guanajuato. CEMH and IEPG were recipients of a fellowship from CONACyT, México.

## References

- Abbott DA, Suir E, Duong GH, de Hulster E, Pronk JT, van Maris AJ (2009) Catalase overexpression reduces lactic acid-induced oxidative stress in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 75:2320–2325
- Braga GU, Destefano RH, Messias CL (1999) Oxygen consumption by *Metarhizium anisopliae* during germination and growth on different carbon sources. *J Invertebr Pathol* 74:112–119
- Brown-Peterson NJ, Salin ML (1993) Purification of a catalase-peroxidase from *Halobacterium halobium*: characterization of some unique properties of the halophilic enzyme. *J Bacteriol* 175:4197–4202
- Crespo R, Pedrini N, Juarez MP, Dal Bello GM (2008) Volatile organic compounds released by the entomopathogenic fungus *Beauveria bassiana*. *Microbiol Res* 163:148–151
- Dutra V, Nakazato L, Broetto L, Silveira Schrank I, Henning Vainstein M, Schrank A (2004) Application of representational difference analysis to identify sequence tags expressed by *Metarhizium anisopliae* during the infection process of the tick *Boophilus microplus* cuticle. *Res Microbiol* 155:245–251
- Fernandez-Abalos JM, Fox H, Pitt C, Wells B, Doonan JH (1998) Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, *Aspergillus nidulans*. *Mol Microbiol* 27:121–130
- Freimoser FM, Screen S, Bagga S, Hu G, St Leger RJ (2003) Expressed sequence tag (EST) analysis of two subspecies of *Metarhizium anisopliae* reveals a plethora of secreted proteins with potential activity in insect hosts. *Microbiology* 149:239–247
- Freimoser FM, Hu G, St Leger RJ (2005) Variation in gene expression patterns as the insect pathogen *Metarhizium anisopliae* adapts to different host cuticles or nutrient deprivation *in vitro*. *Microbiology* 151:361–371
- Garre V, Tenberge KB, Eising R (1998) Secretion of a fungal extracellular catalase by *Claviceps purpurea* during infection of rye: putative role in pathogenicity and suppression of host defense. *Phytopathology* 88:744–753
- Giles SS, Stajich JE, Nichols C, Gerrald QD, Alspaugh JA, Dietrich F, Perfect JR (2006) The *Cryptococcus neoformans* catalase gene family and its role in antioxidant defense. *Eukaryot Cell* 5:1447–1459
- Goettel MS, Leger RJ, Bhaire S, Jung MK, Oakley BR, Roberts D, Staples RC (1990) Pathogenicity and growth of *Metarhizium anisopliae* stably transformed to benomyl resistance. *Curr Genet* 17:129–132
- Hisada H, Hata Y, Kawato A, Abe Y, Akita O (2005) Cloning and expression analysis of two catalase genes from *Aspergillus oryzae*. *J Biosci Bioeng* 99:562–568
- Kawasaki L, Aguirre J (2001) Multiple catalase genes are differentially regulated in *Aspergillus nidulans*. *J Bacteriol* 183:1434–1440
- Kawasaki L, Wysong D, Diamond R, Aguirre J (1997) Two divergent catalase genes are differentially regulated during *Aspergillus nidulans* development and oxidative stress. *J Bacteriol* 179:3284–3292
- Lledias F, Rangel P, Hansberg W (1998) Oxidation of catalase by singlet oxygen. *J Biol Chem* 273:10630–10637
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Michan S, Lledias F, Hansberg W (2003) Asexual development is increased in *Neurospora crassa* cat-3-null mutant strains. *Eukaryot Cell* 2:798–808
- Miller CD, Rangel D, Braga GU, Flint S, Kwon SI, Messias CL, Roberts DW, Anderson AJ (2004) Enzyme activities associated with oxidative stress in *Metarhizium anisopliae* during germination, mycelial growth, and conidiation and in response to near-UV irradiation. *Can J Microbiol* 50:41–49
- Mutoh N, Nakagawa CW, Yamada K (1999) The role of catalase in hydrogen peroxide resistance in fission yeast *Schizosaccharomyces pombe*. *Can J Microbiol* 45:125–129
- Nakazato L, Dutra V, Broetto L, Staats CC, Vainstein MH, Schrank A (2006) Development of an expression vector for *Metarhizium anisopliae* based on the *tef-1 $\alpha$*  homologous promoter. *Appl Microbiol Biotechnol* 72:521–528
- Paris S, Wysong D, Debeaupuis JP, Shibuya K, Philippe B, Diamond RD, Latge JP (2003) Catalases of *Aspergillus fumigatus*. *Infect Immun* 71:3551–3562
- Pedrini N, Juarez MP, Crespo R, de Alaniz MJ (2006) Clues on the role of *Beauveria bassiana* catalases in alkane degradation events. *Mycologia* 98:528–534
- Roberts DW, St Leger RJ (2004) *Metarhizium* spp., cosmopolitan insect-pathogenic fungi: mycological aspects. *Adv Appl Microbiol* 54:1–70
- Sambrook JG, Russell R (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Schliebs W, Wurtz C, Kunau WH, Veenhuis M, Rottensteiner H (2006) A eukaryote without catalase-containing microbodies: *Neurospora crassa* exhibits a unique cellular distribution of its four catalases. *Eukaryot Cell* 5:1490–1502
- Shima S, Netrusov A, Sordel M, Wicke M, Hartmann GC, Thauer RK (1999) Purification, characterization, and primary structure of a monofunctional catalase from *Methanosarcina barkeri*. *Arch Microbiol* 171:317–323
- Skamnioti P, Henderson C, Zhang Z, Robinson Z, Gurr SJ (2007) A novel role for catalase B in the maintenance of fungal cell-wall integrity during host invasion in the rice blast fungus *Magnaporthe grisea*. *Mol Plant Microbe Interact* 20:568–580
- St Leger R, Cooper R, Charnley A (1986) Cuticle-degrading enzymes of entomopathogenic fungi: regulation of production of chitinolytic enzymes. *J Gen Microbiol* 132:1509–1517
- St Leger RJ, Bidochka MJ, Roberts DW (1994) Isoforms of the cuticle-degrading Pr1 proteinase and production of a metalloproteinase by *Metarhizium anisopliae*. *Arch Biochem Biophys* 313:1–7
- St Leger R, Joshi L, Bidochka MJ, Roberts DW (1996) Construction of an improved mycoinsecticide overexpressing a toxic protease. *Proc Natl Acad Sci USA* 93:6349–6354
- St Leger RJ, Joshi L, Roberts DW (1997) Adaptation of proteases and carbohydrates of saprophytic, phytopathogenic and entomopathogenic fungi to the requirements of their ecological niches. *Microbiology* 143(Pt 6):1983–1992
- Staats CC, Junges A, Fitarelli M, Furlaneto MC, Vainstein MH, Schrank A (2007) Gene inactivation mediated by *Agrobacterium tumefaciens* in the filamentous fungi *Metarhizium anisopliae*. *Appl Microbiol Biotechnol* 76:945–950

- Wang C, St Leger RJ (2005) Developmental and transcriptional responses to host and nonhost cuticles by the specific locust pathogen *Metarhizium anisopliae* var. *acridum*. *Eukaryot Cell* 4:937–947
- Wang N, Yoshida Y, Hasunuma K (2007) Loss of Catalase-1 (Cat-1) results in decreased conidial viability enhanced by exposure to light in *Neurospora crassa*. *Mol Genet Genomics* 277:13–22
- Wayne LG, Diaz GA (1986) A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide electrophoresis gels. *Anal Biochem* 157:89–92
- Yamashita K, Shiozawa A, Banno S, Fukumori F, Ichiishi A, Kimura M, Fujimura M (2007) Involvement of OS-2 MAP kinase in regulation of the large-subunit catalases CAT-1 and CAT-3 in *Neurospora crassa*. *Genes Genet Syst* 82:301–310
- Zamocky M, Furtmüller PG, Obinger C (2008) Evolution of catalases from bacteria to humans. *Antioxid Redox Signal* 10:1527–1548