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Modulation of Antioxidant Defense in *Aspergillus parasiticus* Is Involved in Aflatoxin Biosynthesis: a Role for the Ap*yapA* Gene[▽][†]

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Oxidative stress is recognized as a trigger of different metabolic events in all organisms. Various factors correlated with oxidation, such as the β-oxidation of fatty acids and their enzymatic or nonenzymatic by-products (e.g., precocious sexual inducer factors and lipoperoxides) have been shown to be involved in aflatoxin formation. In the present study, we found that increased levels of reactive oxygen species (ROS) were correlated with increased levels of aflatoxin biosynthesis in *Aspergillus parasiticus*. To better understand the role of ROS formation in toxin production, we generated a mutant (ΔAp*yapA*) having the Ap*yapA* gene deleted, given that Ap*yapA* orthologs have been shown to be part of the antioxidant response in other fungi. Compared to the wild type, the mutant showed an increased susceptibility to extracellular oxidants, as well as precocious ROS formation and aflatoxin biosynthesis. Genetic complementation of the ΔAp*yapA* mutant restored the timing and quantity of toxin biosynthesis to the levels found in the wild type. The presence of putative AP1 (ApYapA orthologue) binding sites in the promoter region of the regulatory gene *aflR* further supports the finding that ApYapA plays a role in the regulation of aflatoxin biosynthesis. Overall, our results show that the lack of Ap*yapA* leads to an increase in oxidative stress, premature conidiogenesis, and aflatoxin biosynthesis.

Reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (HO$^{\bullet}$), and lipoperoxides (LOOH), which are formed from unsaturated fatty acids and can be produced in the cell during metabolic processes, can be overproduced following the action of oxidative stressors present in the environment (32, 49, 57). To counteract the potentially dangerous accumulation of ROS, cells have evolved strategies (49, 61) based on enzymatic or nonenzymatic systems (28, 45). The main antioxidant enzymes in cells involved in ROS removal are superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX). If H_2O_2 exceeds the cell-scavenging capacity, it can generate highly reactive HO$^{\bullet}$ through a Fenton reaction, which initiates the formation of LOOH in the membrane lipids (32).

When ROS accumulation occurs, the oxidant/antioxidant balance is perturbed, which can damage the cell membrane and cell metabolism (free-radical theory of aging) (26). ROS produced at certain time points during the cell's life cycle and at low physiological concentrations play a crucial role in the organism's homeostasis and cell functions. As second messengers, ROS take part in the plant's developmental processes (18, 24, 31) and in the defense mechanisms against pathogens and abiotic stress (5, 24, 52, 62). Similar effects have been shown in mammals, where ROS at proper levels stimulate antioxidant reactions, immune system modulation, and regulation of cell proliferation (3, 4, 55, 59, 65). One of the major objectives of studying the biology of stress is to identify the key factors that control the switch from cytoprotective responses to cell dysfunction following oxidative insult (11).

In fungi, recent studies have evaluated the role played by ROS and fatty-acid metabolism in the differentiation process during growth (1, 8, 9, 38, 42, 63). For example, ROS generated by NADPH oxidase, which are partially controlled by SOD and CAT, play an important role in different aspects of fungal development, such as growth and differentiation (35, 36). In particular, in *Neurospora crassa*, the start of the transition from conidia to germination is affected by singlet oxygen-generated redox imbalance (38). Oxylipin formation can occur via dioxygenase (DOX) or lipoxygenase (LOX) action and, to a lesser extent, nonenzymatically (24, 58). In *Aspergillus nidulans*, the presence of the fatty-acid DOXs PpoA, -B, and -C has been reported (63). In *Aspergillus*, compounds produced by LOXs and DOXs, such as hydroperoxyoctadecadienoic acid (HPODE) and precocious sexual inducer factors, which consist of a mixture of hydroxylated oleic, linoleic, and linolenic acids, have all been shown to stimulate conidiogenesis, and in *Aspergillus flavus* this occurrence is related to aflatoxin biosynthesis (7, 10, 12, 13). In *A. nidulans*, ROS can also steer the production of mitospores and meiospores in the regulation of the asexual and sexual phases in development (25, 63). In *Aspergillus parasiticus*, ROS can control sclerotium formation (14), improving the resistance to adverse environmental conditions. In *A. flavus* and *A. nidulans*, the biosynthesis of mycotoxins is closely related to different stages of fungal develop-
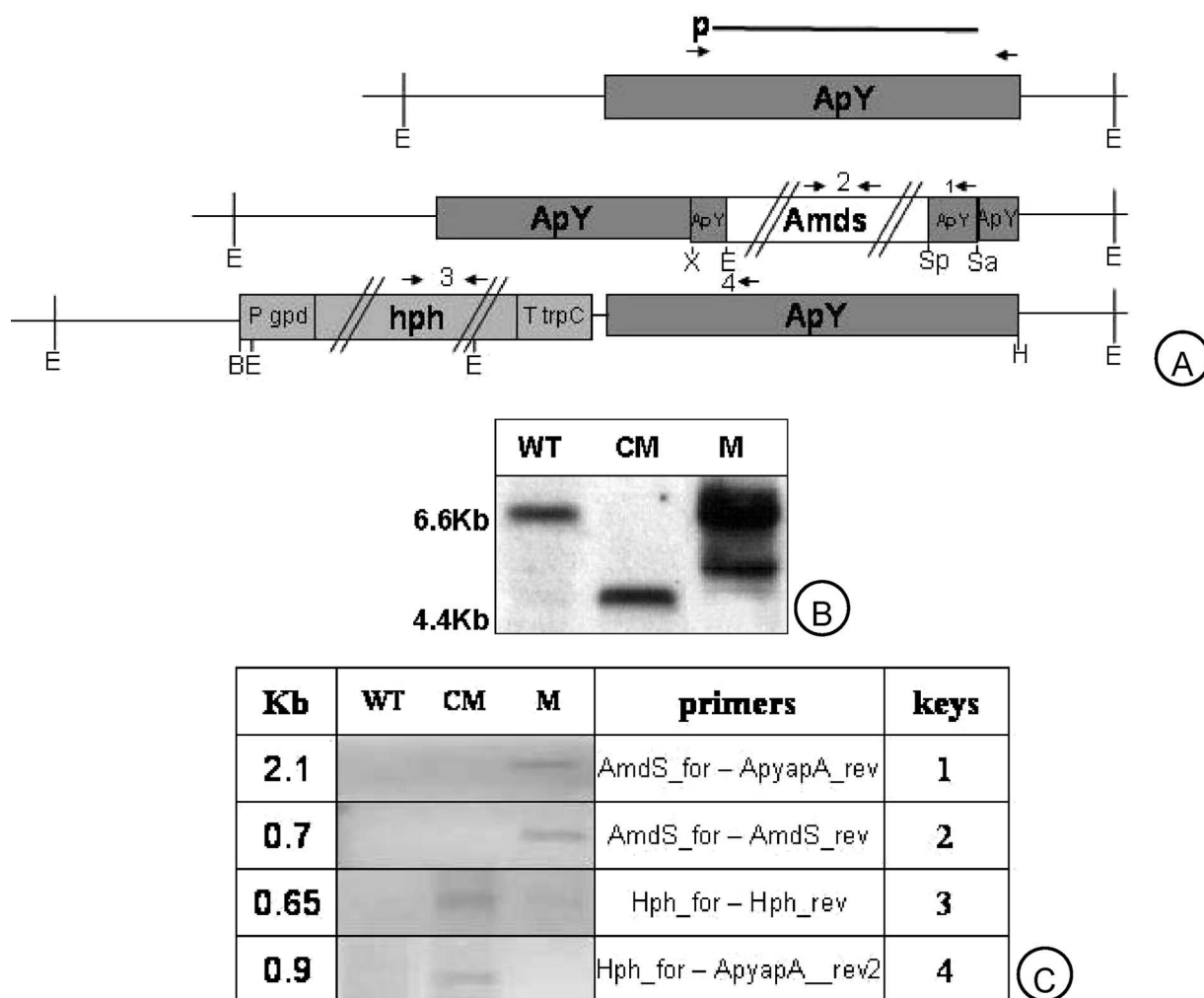
FIG. 1. DNA gel blot analysis of Ap*yapA* gene replacement mutants and complementation. (A) WT Ap*yapA* locus (ApY); final deletion event with construct containing acetamide resistance cassette (AmdS) and Ap*yapA* gene XhoI-EcoRI (X-E) and SphI-SalI (Sp-Sa) fragments used for transforming WT protoplast; the 5.4-kb BglII-HindIII (B-H) fragment which carries also the hygromycin B resistance cassette (*hph*) used for complementing ΔAp*yapA* strains. The probe used for the subsequent Southern blot analysis is indicated (p). Genomic DNA was isolated from the wild-type strain NRRL 2999 (WT), the Ap*yapA* complemented mutant (CM), and the gene replacement transformant (M) and digested with EcoRI (E). (B) The blots were hybridized with 1.9-kb Ap*yapA* PCR DIG-labeled probes. (C) PCR amplification of WT, CM, and M strain genomic DNA using AmdS_for and ApyapA_rev primers (expected size of the PCR fragment, ~2.1 kb) or AmdS_for and AmdS_rev primers (expected size of the PCR fragment, ~0.7 kb) or Hph_for and Hph_rev primers (expected size of the PCR fragment, ~0.65 kb) or Hph_for and ApyapA_rev2 primers (expected size of the PCR fragment, ~0.9 kb). The numbers in the "keys" column indicate the primers used for PCR amplification.

ment, such as conidiogenesis and sclerotium formation (51, 56) in the idiophase, during which an increase in ROS occurs. Other recent studies of different strains of *A. parasiticus*, some of which are producers of aflatoxin, have demonstrated that oxidative stress is important in aflatoxin production (46).

The efficiency of the cell in maintaining safe levels of ROS mainly depends on the effectiveness of its antioxidant system (29, 49). A quick and effective defensive response depends on the cell's efficient perception of the stress, as well as on the transduction of oxidative signals. In fungi, as well as in animal cells, some transcription factors are able to act as sensors of oxidants in the cell (47, 50). In yeast, it has been shown that oxidative stress-related transcription factors (OSRTFs) (e.g., Yap1, Skn7, Hsf1-2, and Msn2-4) are differentially activated by oxidative stimuli provided by peroxides, diamide, and free-

radical generators (45), as well as by antioxidant treatment (34). In particular, Yap1 is a nuclear factor localized in the cytoplasm (where it interacts with the export receptor Crm1) which, under oxidative conditions, migrates to the nucleus, where it binds with responsive elements (TGACTCA). These elements are similar to antioxidant-responsive elements (TGACnnnGC) and promote, together with Skn7 and Hsf1-2, the transcription of many antioxidant-related genes (*gst*, *sod1*, *sod2*, *cta1*, *ctt*, *trr*, and *txl* [30, 45]). Recently, *Saccharomyces cerevisiae* has been used as a model for studying the regulation of the response to oxidative stress in *A. parasiticus*, in particular for investigating the relation between treatment with antioxidant compounds and aflatoxin biosynthesis (34). In *A. parasiticus* and *A. flavus*, for some time a correlation has been known to exist among fungal cell oxidative stress, free-radical forma-
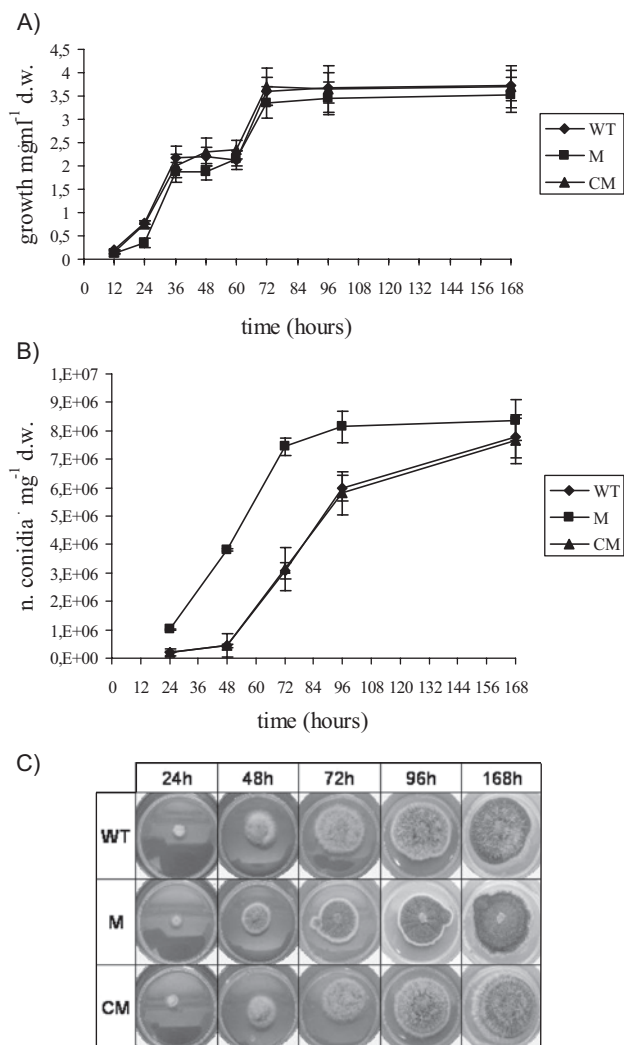
A)

B)

C)

FIG. 2. (A) Mycelial growth (mg [dry weight] · ml^{-1}) of WT, ΔApyapA (M), and ApyapA complemented (CM) strains inoculated in PDB (25 ml) and incubated at 30°C from 12 up to 168 h. (B) Numbers of conidia produced by WT, M, and CM strains cultured under the same experimental conditions. (C) Agar plates (PDA) showing the different timing in conidium formation of WT, M, and CM strains at different time intervals after inoculation (24 to 168 h). The results in panels A and B are the means ± SEMs of three determinations from three separate experiments.

tion, lipoperoxidation, and aflatoxin biosynthesis (17, 19, 20, 22, 48). Based on the huge quantity of data on fungal development and aflatoxin biosynthesis collected in recent years, the formation of this toxin is considered to be closely related to differentiation and senescence in fungi. However, the extent to which the defense against oxidative stress in the fungal cell plays a role in aflatoxin biosynthesis and the mechanisms underlying this role have not been extensively studied. To this end, we conducted a study of *A. parasiticus* wild type (WT) and the ΔApyapA null mutant strains and found that the oxidant/antioxidant balance affects aflatoxin biosynthesis and that oxidative stress is one of the main factors involved in the triggering of aflatoxin biosynthesis.

MATERIALS AND METHODS

Fungal strains and culture conditions. The WT strain used was *Aspergillus parasiticus* NRRL 2999, a producer of the aflatoxins B_1, B_2, G_1, and G_2. ΔApyapA deletion mutants (M) and ApyapA complemented mutants (CM) were generated as described below. The isolates were incubated on potato dextrose agar (PDA; Difco) for 7 days at 30°C, before use. Twenty-five milliliters of potato dextrose broth (PDB; Difco) (which is aflatoxin conducive) in 50-ml Erlenmeyer flasks was inoculated with the WT, M, and CM strains, using 0.2 ml of conidial suspension (~10^6 conidia) for each flask; incubation was performed at 30°C for different time periods (10, 12, 14, 18, 21, 24, 30, 36, 42, 48, 60, 72, 96, 120, 144, and 168 h). In other experiments, 50 ml of Czapek Dox (CD) broth (Difco) (which is low-conducive for aflatoxins) was inoculated with the WT and M strains; after 4 days of incubation at 30°C, 1 mM cumene hydroperoxide (CH), 0.5 mM menadione (Men), and 1 mM and 10 mM hydrogen peroxide (H_2O_2) were added to test the strain's sensitivity to oxidant stressors.

Fungal growth and aflatoxin production in culture media. At each point in time, fungal growth was determined by weighing the mycelium after filtration (Millipore filters; 0.45 μm pore size) and drying it for 48 h at 80°C. To determine the quantity of ROS and LOOH and to perform molecular analyses, the filtered mycelia were lyophilized and weighed.

Aflatoxin production ($B_1 + B_2 + G_1 + G_2$) was analyzed in culture filtrates of the WT, M, and CM strains following extraction with chloroform-methanol (2:1, vol/vol). The extracts were collected, the volume was reduced under a stream of nitrogen, and the quantitative analyses were carried out by high-pressure liquid chromatography, as previously reported (23).

Total hydroperoxides of linoleic acid and percentages of 9- and 13-HODE regioisomers in mycelia. The WT and M strains collected from PDB cultures at different incubation times were homogenized in liquid nitrogen to repress the accidental formation of peroxides. Hydroperoxyoctadecadienoic acids (9- and 13-HPODE) present in the mycelia were analyzed following triple extraction with chloroform-methanol (2:1, vol/vol) in the presence of 100 μg of butylated hydroxytoluene as antioxidant. Peroxides in the extracts were reduced with NaBH$_4$ to obtain hydroxyoctadecadienoic acids (9- and 13-HODE), as previously reported (54). Neither 9-HODE nor 13-HODE was detected in the extracts before reduction. The regioisomers 9-HODE and 13-HODE were analyzed by high-pressure liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry (MS), as reported previously (54). 9-HODE and 13-HODE were identified by comparison with authentic compounds (purchased from Cayman).

Detection and quantification of α-tocopherol. Analyses of α-tocopherol were performed on lyophilized mycelia at different times, as previously reported (52). Samples were extracted three times in chloroform-methanol (2:1, vol/vol) for 1 h in the presence of 100 μg butylated hydroxytoluene, as antioxidant, and 5 μg heptadecanoic acid ($C_{17:0}$), as internal standard. The recovered chloroform-methanol mixture was extracted three times in hexane, filtered, vacuum evaporated, and then silylated with trimethyl-silyl-ether (TMS). The TMS derivatives were analyzed by gas chromatography-MS, as described elsewhere (52). Quantitative analyses were performed in single-ion monitoring mode, selecting the ions having m/z values of 502, 277, and 237 for the TMS derivative of α-tocopherol and ions with m/z values of 342 and 327 for the derivative of $C_{17:0}$. Calibration curves were performed as previously reported; the method was linear ($R >$ 0.99) in the range of 1 to 50 ng α-tocopherol · μl^{-1}.

Levels of anion superoxide and hydrogen peroxide in mycelia. WT and M lyophilized mycelia (10 mg) collected from PDB cultures at different incubation times (from 10 to 168 h) were homogenized as reported above. $O_2^{\cdot-}$ and H_2O_2 accumulation was measured at each point in time. $O_2^{\cdot-}$ was detected by measuring the reduction of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) to formazan, according to a published protocol (60), with slight modifications (10). Absorbance at 490 nm was measured in a microplate reader (μQuant; Bio-Tek instruments). XTT levels were expressed as mean absorbance values mg^{-1} ± standard errors of the means (SEMs).

The quantity of H_2O_2 was measured using an Amplex Red hydrogen peroxide assay kit (Molecular Probes). Amplex Red, in the presence of horseradish peroxidase enzyme (supplied by the kit), reacts with H_2O_2 in a 1:1 stoichiometry to produce resorufin, a red fluorescent compound. Samples were incubated for 30 min, and the absorbance at 570 nm was measured using the microplate reader. A standard curve of H_2O_2 was prepared and used as a reference for quantification.

Activities of SOD, GPX, HPR, and LOX-like enzymes. The activities of SOD, pH 7.8 and 10.0 (EC 1.15.1.1), GPX (EC 1.11.1.9), hydrogen peroxide reducing
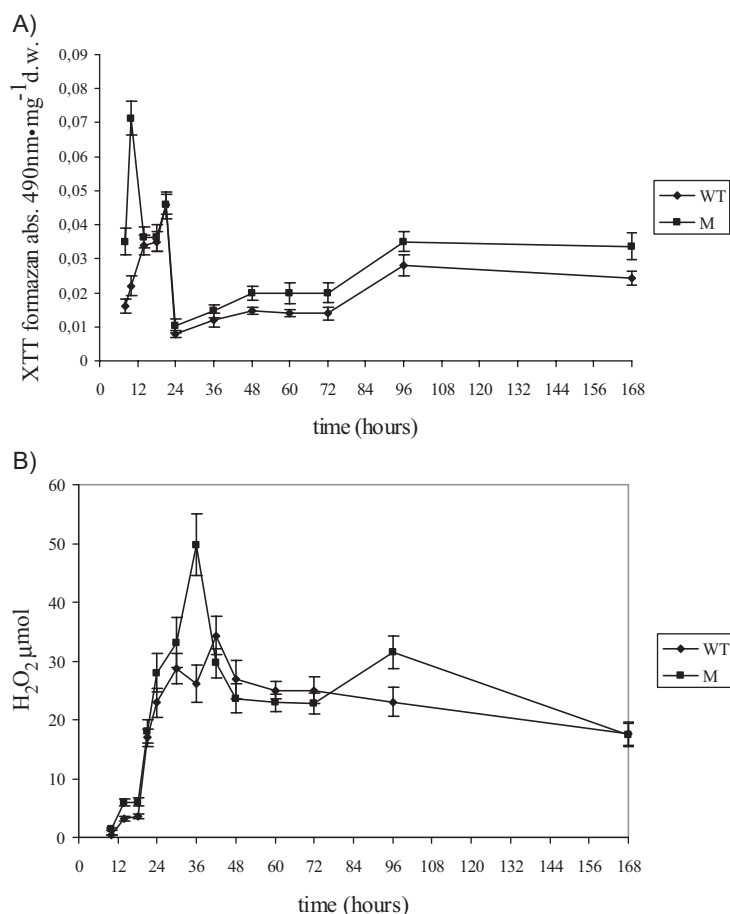
FIG. 3. (A) Detection of superoxide anion formation (reported as absorbance of XTT formazan at 490 nm). (B) H_2O_2 formation (μmol) in mycelia of WT and ΔAp*yapA* (M) strains grown in PDB (25 ml) and incubated at 30°C from 8 to 168 h. The results are the means $\pm$ SEMs of three determinations from three separate experiments.

enzymes (HPR), and LOX-like enzymes (EC 1.13.11.12) were analyzed in the homogenized mycelia of the WT and M strains, as previously described (53).

Zymogram of CAT. CAT is modified by reacting with ROS, giving rise to more-acidic isoforms (38). CAT conformers, which were extracted from homogenized mycelia of the WT and M strains and collected at different times, were analyzed by zymography. As control, an acidic CAT conformer derived from *Aspergillus niger* (C1; Sigma-Aldrich) was used, as was the CAT itself, which was oxidized under an O_2 stream, which produces a more acidic form (C2). Native minigels (8 to 9 cm and 0.75 nm thick) consisting of 8% polyacrylamide and 0.2% bisacrylamide (Bio-Rad) were loaded with mycelium lysates containing 1 U of putative CAT activity in each lane. Gels were run at 200 V for 2 h 45 min at 4°C on a Miniprotean II Bio-Rad apparatus. Native enzyme was detected as described elsewhere (43).

Cloning and sequencing of Ap*yapA* and Ap*skn7*. DNA extracted from *A. parasiticus* NRRL 2999 was amplified in a thermal MasterCycler gradient (Eppendorf) following amplification steps (94°C for 2 min; 35 cycles of 94°C for 30 s, 56°C for 45 s, and 72°C for 1 min; 72°C for 8 min) using the primers Af-yap1 (forward, 5′ TCAC ACCAGTTCCTCTCATC 3′; reverse, 5′ GCGGAACTTCTCCATAGATT 3′) and Af-skn7 (forward, 5′ TTCCCACTCAACAGATTGAC 3′; reverse, 5′ CATGATT GATCTTTTTCC 3′) designed on the expressed sequence tag sequence of *A. flavus* and aligned with the *yap1* and *skn7* gene sequences of *S. cerevisiae*. Amplification under high-stringency conditions produced a unique band for the *yap1*-like fragment (720 bp, GenBank accession number DQ104418) and Ap*skn7* (1,059 bp, GenBank accession number DQ104417). The *yap1*-like fragment was used for searching for the homologue AfYap1. New primers were designed on the codon start (+1; ApyapA_for, ATGGCCGATTACAATACCCTC) and at the 3′ end of the gene sequences (+1882; ApyapA_rev, TCGAGTTATTTGACCGCGACC) to obtain the complete sequence of Ap*yapA*. This gene was cloned in pGEM-T Easy vector (Promega) and sequenced and aligned with the TBLASTX 2.2.17 in the NCBI

website (www.ncbi.nlm.nih.gov/BLAST). The results of BLAST indicated a high homology (amino acid identities of 62% and similarities of 100% [score, 268] with the putative bzip transcription factor [Ap-1] of *Aspergillus fumigatus* XP_750882.1) of the conceptual amino acid translation of Ap*yapA* with the bzip transcription factor Yap-1 of diverse fungal species. Furthermore, Ap*skn7* was observed to share a high homology (amino acid identities of 40% and similarities of 57% with the putative response regulator receiver Skn7p of *Cochliobolus heterostrophus*) with the stress response regulator Skn7 (*srrA* in *A. nidulans*) of diverse fungal species. ApYapA (forward, 5′ GTTCTCCATCATCCTCATCC 3′, +1112; reverse, 5′ TGCGGAAC TTCTCCATAGAC 3′, +1771) and ApSkn7 (forward, 5′ GGGTACACTACAGG TTCAAA 3′; reverse, 5′ ACGCGTCAAAGCTTCTTAAC 3′) primers were designed and used for the subsequent reverse transcription-PCR (RT-PCR) analysis. The 18S primer pair (forward, 5′ ATGGCCGTTCTTAGTTGGTG 3′; reverse, 5′ GTACAAAGGGGCAGGGACGTA 3′) produced a single fragment of 500 bp (internal standard), whereas the primers chosen for the amplification of Ap*yap1* and Ap*skn7* genes produced single fragments of 659 bp and 480 bp, respectively.

Plasmids and transformation. Two fragments of Ap*yapA* were amplified by PCR from genomic DNA of *A. parasiticus* NRRL 2999 (WT) with the primer pairs ApyapAXbaI_for (+1112) and ApyapAEcoRI_rev (+1320) and Apyap-ASphI_for (+1420) and ApyapA_rev (+1772; SalI internal restriction site) and used for the subsequent plasmid transformation. The PCR fragments were eluted from the gel (GelElute extraction kit; Sigma-Aldrich) and cloned in the plasmid pGEM-T (Promega). Plasmid DNA from bacterial colonies containing the resulting pGEM-T::Ap*yapA* constructs was digested with XbaI and EcoRI and with SphI and SalI. The resulting Ap*yapA* fragments were cloned into the vector p3SR2 (8.8 kb) alongside the *amdS* gene of the acetamide cassette (Fig. 1A). The orientation of the insert was determined by PCR with Ap*yapA*- and AmdS-specific primers. Strain NRRL 2999 of *A. parasiticus* was transformed with the fragment XbaI-SalI obtained from vector p3SR2::Ap*yapA* (~9.4 kb), which
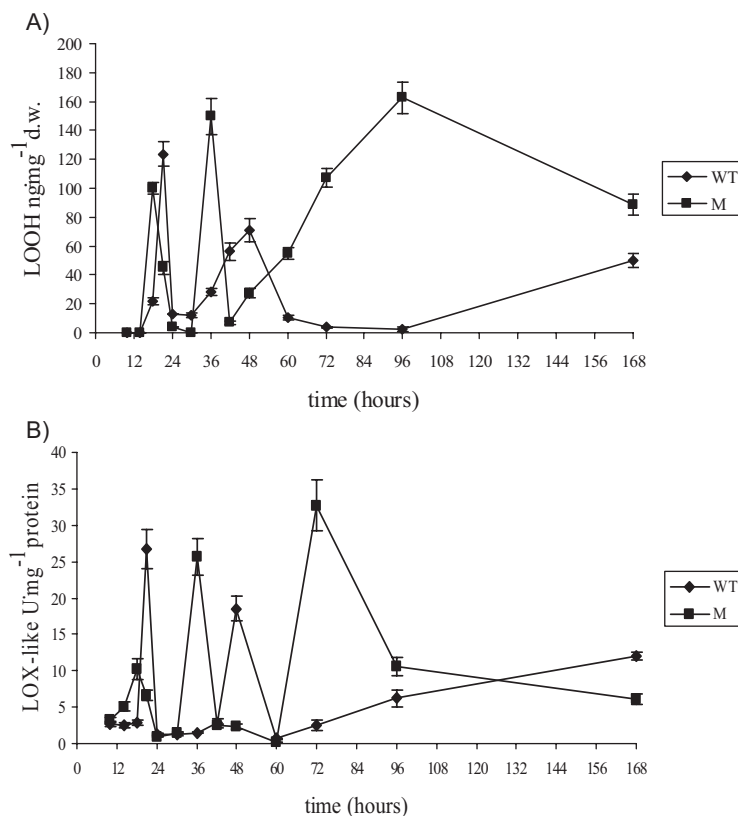
FIG. 4. (A) 9- and 13-HODE (ng · mg^{-1} [dry weight]). (B) LOX-like activity measured as diene conjugate formation at 234 nm (U · mg^{-1} protein) in mycelia of WT and ΔAp$yapA$ (M) strains grown in PDB (25 ml) and incubated at 30°C from 10 to 168 h. The results are the means ± SEMs of three determinations from three separate experiments.

contains two fragments of the Ap$yapA$ gene interrupted by the AmdS cassette ($\sim$4.6 kb) (Fig. 1A). In the *A. flavus* genome (checked at the website www .aspergillusflavus.org), EcoRI presents two restriction sites $\sim$1.6 kb downstream and $\sim$4.0 kb upstream of the Af$yapA$ gene sequence. Thus, considering the high homology of this genome with the *A. parasiticus* genome (and also the alignment of the *A. parasiticus* contig present in the NCBI GenBank with the same contig of *A. flavus* in which the Ap$yapA$ homologue is present), the 6.5-kb fragment that originated in the Ap$yapA$ knockout mutant could be the result of the sum of the

EcoRI downstream fragment ($\sim$1.6 kb), the remaining part of the Ap$yapA$ WT gene ($\sim$0.2 kb), the gene fragment used in the deletion construct cloned along-side the *amdS* resistance cassette ($\sim$0.3 kb), and the *amdS* fragment ($\sim$4.4 kb) that resulted from EcoRI restriction. The $\sim$5.5-kb fragment could be the result of Ap$yapA$ probe hybridization in a sequence constituted by the 4.0-kb EcoRI Ap$yapA$ upstream fragment adjacent to the remaining part of the gene ($\sim$1.1 kb) and to the rest of the deletion construct ($\sim$0.4 kb).

For complementation of the M strain, a 5.4-kb BglII-HindIII fragment resistance cassette was excised from pAN7.1::Ap$yapA$, which carries the hygromycin B resistance selectable marker. The protoplast transformation of the WT and the M strains was performed as described elsewhere (41). As described above, the single $\sim$4.5-kb fragment present in the CM strain can be generated by the hybridization of the Ap$yapA$ probe with a fragment constituted by the $\sim$1.0-kb EcoRI fragment of the *hph* resistance cassette, the $\sim$1.9-kb Ap$yapA$ gene sequence (complemented), and the EcoRI Ap$yapA$ downstream fragment ($\sim$1.6 kb).

Selection of Ap$yapA$ deleted and complemented mutants. The selection of transformants (strains with deleted Ap$yapA$ [M]) was conducted at 30°C on Czapek Dox agar (CDA) containing 30 mM acetamide as the sole nitrogen source; putative transformants were selected, transferred to fresh selective medium, and allowed to sporulate. To obtain homokaryons, single spores were isolated from each selected heterokaryotic transformant and transferred to fresh selective medium. This monoconidial transfer was conducted three times. Finally, 20 homokaryotic progenies were selected and further subcultured to determine the occurrence of abortive transformants. The stability of these transformants was also tested by two additional single-spore transfers on nonselective medium and then again on selective medium and by several mycelial transfers on selective plates. CM ($n = 20$) strains were selected by testing their resistance to both oxidant stressors and hygromycin B. The protoplasts obtained from conidia of stable M strains were transformed with a 5.4-kb BglII-HindIII fragment excised from pAN7.1::Ap$yapA$, which also carries the hygromycin B resistance selectable marker. The protoplasts were plated in CDA in the presence of 1 mM Men and 500 ppm hygromycin B, which at these concentrations completely

TABLE 1. Relative percentages of 9- and 13-HODE in the mycelia of *A. parasiticus* WT and ΔAp$yapA$ mutant (M) strains

| Time (h) | % HODE (mean ± SEM[a]) for strain: | | | |
| | WT | | M | |
	9-HODE	13-HODE	9-HODE	13-HODE
10	3.9 ± 0.5	96.1 ± 11.2	0.2 ± 0.1	99.8 ± 10.2
14	9.1 ± 1.2	90.9 ± 11.5	46.0 ± 5.2	54.0 ± 6.3
18	0.1 ± 0.05	99.9 ± 15.2	31.0 ± 4.2	69.0 ± 7.1
21	3.4 ± 0.4	96.6 ± 9.6	1.5 ± 0.5	98.5 ± 12.2
24	6.3 ± 0.6	93.6 ± 8.4	0.2 ± 0.1	99.8 ± 9.2
30	8.5 ± 0.9	91.5 ± 10.2	0.1 ± 0.03	99.9 ± 8.5
36	10.4 ± 1.1	89.6 ± 8.5	34.0 ± 4.2	66.0 ± 8.5
42	93.9 ± 10.5	6.1 ± 1.2	87.6 ± 8.2	12.4 ± 1.5
48	69.5 ± 6.2	30.5 ± 2.1	87.2 ± 9.6	12.8 ± 1.3
72	36.5 ± 3.9	63.5 ± 5.1	31.0 ± 3.5	69.0 ± 5.2
96	9.80 ± 1.8	90.2 ± 10.5	23.0 ± 2.6	77.0 ± 8.3
168	32.5 ± 4.3	67.5 ± 7.5	70.0 ± 8.2	30.0 ± 5.2

[a] The values represent the means ± SEMs of three replicates from three separate experiments.
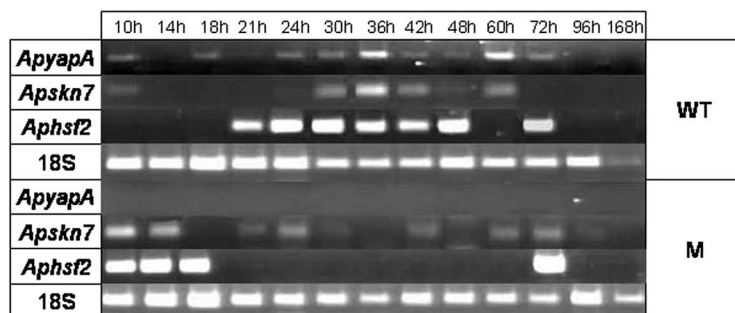
FIG. 5. RT-PCR analysis of oxidative stress transcription factor (Ap*yapA*, Ap*skn7*, and Ap*hsf2*) mRNA in *A. parasiticus* mycelia from WT and M strains, grown in PDB after different periods of incubation. The results are representative of three separate experiments.

inhibited the germination of ΔAp*yapA* conidia and their development. The stability of these CM strains was tested by several single-spore transfers on selective medium (Men [1 mM] plus hygromycin B [500 ppm]), as described above for ΔAp*yapA* selection.

For the selection of the M and CM strains, the following criteria were used: (i) Southern blot hybridization with probe (Fig. 1A) obtained by the amplification of the Ap*yapA* WT with the Ap*yapA* primers (see above paragraph) as described above and testing for the presence of the expected bands after Southern analysis of the fungal DNA digested with EcoRI (which does not restrict Ap*yapA* but cuts the *amdS* sequence once at +4361 and the Hph coding sequence at +2561); the hybridization of the probe is expected to generate a double band in M strain DNA and a single band in the WT and in positive CM strains; (ii) presence/absence of the *amdS* cassette in the fungal genome, which was tested by PCR with primers AmdS for and AmdS rev; (iii) presence/absence of the Hph cassette in the fungal genome, which was tested by PCR with primers Hph_for (5′ CTTGTATGGAGCAGGAGACC 3′) and Hph_rev (5′ ATTTGTGTACGCCCGACAGC 3′); (iv) PCR amplification of the genomic DNA of the WT, M, and CM strains using AmdS_for and ApyapA_rev primers (expected size of the PCR fragment, ~2.1 kb); (v) PCR amplification of the genomic DNA of the WT, M, and CM strains using Hph_for and ApyapA_rev2 (5′ GAGGGCCTTCTGCAACTCAAG 3′) primers (expected size of the PCR fragment, ~0.9 kb); (vi) RT-PCR products of cDNA from all of the strains were amplified by using ApyapA_for and ApyapA_rev (in the figures, only one representative strain is shown for the WT, M, and CM strains); and (vii) the ability of the M strain to grow and sporulate similarly to the WT on CDA with or without acetamide and the ability of the CM strains to grow and sporulate similarly to the WT on CDA with or without Men (1 mM) and hygromycin B (500 ppm). Strain development was determined by measuring the growth rate and spore counts of cultures grown on plates of PDA.

***aflR*, *norA*, Ap*yapA*, Ap*hsf2*, and Ap*skn7* semiquantitative RT-PCR analysis.** Total RNA from 100 mg of freeze-dried mycelia was extracted using the Tri-Reagent protocol (Sigma) and was quantified by spectrophotometry, determining the optical density at 260 nm. RNA was treated with RNase-free DNase I and then resuspended in 20 μl of diethyl pyrocarbonate-treated water. RNA was extracted at different points in time (from 10 to 168 h; three tubes for each point in time) from *A. parasiticus* PDB culture and was used to develop an *aflR*, *norA*, Ap*yapA*, Ap*hsf2*, and Ap*skn7* RT-PCR assay, as previously reported (54). A semiquantitative analysis was conducted by performing RT-PCR amplification under several different conditions, in which the annealing temperature (from 55°C to 65°C), amplification cycles (from 20 to 35), and cDNA quantity (1 to 25 ng) were optimized to obtain a reproducible and reliable amplification curve capable of indicating the best conditions for revealing significant differences in the level of mRNA expression in comparing the various points in time and the different strains. The ratio of gene-target expression to 18S rRNA (used here as an internal standard), which indicates a semiquantitative analysis of mRNA expression, was calculated using quanti-doc, a tool present in the UVI-doc software package.

**Southern hybridization.** For Southern blot analysis, 10 μg of genomic DNA from *A. parasiticus* NRRL 2999 and each M and CM strain was completely digested with EcoRI (10 U) at 37°C for 4 h in the manufacturer's buffer at the recommended concentrations (Fermentas). EcoRI-digested DNA fragments were separated by electrophoresis for 3 h 30 min at 40 V on an 0.8% (wt/vol) agarose gel in 1× Tris-acetate-EDTA buffer. Digoxigenin (DIG)-labeled HindIII cut lambda (λ) (Roche) was used as molecular weight standard. Before blotting, the gel containing the DNA was washed twice with denaturation buffer (0.5 N NaOH and 1.5 M NaCl) for 30 min and twice with neutralization buffer (0.5 M Tris-HCl and 3 M NaCl, pH 7.5) for 15 min. The nucleic acids were transferred

to a Hybond-N$^+$ nylon membrane (Roche) using 10× SSC (1.5 M NaCl and 0.15 M sodium citrate) and fixed by UV illumination, in accordance with the Roche method, after overnight blotting. Fluorescent DNA probes were prepared according to the PCR DIG labeling mix method (Roche). The membranes were prehybridized according to the instructions of the manufacturer of the DIG detection kit, at 64°C in DIG Easy buffer (Roche); they were then hybridized for 12 to 16 h in the same buffer containing 250 ng of freshly denatured DIG Ap*yapA* probe at the same temperature.

Statistics. Data are presented as the mean value (±SEM) of three independent determinations from three separate experiments. In all experiments, data sets were pooled and compared using Student's *t* test, and the differences were considered significant when the *P* value was <0.05.

RESULTS

Ap*yapA* deleted and complemented mutant generation. In yeast, Yap1 modulates the expression of many antioxidant-related genes (2, 16, 45). The expression of the Ap*yapA* gene (Yap1 orthologue) is correlated with responsiveness to oxidative stress (54). Mutants (*n* = 20) with the Ap*yapA* gene deleted were generated to assess whether ApYapA acts as a sensor of oxidative stress and a modulator of cell antioxidative responses also in *A. parasiticus* NRRL 2999. ΔAp*yapA* (M), CM, and WT mycelia grown in aflatoxin-conducive medium were analyzed by Southern blotting (Fig. 1B) and PCR analysis (Fig. 1C). As expected, the M strain presented positive hybridization in two fragments of ~5.5 to 6.5 kb when the Ap*yapA* probe was used (Fig. 1B). This indicates that the Ap*yapA* gene sequence in the M strain was replaced by the deletion cassette that presents an EcoRI restriction site, which is absent in the Ap*yapA* sequence of the WT (Fig. 1A). A unique hybridization signal for a CM strain at a molecular size (~4.5 kb) lower than that of the WT (~6.5 kb) is also shown in Fig. 1A. The different size is due to the presence of an EcoRI restriction site in the Hph cassette (Fig. 1A) and indicates (as extensively explained in Materials and Methods) that Ap*yapA* has been correctly reinserted in its locus. The growth of the CM strain (*n* = 20) treated with 1 mM Men (which severely affected the growth of the M strain) and with hygromycin B (500 ppm) (which was restrictive for the growth of WT and M strains) was not significantly different from that of WT. This confirms the presence of a functioning ApYapA and hygromycin B phosphotransferase in the CM strain. Furthermore, the combination of the primers AmdS_for and ApYapA_rev and of Hph_for and ApyapA_rev2 was positive for PCR amplification (2.1 kb and 0.9 kb in the M and CM strains, respectively) (Fig. 1C). When *amdS* and *hph* primers were used, positive amplification

A)

B)

C)

D1)

D2)
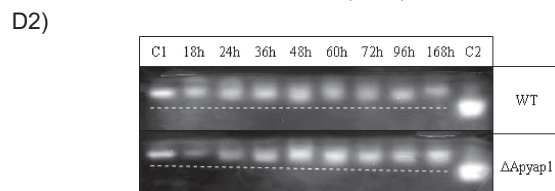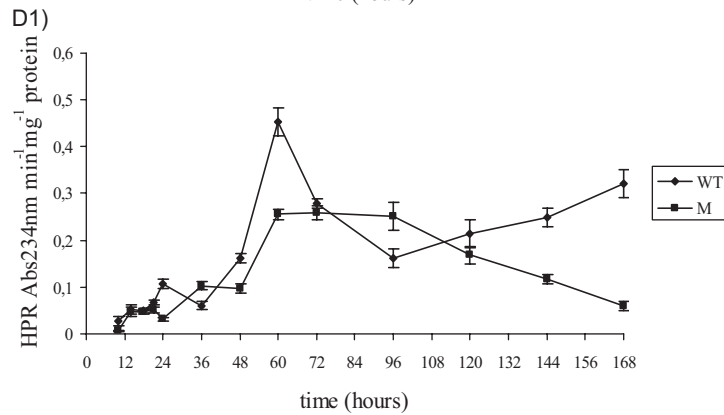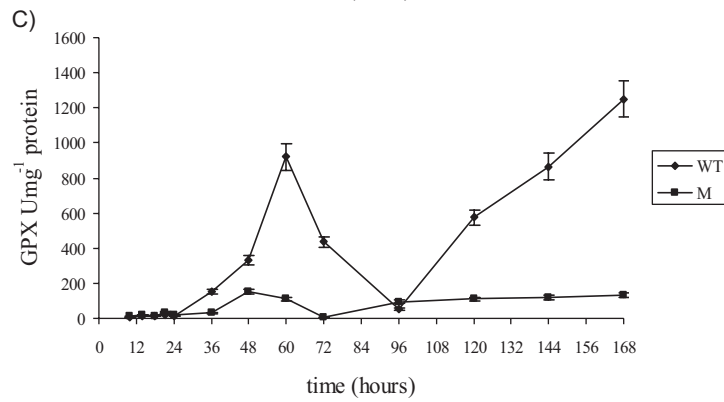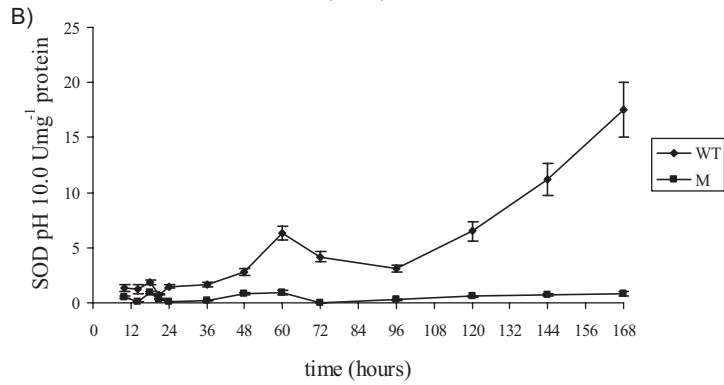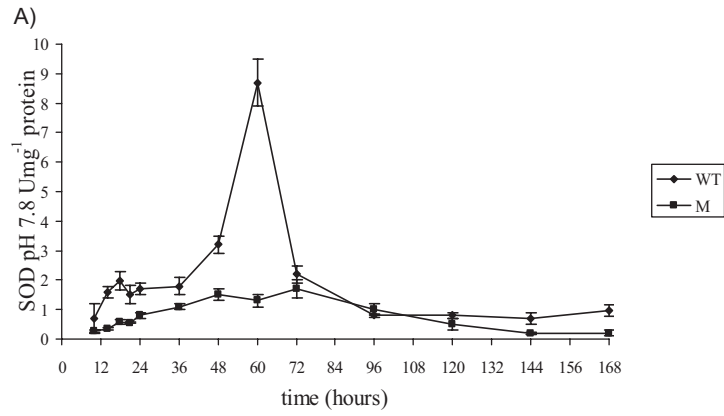
(0.7 and 0.65 kb, respectively) appeared only in the M and CM strains, respectively (Fig. 1C).

ΔApyapA mutant showed earlier conidium formation than did WT. The deletion of ApyapA was not directly involved in fungal growth, and yet conidiogenesis was significantly affected. The growth rates of the WT and M strains after 24 h of incubation (Fig. 2A) showed only slight differences. For both, the growth curve presented a biphasic profile: it decreased between 36 and 60 h, whereas the stationary phase was reached 72 h after inoculation. The growth curve of the CM strain did not differ from that of WT (Fig. 2A). With regard to conidiogenesis, the M strain showed a higher number of conidia than did the WT and CM strains, especially between 24 h and 96 h (Fig. 2B and C). However, at 168 h, all three strains had almost the same quantity of conidia. A similar trend has been observed in *N. crassa* and other fungi, where the undifferentiated vegetative growth is followed by a differentiated status (stimulated by a hyperoxidant condition), in which growth slows down and conidia are formed (1, 2).

ROS are formed soon after conidium germination and during fungal growth. Oxidative stress (i.e., $O_2^{\cdot-}$ and H_2O_2 production, the formation of 9- and 13-HODE, and LOX-like activity) was monitored in the mycelia of the WT and M strains between 10 and 168 h (Fig. 3 and 4A and B; Table 1). In both the WT and M strains, ROS production occurred quite early (i.e., in the first 24 h of growth), although in the M strain, production occurred earlier and the $O_2^{\cdot-}$ levels were higher than in the WT strain (Fig. 3A). In the time interval 24 to 60 h, the M strain also had higher levels of H_2O_2 (about 50 μmol at 36 h, compared to 28 μmol for the WT) and LOOH (about 160 ng · mg^{-1} at 36 h versus about 80 ng · mg^{-1} at 48 h in the WT) (Fig. 3B and 4A). After 60 h, for both $O_2^{\cdot-}$ and H_2O_2, a slight or nonsignificant difference was observed between the M and WT strains, whereas the amount of LOOH was significantly higher for the M strain than for the WT. The trend in LOX-like activity was similar to that for LOOH formation throughout nearly the entire incubation for both the M and the WT strains (Fig. 4A and B).

In fungi, 9- and 13-HPODE can play different physiological roles (63). The 13-HPODE produced by maize LOX inhibits the expression of aflatoxin-related genes in *Aspergillus*, whereas 9-HPODE stimulates expression (6). To determine whether these two regioisomers were present in *A. parasiticus* mycelia and played a differential role in regulating aflatoxin biosynthesis, their amount (relative percentage) was analyzed by liquid chromatography-MS (Table 1). At 42 to 48 h, the amount of 9-HODE in both the WT and M strains was significantly higher than that of 13-HODE. However, after 48 h, the amount was higher for 13-HODE.

ΔApyapA inefficiently scavenged ROS in comparison with WT. In previous studies (53, 54), we showed that fungal cells

activated antioxidant systems in response to oxidative stressors and cell aging. In the present study, to demonstrate the role of ApYapA in the control of antioxidant defenses, we carried out a comparison between the WT and M strains, performing experiments with a narrower time interval than that used in the previous studies.

Expression of mRNA was analyzed for three genes (ApyapA, Apskn7, and Aphsf2) whose orthologues are involved in the response to oxidative stress in other fungi (16). In the WT, ApyapA mRNA was expressed from 10 h to 72 h and Apskn7 mRNA was expressed at 10 h and from 30 h to 60 h, whereas Aphsf2 mRNA was expressed from 21 to 48 and 72 h (Fig. 5). In the M strain, at 10 h, there was marked expression of Apskn7 and Aphsf2 mRNA (Fig. 5), which could be the result of an attempt to make up for the lack of ApYapA; in fact, Skn7 can contribute to enhancing CAT gene expression (39), whereas Hsf2 can promote the expression of metallothioneins, which are also involved in ROS scavenging (16). The CM strain showed an amplification profile of ApyapA mRNA that was not significantly different from that of the WT (data not shown). Interestingly, from 0 h to 72 h, the expression of these transcription factors was correlated with antioxidant enzyme activation (SOD, HPR, and GPX) in the fungal cell, and all of the enzyme activities were lower in the M strain than in the WT (Fig. 6A to D1). In the WT, SOD (pH 7.8 and 10.0) and HPR were already activated at 10 h and showed higher levels than those in the M strain. SOD, GPX, and HPR activities peaked at 60 h (Fig. 6A to D1); afterwards, SOD activity at pH 7.8 decreased, whereas SOD at pH 10.0 showed a steep increase beginning at 96 h. SOD activity at basic pH can be peroxisomal or mitochondrial (64). The insufficient response of antioxidant enzymes to the LOOH (very low GPX activity) led to the use of α-tocopherol in the WT mycelia at early points in time. In fact, the amount of α-tocopherol was 12.5 ng · mg^{-1} at 24 h, followed by a progressive decrease. This compound, which is the most prominent lipophilic antioxidant (66), is thus able to scavenge the excess of LOOH produced at early points in time. In the WT, the marked increase in SOD at pH 10.0 after 96 h could reflect a strategy of the cell to defend mitochondria and peroxisomes from the ROS attack. GPX activity in the WT decreased after 60 h and increased between 96 and 168 h. HPR activity decreased after peaking at 60 h, with a slight increase at 96 h. In general, it was evident that the rate of increase in enzymatic activities was lower from 96 to 168 h than that from 36 to 72 h. In the M strain, between 48 and 72 h, SOD and GPX activity (Fig. 6A to C) and α-tocopherol content (5.8 ng · mg^{-1} at 24 h) were significantly lower than those in the WT, especially at 60 h, when a difference in HPR activity was also observed (Fig. 6D1). In the M strain, the defective perception of oxidative stress resulted in a less efficient defensive response in the fungal cell.

FIG. 6. Antioxidant enzyme SOD, GPX (U · mg^{-1} protein), and HPR (absorbance at 234 nm min^{-1} · mg^{-1} protein) activities in mycelia of *A. parasiticus* WT and ΔApyapA (M) strains grown in PDB at different time intervals of incubation at 30°C. (A and B) SOD activity at pH 7.8 (A) and pH 10.0 (B); (C) GPX activity; (D1) HPR activity; (D2) zymogram analysis of WT and M strain protein extracts obtained from mycelia grown for different time periods at 30°C in PDB and fractionated in a native polyacrylamide gel stained for CAT activity. C1 represents a low-acidic conformer of CAT derived from *A. niger*, and C2 represents the same CAT oxidized under an O_2 stream, giving a more-acidic form. The dashed white line represents the trend (from low- to high-acidic form) of CAT conformers during the different time intervals. The results in panels A to D1 are the means ± SEMs of three determinations from three separate experiments.

FIG. 7. (A to D) Ratios between the fungal growth (mg [dry weight] $\cdot$ ml^{-1}) of WT and ΔAp*yapA* (M) strains in CD amended with CH (1 mM) (A) or 1 (B) or 10 (C) mM H$_2$O$_2$ or Men (0.5 mM) (D) and their growth in CD (control). (E) Aflatoxin (AFTX) production in culture filtrate (μg $\cdot$ ml^{-1}) of WT and M strains grown in media amended with different stressors (CH, 1 mM; Men, 0.5 mM; H$_2$O$_2$, 1 and 10 mM), at different time intervals (4 to 11 days). All the compounds were added after 4 days of incubation at 30°C in CD medium. The results are the means ± SEMs of three determinations from three separate experiments.

H$_2$O$_2$ and LOOH levels after 18 h. LOOH formation can be ascribed both to a LOX-like activity (which also showed a very early activation) and, indirectly, to H$_2$O$_2$ (via a Fenton reaction). In fungi, LOOH could act as a modulator of differentiation events. In other studies of *Aspergillus* spp. (6, 67), 9-HODE and 13-HODE have been reported to have different physiological roles, although the intracellular detection of regioisomers in relation to aflatoxin biosynthesis has not yet been studied. In our study, the correlation between endogenous levels of 9- and 13-HODE and aflatoxin biosynthesis was not straightforward and needs to be investigated further.

The early accumulation of ROS results in the activation of antioxidant defense mechanisms, through the expression of Ap*yapA*, Ap*skn7*, and Ap*hsf2* mRNA. In *A. parasiticus*, it is likely that the putative activation of the above transcription factors organizes intracellular defensive machinery, which includes antioxidant enzymes, such as SODs, HPR, and GPX (which were prevalent at 60 h), and α-tocopherol (at early points in time). In *N. crassa*, antioxidant activities inhibit cell differentiation, whereas high levels of ROS are required to trigger this process (1). In yeast, the ROS which overwhelmed the endogenous antioxidant system are able to regulate cell aging (66). In our study, in the WT, antioxidant enzyme activity peaked between 48 and 60 h, soon after the increase of oxi-

A)

B)

FIG. 8. (A) Aflatoxin (AFTX) production in culture filtrate (µg ml^{-1}) of *A. parasiticus* WT, ΔAp*yapA* (M), and complemented Ap*yapA* (CM) strains inoculated into aflatoxin-conducive medium (PDB). (B) *aflR* and *norA* mRNA RT-PCR analysis in *A. parasiticus* mycelia after different time intervals. The aflatoxin results are the means ± SEMs of three determinations from three separate experiments.

dants within the cell (36 to 48 h), though a peak in α-tocopherol was present at 24 h. In the same time interval, the hyphal gro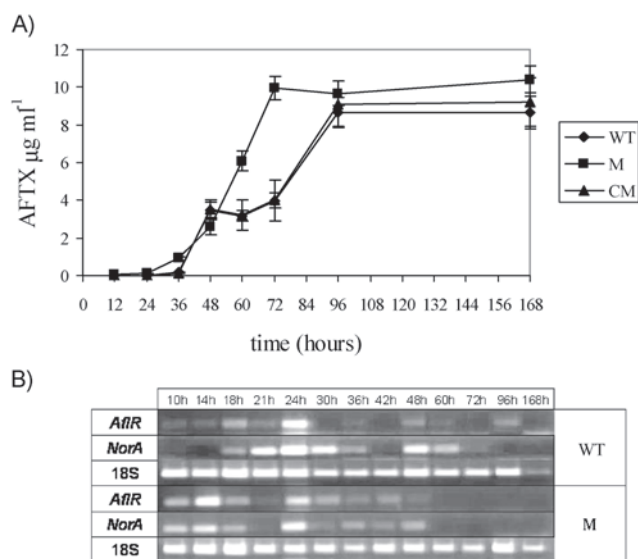wth rate decreased (36 to 60 h), cell differentiation occurred (as demonstrated by the appearance of conidia [48 h]), and secondary metabolism (36 h) switched on, leading to aflatoxin biosynthesis.

In previous studies (21, 53, 54), we investigated the associations among the expression of OSRTFs, antioxidant responses, and aflatoxin biosynthesis. The results showed that antioxidants inhibited aflatoxin biosynthesis and enhanced Ap*yapA* mRNA expression. In the present study, we describe the role of the Yap1 orthologue, ApYapA, in regulating cell differentiation and aflatoxin biosynthesis following ROS formation and the activation of antioxidant defensive mechanisms. The theoretical translation of the gene sequence of Ap*yapA* shares high similarities with orthologues in *A. fumigatus* (62% identities and 100% similarities, score of 268), with CHAP1 in *C. heterostrophus* (53% identities and 79% similarities, score of 128), and with Yap1 in *S. cerevisiae* (54% identities and 73% similarities, score of 48.5) (see Fig. S1A in the supplemental material).

The presence of regulatory elements in the *aflR* promoter responsive to AP1 (the human orthologue of Yap1) may suggest that oxidative stress exerts control in the modulation of aflatoxin biosynthesis. Thus, while ApYapA activates antioxidant defenses, it can contribute to the early decrease in *aflR* and *norA* mRNA expression. In relation to this, the lack of ApYapA could lead to the enhancement of the expression of aflatoxin-related genes in the mutant strain at the same points in time. In fission yeast, some transcription factors related to sexual differentiation can function both as activators of the expression of specific genes and simultaneously as repressors of the expression of others. In this way, the regulatory options of the cell to express specific gene sets to face different meta-

bolic situations are enhanced (44). Furthermore, the similarity between the *A. parasiticus* Ap*yapA* and *S. cerevisiae* *yap1* suggests that the gene is involved in modulating the response to oxidative stress. This similarity was also suggested by the presence of 10 well-conserved cysteine residues in the Ap*yapA* AA-deduced sequence, revealed by the Softberry P_SITE analysis. To support this evidence, we used a loss-of-function approach by producing a ΔAp*yapA* mutant of *A. parasiticus* which strengthens the relationship among oxidative stress, the activation of antioxidant defense mechanisms, cell differentiation, and mycotoxin biosynthesis. In the M strain, ROS production occurred earlier and to a greater extent than in the WT. Consistently, the oxidative signal triggered modest antioxidant defense. The residual antioxidant activity was probably the result of the early enhancement of Ap*skn7* and Ap*hsf2* mRNA transcription. In *S. cerevisiae*, other transcription factors (e.g., Prr1, a homologue of Skn7) are regulated by oxidative stress and, in turn, induce the expression of antioxidant enzyme activities, such as the CAT CTT1 and the SOD SOD1 (39). Moreover, in our study, the CAT zymogram showed that in the M strain the enzyme seems to undergo some oxidation, which is responsible for the slight alteration of the EM during fungal growth. Although the zymogram results did not allow us to reach definitive conclusions concerning the role of the CAT in total HPR activity during oxidative stress, the total HPR activity was less affected than were the activities of other antioxidant enzymes in the M strain; in fact, it has been hypothesized that the oxidation of the tetrapyrrolic ring does not alter CAT activity (37).

Nonetheless, the remaining antioxidant activities were insufficient to scavenge all of the ROS formed, forcing the cell to face a hyperoxidant status and respond by activating earlier cell differentiation and secondary metabolism. In fact, in ΔAp*yapA* *aflR* and *norA*, mRNA transcription was enhanced soon after conidium germination, conidiogenesis occurred early and increased starting at 24 h, and aflatoxins were already detectable in culture medium at 12 h. The formation of ROS was slightly delayed in the WT compared to the M strain (delay of 6 to 12 h), which had effects on gene activation, such as a delay in the OSRTF mRNA expression (Ap*skn7* and Ap*hsf2*) and in the transcription of aflatoxin-related genes (*aflR* and *norA*). In relation to this, aflatoxins did not appear before 36 h.

In the M strain, an impaired perception of oxidative stress and a malfunctioning of its signaling led to a defective antioxidant defense response and the consequent stimulation of aflatoxin biosynthesis. This could explain why this strain is more sensitive to different stressors, such as CH and Men, as well as hydrogen peroxide.

Considering our results as whole, similar phases can be envisioned during the growth of *A. parasiticus* NRRL 2999. Each phase consists of an oxidative burst which triggers the expression of Ap*yapA*, whose product modulates the activation of antioxidant enzymes. Excessive ROS are probably able to trigger aflatoxin biosynthesis, though the underlying mechanism is still unclear. During the growth phase, as soon as the activity of antioxidant enzymes decreases, aflatoxin biosynthesis begins. This hypothesis is supported by our finding that this kind of modulation was not found in ΔAp*yapA*. In this strain, the antioxidant defenses were less effective throughout the time considered.

We demonstrated that oxidative stress, generated either within or outside of the cell, affects aflatoxin formation in *A. parasiticus*. Ap*yapA* appears to play a significant role in the modulation and maintenance of an appropriate balance between oxidant and antioxidant species and aflatoxin biosynthesis. A complete comprehension of the mechanism by which the carcinogenic aflatoxins are synthesized by the fungus is instrumental in designing appropriate strategies for controlling their production and release into the environment.

REFERENCES

1. **Aguirre, J., M. Rios-Momberg, D. Hewitt, and W. Hansberg.** 2005. Reactive oxygen species and development in microbial eukaryotes. Trends Microbiol. **13**:111–118.
2. **Aguirre, J., W. Hansberg, and R. Navarro.** 2006. Fungal responses to reactive oxygen species. Med. Mycol. **44**(Suppl.):101–107.
3. **Arnold, R. S., J. Shi, E. Murad, A. M. Whalen, C. Q. Sun, R. Polavarapu, S. Parthasarathy, J. A. Petros, and J. D. Lambeth.** 2001. Hydrogen peroxide mediates the cell growth and transformation caused by mitogenic oxidase Nox1. Proc. Natl. Acad. Sci. USA **98**:5550–5555.
4. **Bokoch, G. M., and U. G. Knaus.** 2003. NADPH oxidase: not just for leukocytes anymore! Trends Biochem. Sci. **28**:502–508.
5. **Bolwell, G. P., L. V. Bindschedler, K. A. Blee, V. S. Butt, D. R. Davies, S. L. Gardner, C. Gerrish, and F. Minibayeva.** 2002. The apoplastic oxidative burst in response to biotic stress in plants: a three-component system. J. Exp. Bot. **53**:1367–1376.
6. **Burow, G. B., T. C. Nesbitt, J. Dunlap, and N. P. Keller.** 1997. Seed lipoxygenase products modulate Aspergillus mycotoxin biosynthesis. Mol. Plant-Microbe Interact. **10**:380–387.
7. **Calvo, A. M., L. L. Hinze, H. W. Gardner, and N. P. Keller.** 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. Appl. Environ. Microbiol. **65**:3668–3673.
8. **Calvo, A. M., H. W. Gardner, and N. P. Keller.** 2001. Genetic connection between fatty acid metabolism and sporulation in *Aspergillus nidulans*. J. Biol. Chem. **276**:25766–25774.
9. **Calvo, A. M., R. A. Wilson, J. W. Bok, and N. P. Keller.** 2002. Relationship between secondary metabolism and fungal development. Microbiol. Mol. Biol. Rev. **66**:447–459.
10. **Castoria, R., L. Caputo, F. De Curtis, and V. De Cicco.** 2003. Resistance of postharvest biocontrol yeast to oxidative stress: a possible new mechanism of action. Phytopathology **93**:564–572.
11. **Ceaser, E. K., D. R. Moellering, S. Shiva, A. Ramachandran, A. Landar, A. Venkartraman, J. Crawford, R. Patel, D. A. Dichinson, E. Ulasova, S. Ji, and V. M. Darley-Usmar.** 2004. Mechanisms of signal transduction mediated by oxidated lipids: the role of the electrophile-responsive proteome. Biochem. Soc. Trans. **32**:151–155.
12. **Champe, S. P., P. Rao, and A. Chang.** 1987. An endogenous inducer of sexual development in *Aspergillus nidulans*. J. Gen. Microbiol. **133**:1383–1387.
13. **Champe, S. P., and A. A. el-Zayat.** 1989. Isolation of a sexual sporulation hormone from *Aspergillus nidulans*. J. Bacteriol. **171**:3982–3988.
14. **Chang, P. K., J. W. Bennett, and P. J. Cotty.** 2002. Association of aflatoxin biosynthesis and sclerotial development in *Aspergillus parasiticus*. Mycopathologia **153**:41–48.
15. **Delaunay, A. D., M. Pflieger, M. Barrault, J. Vinh, and M. Toledano.** 2002. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell **111**:471–481.
16. **Estruch, F.** 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol. Rev. **24**:469–486.
17. **Fabbri, A. A., C. Fanelli, G. Panfili, S. Passi, and P. Fasella.** 1983. Lipoperoxidation and aflatoxin biosynthesis by *Aspergillus parasiticus* and *A. flavus*. J. Gen. Microbiol. **129**:3447–3452.
18. **Fabbri, A. A., C. Fanelli, M. Reverberi, A. Ricelli, E. Camera, S. Urbanelli, A. Rossini, M. Picardo, and M. M. Altamura.** 2000. Early physiological and cytological events induced by wounding in potato tuber. J. Exp. Bot. **51**:1267–1275.
19. **Fanelli, C., A. A. Fabbri, E. Finotti, and S. Passi.** 1983. Stimulation of aflatoxin biosynthesis by lipophilic epoxides. J. Gen. Microbiol. **129**:1721–1723.
20. **Fanelli, C., A. A. Fabbri, E. Finotti, P. Fasella, and S. Passi.** 1984. Free radicals and aflatoxin biosynthesis. Experientia **40**:191–193.
21. **Fanelli, C., A. A. Fabbri, S. Pieretti, E. Finotti, and S. Passi.** 1985. Effect of different antioxidants and free radical scavengers on aflatoxin production. Mycol. Res. **1**:65–69.
22. **Fanelli, C., and A. A. Fabbri.** 1989. Relationship between lipids and aflatoxin biosynthesis. Mycopathologia **107**:115–120.
23. **Fanelli, C., V. Tasca, A. Ricelli, M. Reverberi, S. Zjalic, E. Finotti, and A. A. Fabbri.** 2000. Inhibiting effect of medicinal mushroom *Lentinus edodes*
24. **Feussner, I., and C. Wasternack.** 2002. The lipoxygenase pathway. Annu. Rev. Plant Biol. **53**:275–297.
25. **Han, K. H., K. Y. Han, J. H. Yu, K. S. Chae, K. Y. Jahng, and D. M. Han.** 2001. The *nsdD* gene encodes a putative GATA-type transcription factor necessary for sexual development of *Aspergillus nidulans*. Mol. Microbiol. **41**:299–309.
26. **Harman, D.** 1956. Aging, a theory based on free radical and radiation chemistry. J. Gerontol. **11**:298–300.
27. **Hicks, J. K., J. H. Yu, N. P. Keller, and T. H. Adams.** 1997. *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signaling pathway. EMBO J. **16**:4916–4923.
28. **Itoh, K., K. I. Tong, and M. Yamamoto.** 2004. Molecular mechanism activating Nrf2-Keap1 pathway in regulation of adaptive response to electrophiles. Free Radic. Biol. Med. **36**:1208–1213.
29. **Jayashree, T., and C. Subramanyam.** 2000. Oxidative stress as a prerequisite for aflatoxin production by *Aspergillus parasiticus*. Free Radic. Biol. Med. **29**:981–985.
30. **Jimenez, A., L. Mateos, J. R. Pedrajas, A. Miranda-Vizuete, and J. L. Revuelta.** 2007. The txl1(+) gene from *Schizosaccharomyces pombe* encodes a new thioredoxin-like 1 protein that participates in the antioxidant defence against tert-butyl hydroperoxide. Yeast **24**:481–490.
31. **Jones, A. M.** 1994. Surprising signalling in plant cells. Science **263**:183–184.
32. **Kappus, H.** 1985. Lipid peroxidation: mechanism, analysis, enzymology and biological relevance, p. 273–310. *In* H. Sies (ed.), Oxidative stress. Academic Press, London, United Kingdom.
33. **Kawasaki, L., and J. Aguirre.** 2001. Multiple catalase genes are differentially regulated in *Aspergillus nidulans*. J. Bacteriol. **183**:1434–1440.
34. **Kim, J. H., B. C. Campbell, J. Yu, N. Mahoney, K. L. Chan, R. J. Molyneux, D. Bhatnagar, and T. E. Cleveland.** 2005. Examination of fungal stress response genes using *Saccharomyces cerevisiae* as a model system: targeting genes affecting aflatoxin biosynthesis by *Aspergillus flavus* Link. Appl. Microbiol. Biotechnol. **67**:807–815.
35. **Lambeth, J. D.** 2004. Nox enzymes and the biology of reactive oxygen. Nat. Rev. Immunol. **4**:181–189.
36. **Lara-Ortiz, T., H. Riveros-Rosas, and J. Aguirre.** 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Mol. Microbiol. **50**:1241–1255.
37. **Lledias, F., P. Rangle, and W. Hansberg.** 1998. Oxidation of catalase by singlet oxygen. J. Biol. Chem. **273**:10630–10637.
38. **Lledias, F., P. Rangle, and W. Hansberg.** 1999. Singlet oxygen is part of an hyperoxidant state generated during spore germination. Free Radic. Biol. Med. **26**:1396–1404.
39. **Lee, J., C. Godon, G. Lagniel, D. Spector, J. Garin, J. Labarre, and M. B. Toledano.** 1999. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J. Biol. Chem. **274**:16040–16046.
40. **Lev, S. R., P. Hadar, S. E. A. Baker, O. C. Yoder, and B. A. Horwitz.** 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.
41. **Lorito, M., C. K. Hayes, A. Di Pietro, and G. E. Harman.** 1993. Biolistic transformation of *Trichoderma harzianum* and *Gliocladium virens* using plasmid and genomic DNA. Curr. Genet. **24**:349–356.
42. **Malagnac, F., H. Lalucque, G. Lepere, and P. Silar.** 2004. Two NADPH oxidase isoforms are required for sexual reproduction and ascospore germination in the filamentous fungus *Podospora anserina*. Fungal Genet. Biol. **41**:982–997.
43. **Maresca, V., E. Flori, S. Briganti, E. Camera, M. Cario-Andre, M. Taıeb, and M. Picardo.** 2006. UVA-induced modification of catalase charge properties in the epidermis is correlated with the skin phototype. J. Investig. Dermatol. **126**:182–190.
44. **Mata, J., A. Wilbrey, and J. Bahler.** 2007. Transcription regulatory networks for sexual differentiation in fission yeast. Genome Biol. **8**:R217.
45. **Moye-Rowley, W. S.** 2003. Regulation of transcriptional response to oxidative stress in fungi: similarities and differences. Eukaryot. Cell **2**:381–389.
46. **Narasaiah, K. V., R. B. Sashidhar, and C. Subramanyam.** 2006. Biochemical analysis of oxidative stress in the production of aflatoxin and its precursor intermediates. Mycopathologia **162**:179–189.
47. **Nguyen, T., P. J. Sherratt, and C. B. Pickett.** 2003. Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. Annu. Rev. Pharmacol. Toxicol. **43**:233–260.
48. **Passi, S., C. Fanelli, A. A. Fabbri, E. Finotti, G. Panfili, and M. Nazzaro-Porro.** 1985. Effect of halomethanes on aflatoxin induction in cultures of *Aspergillus parasiticus*. J. Gen. Microbiol. **131**:687–691.
49. **Passi, S., R. Ricci., E. Aleo, and M. Cocchi.** 2005. Oxidative stress, aging and aging-related diseases. Progr. Nutr. **7**:3–22.
50. **Pinkus, R., L. M. Weiner, and V. Daniel.** 1996. Role of oxidants and antioxidants in the induction of AP-1, NF-κB, and glutathione *S*-transferase gene expression. J. Biol. Chem. **271**:13422–13429.
51. **Prade, R. A., and W. E. Timberlake.** 1993. The *Aspergillus nidulans nbrlA*

(Berk.) Sing (Agaricomycetideae) on aflatoxin production by *Aspergillus parasiticus* Speare. Int. J. Med. Mushrooms **2**:229–236.

regulatory locus consists of overlapping transcription units that are individually required for conidiophore development. EMBO J. **12:**2439–2447.

52. **Reverberi, M., M. Picardo, A. Ricelli, E. Camera, C. Fanelli, and A. A. Fabbri.** 2001. Oxidative stress, growth factor production and budding in potato tubers during cold storage. Free Radic. Res. **35:**833–841.

53. **Reverberi, M., A. A. Fabbri, S. Zjalic, A. Ricelli, F. Punelli, and C. Fanelli.** 2005. Antioxidant enzymes stimulation in *Aspergillus parasiticus* by *Lentinula edodes* inhibits aflatoxin production. Appl. Microbiol. Biotechnol. **69:**207–215.

54. **Reverberi, M., S. Zjalic, A. Ricelli, A. A. Fabbri, and C. Fanelli.** 2006. Oxidant/antioxidant balance in *Aspergillus parasiticus* affects aflatoxin biosynthesis. Mycotox. Res. **22:**39–47.

55. **Shackelford, R. E., W. K. Kaufmann, and R. S. Paules.** 2000. Oxidative stress and cell cycle check point function. Free Radic. Biol. Med. **28:**1387–1404.

56. **Shimizu, K., and N. P. Keller.** 2001. Genetic involvement of cAMP-dependent protein kinase in a G protein signaling pathway regulating morphological and chemical transitions in *Aspergillus nidulans*. Genetics **157:**591–600.

57. **Sies, H.** 1985. Oxidative stress: introductory remarks, p. 1–8. *In* H. Sies (ed.), Oxidative stress. Academic Press, London, United Kingdom.

58. **Spiteller, G.** 2001. Peroxidation of linoleic acid and its relation to aging and age dependent diseases. Mech. Ageing Dev. **122:**617–657.

59. **Suh, Y. A., R. S. Arnold, B. Lassegue, J. Shi, X. Xsu, D. Sorescu, A. B. Chung, K. K. Griendling, and J. D. Lambeth.** 1999. Cell transformation by the superoxide-generating oxidase Mox1. Nature **401:**79–82.

60. **Sutherland, M. W., and B. A. Learmonth.** 1997. The tetrazolium dyes MTS

and XTT provide new quantitative assays for superoxide and superoxide dismutase. Free Radic. Res. **27:**283–289.

61. **Talalay, P., A. T. Dinkova-Kostova, and W. D. Holzelaw.** 2003. Importance of phase 2 regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv. Enzyme Regul. **43:**121–134.

62. **Torres, M. A., J. L. Dangl, and J. D. Jones.** 2002. *Arabidopsis gp91phox* homologues *AtrbohD* and *AtrbohF* are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. USA **99:**517–522.

63. **Tsitsigiannis, D., and N. P. Keller.** 2007. Oxylipins as developmental and host-fungal communication signals. Trends Microbiol. **15:**109–118.

64. **Van Roermund, W. T., M. De Jong, L. Ijlst, J. van Marie, T. B. Dansen, R. J. A. Wanders, and H. R. Waterham.** 2004. The peroxisomal lumen in *Saccharomyces cerevisiae* is alkaline. J. Cell Sci. **117:**4231–4237.

65. **Vignais, P. V.** 2002. The superoxide-generating NADPH oxidase: structural aspects and activation mechanism. Cell. Mol. Life Sci. **59:**1428–1459.

66. **Wilhelm, J., H. Fuksova, Z. Shwippelova, R Vytasek, and A. Pichova.** 2006. The effects of reactive oxygen and nitrogen species during yeast replicative ageing. Biofactors **27:**185–193.

67. **Wilson, R. A., H. W. Gardner, and N. P. Keller.** 2001. Cultivar-dependent expression of a maize lipoxygenase responsive to seed infesting fungi. Mol. Plant-Microbe Interact. **14:**980–987.

68. **Yu, J., and N. P. Keller.** 2005. Regulation of secondary metabolism in filamentous fungi. Annu. Rev. Phytopathol. **43:**437–458.