Modulation of Antioxidant Defense in Aspergillus parasiticus Is Involved in Aflatoxin Biosynthesis: a Role for the ApyapA Gene

Massimo Reverberi,1 Slaven Zjalic,1 Alessandra Ricelli,2 Federico Punelli,1 Emanuela Camera,3 Claudia Fabbri,3 Mauro Picardo,3 Corrado Fanelli,3 and Anna A. Fabbri1*

Dipartimento di Biologia Vegetale, Università La Sapienza, L.go Cristina di Svezia, 24 00165 Roma, Italy; Istituto di Scienze delle Produzioni Alimentari, CNR, Via G. Amendola, 122/O, 70126 Bari, Italy; and Istituto Dermatologico San Gallicano, IRCCS, Via S. Gallicano 25, 00153 Roma, Italy

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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 (ΔApyapA) having the ApyapA gene deleted, given that ApyapA 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 ΔApyapA 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 ApyapA leads to an increase in oxidative stress, premature conidiogenesis, and aflatoxin biosynthesis.

Reactive oxygen species (ROS), such as superoxide anion (O2•−), hydrogen peroxide (H2O2), hydroxyl radical (HO•), 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 H2O2 exceeds the cell-scavenging capacity, it can generate highly reactive HO• 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 lipoxigenase (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 mitosporic and meioспорes 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-
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 trd [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-

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 SpeI-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.
tion, liperoxidation, 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 ΔAppuA 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.

**FIG. 2.** (A) Mycelial growth (mg [dry weight] · ml⁻¹) of WT, ΔAppuA (M), and AppuA 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 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.
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 O2 stream, which produces a more acidic form (C2). Native minigel (8 to 9 cm and 0.75 mm 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 2h 45 min at 4°C and stained with Coomassie Blue. Native enzyme was detected as de-phosphorylated under high-stringency conditions produced a unique band for the putative bzip transcription factor [Ap-1] of diverse fungal species. Furthermore, ApYap1 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 Skn7p (areA in A. nidulans) of diverse fungal species. ApYapA (forward, 5′ GGTCTCCCATCATCCTCATCC 3′; reverse, 5′ TGCCGGAAC TTCTCCATAAC 3′, +1771) and ApSkn7 (forward, 5′ GGGTACTACAGG TTCAAA 3′; reverse, 5′ AGCCGTCGAAGCTCTTAAAC 3′) primers were designed and used for the subsequent reverse transcription-PCR (RT-PCR) analysis. The 18S primer pair (forward, 5′ ATGGCCGTTCTCTTGTTG 3′; reverse, 5′ TGACAAAGGGGCAGGGACGTA 3′) produced a single fragment of 500 bp (internal standard), whereas the primers chosen for the amplification of Apyap1 and ApSkn7 genes produced single fragments of 659 bp and 480 bp, respectively.

Plasmids and transformation. Two fragments of Apyap1 were amplified by PCR from genomic DNA of A. parasiticus NRRL 2999 (WT) with the primer pairs ApyapAXbaI_for (+1112) and ApyapAXbaI_rev (+1320) and Apyap1-EcoRI_rev (+1420) and Apyap1-EcoRI_for (+1112) and Apyap1-EcoRI_rev (+1420) and Apyap1-EcoRI_for (+1772) and Apyap1-AphI_for (+1772; SalI internal restriction site) and Apyap1-AphI_rev, which produced single fragment of 900 bp (internal standard), whereas the primers chosen for the amplification of Apyap1 and ApSkn7 genes produced single fragments of 659 bp and 480 bp, respectively.
contains two fragments of the Apsup4 gene interrupted by the AmdS cassette (~4.6 kb) (Fig. 1A). In the A. flavus genome (checked at the website www.aspergillusflavus.org), EcoRI presents two restriction sites ~1.6 kb downstream and ~4.0 kb upstream of the Apsup4 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 Apsup4 homologue is present), the 6.5-kb fragment that originated in the Apsup4 knockout mutant could be the result of the sum of the two fragments.

![Graph](image)

**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 ΔApsup4 (M) strains grown in PDB (25 ml) and incubated at 30°C from 10 to 168 h. The results are the means ± SEM of three determinations from three separate experiments.

**TABLE 1. Relative percentages of 9- and 13-HODE in the mycelia of A. parasiticus WT and ΔApsup4 mutant (M) strains**

| Time (h) | 9-HODE | 13-HODE | 9-HODE | 13-HODE |
|---------|--------|---------|--------|---------|
|         | WT     | M       | WT     | M       |
| 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 |

*The values represent the means ± SEMs of three replicates from three separate experiments.

For complementation of the M strain, a 5.4-kb BglII-HindIII fragment resistance cassette was excised from pAN7.1::Apsup4, 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 ~4.5-kb fragment present in the CM strain can be generated by the hybridization of the Apsup4 probe with a fragment constituted by the ~1.0-kb EcoRI fragment of the lhp resistance cassette, the ~1.9-kb Apsup4 gene sequence (complemented), and the EcoRI Apsup4 downstream fragment (~1.6 kb).

Selection of Apsup4 deleted and complemented mutants. The selection of transformants (strains with deleted Apsup4 [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 monokaryotic 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 (~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::Apsup4, 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
inhibited the germination of ΔApypA 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 ΔApypA 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 ApypA WT with the ApypA 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 ApypA 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 amphid 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’ CTTGTATGGACGAGGAGACC 3’) and Hph_rev (5’ ATTTGTTGACCGCCGGAACG 3’); (iv) PCR amplification of the genomic DNA of the WT, M, and CM strains using AmDS_for and ApypA_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 ApypA_rev2 (5’ GAGGGCTTCTTGAGACAGCTGG 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 ApypA_for and ApypA_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). Selection of the strains was determined by measuring the growth rate and spore counts of cultures grown on plates of PDA.

**RESULTS**

ApypA deleted and complemented mutant generation. In yeast, Yap1 modulates the expression of many antioxidant-related genes (2, 16, 45). The expression of the ApypA gene (Yap1 orthologue) is correlated with responsiveness to oxidative stress (54). Mutants (n = 20) with the ApypA gene deleted were generated to assess whether ApYapA acts as a sensor of oxidative stress and a modulator of cell antioxidant responses also in *A. parasiticus* NRRL 2999. ΔApypA (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 ApypA probe was used (Fig. 1B). This indicates that the ApypA gene sequence in the M strain was replaced by the deletion cassette that presents an EcoRI restriction site, which is absent in the ApypA 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 ApypA 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...
(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$_2^·$ and H$_2$O$_2$ 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$_2^·$ 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$_2$O$_2$ (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$_2^·$ and H$_2$O$_2$, 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 production of 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. 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 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.
TABLE 2. Fungal growth of the WT and Δ YapA (M) strains in CD and in CD amended with 1 mM CH, 0.5 mM Men, or 1 and 10 mM H2O2.

| Strain type and time (h) | Fungal growtha (mg [dry wt] ml−1) in CD amended with: |
|-------------------------|--------------------------------------------------|
|                         | Control | 1 mM CH | 1 mM H2O2 | 10 mM H2O2 | 0.5 mM Men |
| WT 18                   | 2.4 ± 0.3 | 2.3 ± 0.5 | 1.6 ± 0.1 | 1.4 ± 0.1 | 2.1 ± 0.1 |
| 48                      | 6.0 ± 0.5 | 2.7 ± 0.4 | 3.7 ± 0.5 | 5.0 ± 0.6 | 4.8 ± 0.2 |
| 96                      | 7.2 ± 0.7 | 7.1 ± 0.8 | 7.3 ± 0.5 | 8.1 ± 0.8 | 7.5 ± 0.8 |
| 168                     | 7.0 ± 0.8 | 8.5 ± 0.9 | 8.6 ± 0.8 | 7.5 ± 0.8 | 6.0 ± 0.5 |
| 264                     | 0.3 ± 1.2 | 7.0 ± 1.5 | 6.1 ± 1.2 | 6.8 ± 0.5 | 5.8 ± 1.0 |
| M 18                    | 1.6 ± 0.2 | 1.5 ± 0.2 | 1.8 ± 0.1 | 2.1 ± 0.4 | 0.8 ± 0.1 |
| 48                      | 2.0 ± 0.5 | 2.0 ± 0.1 | 4.8 ± 0.2 | 4.6 ± 0.4 | 2.2 ± 0.1 |
| 96                      | 9.4 ± 0.8 | 5.9 ± 0.2 | 4.6 ± 0.4 | 9.1 ± 0.4 | 9.4 ± 1.2 |
| 168                     | 9.4 ± 1.0 | 8.9 ± 0.9 | 9.4 ± 0.8 | 8.8 ± 0.8 | 9.6 ± 0.7 |
| 264                     | 8.3 ± 1.5 | 8.1 ± 0.6 | 7.6 ± 1.0 | 7.1 ± 1.1 | 7.9 ± 1.2 |

* The values represent the means ± SEMs of three replicates from three separate experiments.

The zymograms of the major CAT in the WT and M mycelia are shown in Fig. 6D2. According to other studies (33, 37), CATs are present in fungal mycelia, and their electrophoretic mobility (EM) slightly changes during fungal development and in the presence of oxidative stress in the cell. In our study, one CAT isoform, which was faintly detectable and had a very high molecular weight, did not seem to have been affected by oxidants during fungal growth in either of the strains, whereas the major isoform, which had a higher EM, seemed to have been slightly altered by oxidants. According to a previous study (37), the CAT can be oxidized by ROS and the oxidized form presents a different EM. In our study, in the M strain at 18 h, the EM of the CAT was similar to that of the standard CAT C1; afterwards, the EM increased. In the WT, at 18 h, the CAT had the same EM as that of C1, and at 48 h, a more-acidic form was observed, which could represent a partially oxidized form. At 168 h, the EM of the CAT was very similar to that at 18 h (Fig. 6D2).

Δ YapA is more sensitive to oxidative stressors than the WT is. To assess whether the M strain, which lacks an efficient antioxidant defense, was more sensitive than the WT to the presence of oxidants in the environment, several oxidants were added to liquid media. The M strain was more susceptible than the WT to all of the oxidants tested, in terms of both growth and aflatoxin biosynthesis. Growth was most influenced by CH (1 mM) and Men (0.5 mM) (Table 2; Fig. 7A to D). Regarding aflatoxin biosynthesis, the M strain was more influenced than the WT at all points in time (4, 7, and 11 days), and the compounds with the greatest stimulating effect were 1 mM CH, 0.5 mM Men, and the highest concentration of hydrogen peroxide (10 mM [Fig. 7E]). These results confirm that the alteration of oxidant perception and the absence of a substantial antioxidant defense are closely related to aflatoxin stimulation.

Δ YapA affects aflatoxin biosynthesis. In the WT, between 12 h and 36 h, aflatoxin biosynthesis was lacking or very low, whereas in the M strain it was already observed at 12 h (42 ng·ml−1) (Fig. 8A). Between 48 h and 72 h, the concentration of aflatoxins was significantly higher in the M strain than in the WT. Between 96 and 168 h, although the concentration remained higher for the M strain, the difference was not always significant. In the CM strain, aflatoxin biosynthesis followed the same trend as that in the WT. In the M strain, the expression of aflR and norA mRNAs occurred earlier than in the WT (Fig. 8B) and it was highly correlated with the early accumulation of aflatoxin in the medium (Fig. 7E and 8A). In the WT, aflatoxin biosynthesis decreased between 48 and 72 h, soon after fungal vegetative growth began to decline (36 to 60 h) (Fig. 2A). At the same time (36 to 60 h), a higher activity level of antioxidant enzymes was observed (Fig. 6A to D). At 60 h, all of the enzymes showed a peak in their activities, and at the same time aflatoxin biosynthesis decreased in the WT but not in the M strain, where the enzymatic activities were significantly lower. That there exists an association between the antioxidant/oxidant balance and aflatoxin biosynthesis, probably driven by ApYapA, was also suggested by the results of the in silico analysis of the aflR promoter sequence. This analysis was performed using the N_SITE tool in the Softberry software package, which allowed us to reveal all of the putative regulatory elements present in the promoter region, which were compared with the human N_SITE database. The results obtained showed that the aflR promoter presented diverse regulatory elements, which were similar to those recognized by some OSRTFs in humans, such as AP1 (SiteID, S02349; SiteName, ENKCRE-2, P < 0.05), NF-κB (SiteID, S05669; SiteName, NF-κB-E-selectin, P < 0.05), and Rox1 (SiteID, S06246; SiteName, Rox1-HEM13-3, P < 0.01). This suggests that the aflatoxin regulator gene expression could also be affected by oxidative stress.

DISCUSSION

In recent years, many authors have found evidence of a close association among oxidative stress, development, differentiation, and secondary metabolism in fungi (1, 2, 27, 54, 68). From these studies it has emerged that the levels of oxidants are finely regulated in fungal cells. In yeast, the maintenance of a favorable redox balance is under the control of Yap1. This factor acts as a sensor of the cell’s redox state through a cysteine-rich domain, and it regulates the activation of different antioxidant defense-related genes, such as sod, cat, and gpx (16, 39). In other fungi, such as Candida albicans and Cochliobolus heterostrophus, proteins that are orthologues to Yap1 control the expression of a similar set of genes that are needed to respond to oxidative stress (15, 40).

In A. parasiticus, we found that oxidative stress (i.e., O2−, H2O2, and LOOH formation) occurred soon after conidium germination and during growth. Oxidative perturbation promotes defensive responses such as the expression of OSRTFs mRNA, enzymes, and compounds with antioxidant activity. Their modulation leads to metabolic consequences, including an effect on aflatoxin biosynthesis. In A. parasiticus NRRL 2999, ROS production was evident quite early (i.e., 8 to 10 h after inoculation in liquid medium), which is consistent with the findings of a study of N. crassa (1), though different ROS were considered. The production could be due to the emergence of germination tubes which suddenly expose conidia to O2 and/or to the marked increase in metabolic activity. In our study, O2− was formed early on, followed by an increase in
H₂O₂ 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 H₂O₂ (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 Apyap4, Apskn7, and Aphps2 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-
dants within the cell (36 to 48 h), though a peak in α-tocopheryl was present at 24 h. In the same time interval, the hyphal growth 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 ApypA 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 ApypA 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 metabolic situations are enhanced (44). Furthermore, the similarity between the A. parasiticus ApypA 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 ApypA AA-deduced sequence, revealed by the Softberry P_SITE analysis. To support this evidence, we used a loss-of-function approach by producing a ΔApypA 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 Apysn7 and Apphs2 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 CATzymogram 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 ΔApypA 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 (Apysn7 and Apphs2) 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 ApypA, 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 ΔApypA. 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 Aspergillus parasiticus. AwapA 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 carboxinogenic aflatoxins are synthesized by the fungus is instrumental in designing appropriate strategies for controlling their production and release into the environment.

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