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Redox Regulation of an AP-1-Like Transcription Factor, YapA, in the Fungal Symbiont *Epichloë festucae*

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One of the central regulators of oxidative stress in *Saccharomyces cerevisiae* is Yap1, a bZIP transcription factor of the AP-1 family. In unstressed cells, Yap1 is reduced and cytoplasmic, but in response to oxidative stress, it becomes oxidized and accumulates in the nucleus. To date, there have been no reports on the role of AP-1-like transcription factors in symbiotic fungi. An ortholog of Yap1, named YapA, was identified in the genome of the grass symbiont *Epichloë festucae* and shown to complement an *S. cerevisiae* Δ*yap1* mutant. Hyphae of the *E. festucae* Δ*yapA* strain were sensitive to menadione and diamide but resistant to H_2O_2, KO_2, and *tert*-butyl hydroperoxide (*t*-BOOH). In contrast, conidia of the Δ*yapA* strain were very sensitive to H_2O_2 and failed to germinate. Using a P*catA-eGFP* degron-tagged reporter, YapA was shown to be required for expression of a spore-specific catalase gene, *catA*. Although YapA-EGFP localized to the nucleus in response to host reactive oxygen species during seedling infection, there was no difference in whole-plant and cellular phenotypes of plants infected with the Δ*yapA* strain compared to the wild-type strain. Homologs of the *S. cerevisiae* and *Schizosaccharomyces pombe* redox-sensing proteins (Gpx3 and Tpx1, respectively) did not act as redox sensors for YapA in *E. festucae*. In response to oxidative stress, YapA-EGFP localized to the nuclei of *E. festucae* Δ*gpxC*, Δ*tpxA*, and Δ*gpxC* Δ*tpxA* cells to the same degree as that in wild-type cells. These results show that *E. festucae* has a robust system for countering oxidative stress in culture and *in planta* but that Gpx3- or Tpx1-like thiol peroxidases are dispensable for activation of YapA.

Reactive oxygen species (ROS) play a very important biological role in fungus-plant interactions. Production of ROS by plasma membrane-localized NADPH oxidases at sites of pathogen invasion is one of the early defense responses of the plant host (1–3). This oxidative burst induces programmed cell death but also serves as a second messenger to activate the expression of various plant defense genes (4–6). Detoxification of this burst of ROS is vital for maintaining plant cellular integrity. Likewise, fungal plant pathogens must have efficient mechanisms for ROS detoxification if they are to successfully colonize the host (7, 8). Two strategies that are commonly used by plants and fungi to detoxify ROS are the use of antioxidants, such as ascorbate, glutathione, and carotenoids, and enzymatic scavenging of ROS by superoxide dismutase, catalase, ascorbate peroxidase, or glutathione peroxidase (9, 10). Production of "bursts" of ROS by specific NADPH oxidase isoforms are also important for polarized growth and multicellular development in both plants and fungi (11–16). As with the defense response, restoration of ROS homeostasis following differentiation signaling is vital for maintenance of cellular integrity. One of the key regulators mediating an oxidative stress response is the AP-1 class of basic leucine zipper (bZIP) transcription factors.

The *Saccharomyces cerevisiae* AP-1-like transcription factor Yap1 is the best-characterized member of the bZIP family of transcription factors. Yap1 contains two cysteine-rich domains (CRDs), at the N-distal (n-CRD) and C-proximal (c-CRD) termini, which are fundamental to its activation. In the absence of oxidative stress, the nuclear export sequence (NES) is able to interact with the Crm1 nuclear exportin, resulting in export of the protein from the nucleus to the cytoplasm. In cells exposed to H_2O_2 stress, two disulfide bonds are formed between the n- and c-CRDs, resulting in a conformational change that masks the interaction of the NES with Crm1 (17–19). As a result, oxidized Yap1 is retained in the nucleus and activates the expression of numerous genes involved in antioxidant defense (20).

Interdomain disulfide bond formation between the n- and c-CRDs after exposure to H_2O_2 is by far the most commonly recognized mechanism of Yap1 activation. However, sensing of the redox signal (H_2O_2) is mediated by the glutathione peroxidase Gpx3 (also known as Hyr1 [hydrogen peroxide resistance protein 1] or ORP1 [oxidant receptor peroxidase 1]) rather than by Yap1 itself, resulting in oxidation of a conserved Cys in Gpx3 to a sulfenic acid (21). The Cys-SOH then condenses with a Cys in the c-CRD of Yap1 to form an intermolecular disulfide bond, which is resolved by thiol disulfide exchange, resulting in release of Gpx3 and formation of an intramolecular disulfide bond between conserved cysteines in the c- and n-CRDs of Yap1 (21). The second disulfide bond, between two other conserved cysteines in the n- and c-CRDs, is formed by a second oxidation cycle with Gpx3. Transduction of the H_2O_2 signal requires formation of multiple interdomain disulfide bonds to generate a reduction-resistant Yap1 protein (22). In addition, Ybp1 (Yap1-binding protein) has an important role, together with Gpx3, in the cytoplasmic oxidation of Yap1 in response to H_2O_2 stress and in promotion of nuclear accumulation (23). In *Schizosaccharomyces pombe*, the thioredoxin peroxidase Tpx1, rather than a Gpx protein, is used as the redox sensor to regulate Pap1, the Yap1 homolog (24, 25). Acti-

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vation of Pap1 occurs by a catalytic mechanism slightly different from that described for *S. cerevisiae*, as both catalytic cysteine residues are used in Tpx1, whereas just one is used in Gpx3. Whether homologs of Gpx3 or Tpx1 act as redox sensors for activation of AP-1-like proteins in filamentous fungi is not known. Yap1 and Pap1 can also be modified covalently by thiol-reactive electrophiles, such as diamide, *N*-ethylmaleimide, and diethylmaleate, independently of Gpx3 or Tpx1. Oxidation of conserved cysteines in the c-CRD, without the formation of disulfide bonds, is sufficient to disrupt interaction between Crm1 and either Yap1 or Pap1, promoting retention of the transcription factor in the nucleus (26–28).

Yap1 homologs have been identified in a number of other fungi, including *Candida albicans* (29), *Aspergillus fumigatus* (30), *Cochliobolus heterostrophus* (31), *Alternaria alternata* (32), *Botrytis cinerea* (33), *Neurospora crassa* (34), *Magnaporthe oryzae* (8), and *Ustilago maydis* (7). Disruption of these transcription factors commonly results in hyphal and conidial sensitivity to H_2O_2 and other oxidative stress-inducing compounds, such as *tert*-butyl hydroperoxide (*t*-BOOH), potassium superoxide (KO_2), menadione, and diamide. Regulation through control of nuclear import and export is a shared feature of these AP-1-like proteins, a property that presumably reflects the conserved domain (bZIP, n-CRD, and c-CRD) and motif (NES and nuclear localization sequence [NLS]) structure of this family of proteins.

AP-1-like transcription factors have a key role in protecting phytopathogenic fungi from plant-generated ROS during host colonization, a function highlighted by retention of these proteins in the nucleus upon contact with the leaf surface and during appressorium-mediated penetration of the host (7, 31). While AP-1 deletion mutants of fungi are sensitive to H_2O_2 in culture, the plant colonization phenotype is very dependent on the growth lifestyle. AP-1 mutants of the biotrophic fungi *U. maydis* and *M. oryzae* are sensitive to a host oxidative burst and have reduced virulence (7, 8). In contrast, the AP-1 mutants of the necrotrophic fungi *B. cinerea* and *C. heterostrophus* are as virulent as the wild-type strains (31, 33). *A. alternata* is an exception to the generalization that lifestyle determines the host colonization outcome, as AP-1 mutants of this necrotrophic pathogen are defective in host colonization (32).

In *M. oryzae*, the homolog of *GPX3/HYR1* also has a role in host virulence; the *hyr1* mutant was shown to be less tolerant to ROS generated by a susceptible plant and formed significantly smaller lesions on both barley and rice (35). However, the study did not establish whether these effects were due solely to the antioxidant activity of this protein or because of a defect in redox signaling.

To date, there have been no reports on the role of AP-1-like transcription factors in fungus-plant symbiotic interactions, such as the mutualistic symbiotic interaction between the fungus *Epichloë festucae* (Ascomycota, Clavicipitaceae) and the plant *Lolium perenne* (36, 37). This biotrophic fungus systemically colonizes the vegetative and reproductive aerial tissues but not the roots of the plant. The growth of this fungus within the leaves is tightly regulated, with usually just a single hypha found between adjacent columns of plant cells. Hyphae that colonize the leaves are firmly attached to the plant cells and extend by intercalary division and extension, a mechanism of growth that synchronizes hyphal growth with plant leaf growth (38). Using both forward and reverse genetic strategies, we previously identified genes crucial for establishment and maintenance of this symbiosis, including genes encoding components of the NADPH oxidase and mitogen-activated protein kinase (MAPK) signaling complexes (15, 39–42).

The aim of this study was to test whether YapA-mediated signaling is required for maintenance of a mutualistic symbiotic interaction between *E. festucae* and *L. perenne* and to determine whether *E. festucae* GpxC and TpxA, which are homologs of Gpx3 and Tpx1, respectively, are redox sensors of H_2O_2 stress resulting in nuclear retention and activation of *E. festucae* YapA.

MATERIALS AND METHODS

Strains and growth conditions. *Escherichia coli* cultures (see Table S1 in the supplemental material) were grown overnight in Luria-Bertani (LB) broth or on LB agar containing 100 μg/ml ampicillin as previously described (43).

E. festucae cultures (see Table S1 in the supplemental material) were grown on 2.4% (wt/vol) potato dextrose (PD) agar (44) under previously described conditions (45, 46). Liquid cultures were grown for 5 days at 22°C in 250-ml conical flasks containing 50 ml medium on an orbital shaker at 200 rpm. For collection of spores, *E. festucae* cultures were grown on PD agar for 10 days at 22°C. The spores were vigorously washed from the surface of the colony with PD broth, and the spore suspension was filtered through sterile glass wool.

S. cerevisiae cultures for complementation (see Table S1 in the supplemental material) were grown on synthetic complete (SC) medium (0.67% yeast nitrogen base without amino acids, 2% galactose, 1% raffinose, 0.01% [each] leucine, tryptophan, and uracil, 0.005% [each] histidine and methionine, and 2% agar) at 30°C for 3 days. For yeast recombinational cloning, strain FY834 was grown on YPD (1% yeast extract, 2% peptone, 2% D-glucose, 2% agar), and transformants were selected on synthetic dropout (SD) medium (1 M sorbitol, 0.67% yeast nitrogen base without amino acids, 0.08% uracil dropout supplement [Clontech], 2% glucose, and 2% agar).

DNA isolation, PCR, and sequencing. Fungal genomic DNA was extracted from freeze-dried mycelium by the method of Byrd et al. (47). For small-scale experiments, rapid extracts of genomic DNA mycelia were grown in PD broth for 1 to 3 days, transferred to lysis buffer (150 mM EDTA, 50 mM Tris-HCl, 1% sodium lauroyl sarcosine), and incubated at 70°C for 30 min. DNA was isolated from the aqueous phase by sequential precipitations with 5 M potassium acetate, isopropanol, and 70% ethanol and then was resuspended in 20 μl H_2O. Isolation of plasmid and cosmid DNAs was performed by alkaline lysis and extraction using a High Pure plasmid isolation kit (Roche) according to the manufacturer's instructions.

Standard PCR amplification was performed with *Taq* DNA polymerase (Roche). Where proofreading activity was required, the Expand High Fidelity PCR system (Roche) was used. Each reaction mixture (50 μl) contained 1× *Taq* reaction buffer or 1× Expand High Fidelity buffer, 0.2 μM (each) forward and reverse primers, a 200 μM concentration of each deoxynucleoside triphosphate (dNTP), 1 ng template DNA, and 1 U of *Taq* polymerase or 1.75 U of Expand High Fidelity enzyme mix (Roche). The following cycle conditions were used: 1 cycle of 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 45 to 68°C for 30 s, and 72°C for 1 min per kb of sequence. The final extension consisted of one cycle at 72°C for 10 min, and then the tubes were stored at 4°C until analysis. The sequences of PCR primers are provided in Table S2 in the supplemental material.

DNA fragments were sequenced using the dideoxynucleotide chain termination method with a Big Dye Terminator ready reaction cycle sequencing kit, version 3.1 (Applied Biosystems), and were separated using an ABI3730 genetic analyzer (Applied Biosystems). Sequence data were assembled and analyzed using MacVector sequence assembly software, version 12.0.5.

DNA hybridizations. *E. festucae* genomic digests were transferred to positively charged nylon/nitrocellulose membranes (Roche) by the

method described by Southern (48). DNA was fixed by UV light cross-linking in a Cex-800 UV light cross-linker (Ultra-Lum) at 254 nm for 2 min. The DNA probes were synthesized by random priming with Klenow DNA polymerase and [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham Biosciences), using a High Prime DNA labeling kit (Roche), or by the incorporation of digoxigenin-11-dUTP (DIG-11-dUTP) into DNA by PCR, using a PCR DIG probe synthesis kit (Roche). Hybridizations were performed according to the manufacturer's instructions and visualized by either autoradiography (^{32}P-labeled probes) or nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) color detection (DIG-labeled probes).

Cosmid clone 28E7 (*yapA*) was isolated from an Fl1 cosmid library (49) by colony hybridization as previously described (39), using a 1.7-kb fragment of *yapA*, amplified using the Expand High Fidelity PCR system and the yap5-yap6 primer pair, as a probe. The probe was labeled with [α-^{32}P]dCTP as described above.

Preparation of complementation, deletion, and expression constructs. The *S. cerevisiae YAP1* (pGC8) and *GPX3* (pGC7) genomic complementation constructs were prepared by PCR amplification of the 1.95-kb *YAP1* gene and the 0.5-kb *GPX3* gene, using the yap7-yap8 and gpx7-gpx8 primers, respectively, with the Expand High Fidelity PCR system. The *E. festucae yapA* (pGC6) and *gpxC* (pGC5) cDNA complementation constructs were prepared by PCR amplification of the 1.7-kb *yapA* cDNA and 0.5-kb *gpxC* cDNA from *E. festucae* total mRNA, using the yap5-yap6 and gpx5-gpx6 primer pairs, respectively. These fragments were then ligated into pCR4-TOPO (Invitrogen), sequenced, and subcloned into the pYES2 vector (Invitrogen).

The *yapA* replacement construct (pGC2) was prepared by sequentially ligating into pSF15.15 (a pII99-based vector containing the *hph* cassette) a 1.1-kb BglII/KpnI fragment 5′ of *yapA*, generated by PCR amplification using the yap1-yap2 primer pair, and a 1.2-kb HindIII/XhoI fragment 3′ of *yapA*, generated by PCR amplification using the yap3-yap4 primer pair, as shown in Fig. S1 in the supplemental material.

The *gpxC* replacement construct, plasmid pGC4, was prepared by sequentially ligating into pSF17.8 (a pII99-based vector containing the *nptII* cassette) a 1.1-kb BglII/KpnI fragment 5′ of *gpxC*, generated by PCR amplification using the gpx1-gpx2 primer pair, and a 1.4-kb SalI/SalI fragment 3′ of *gpxC*, generated by PCR amplification using the gpx3-gpx4 primer pair, as shown in Fig. S1 in the supplemental material.

The *tpxA* replacement construct, plasmid pGC12, was prepared by sequentially ligating into pSF15.15 a 2.3-kb BglII/KpnI fragment 5′ of *tpxA*, generated by PCR amplification using the tpx1-tpx2 primer pair, and a 2.45-kb fragment 3′ of *tpxA*, generated by PCR amplification using the tpx3-tpx4 primer pair, as shown in Fig. S1 in the supplemental material.

The 3.8-, 4.3-, and 6.2-kb linear products of pGC2 (*yapA*), pGC4 (*gpxC*), and pGC12 (*tpxA*) used for transformation were amplified with the yap1-yap4, gpx1-gpx4, and tpx1-tpx4 primer pairs, respectively, using the Expand High Fidelity PCR system (Roche) according to the manufacturer's instructions. A 3.7-kb EcoRV/EcoRI fragment extending 740 bp upstream from the transcription start site of *yapA* and 639 bp downstream from the transcription termination site was digested from cosmid 28E7 and subcloned into pSF17.8 to generate the pGC11 *yapA* complementation vector.

A YapA-EGFP fusion construct, pGC9, was prepared by cloning an EcoRI/ClaI fragment generated with the yap27-yap28 primer pair into the pPN94 vector. The translational stop codon of *yapA* was removed and replaced by the enhanced green fluorescent protein (EGFP) coding region, with its stop codon generated with the GCGFP1-GCGFP2 primer pair, creating a C-terminal in-frame fusion with the *yapA* gene.

A Yap1-EGFP fusion construct, pGC19, was prepared by amplifying overlapping Yap1 and EGFP fragments, using the scyap16-scyap18 and GCGFP3-GCGFP4 primer pairs, respectively, recombining them in yeast, and cloning the EcoRI/NotI Yap1-EGFP fragment into pPN94.

A P*catA-eGFP* reporter construct, pGC13, was prepared by sequentially ligating into pSF17.8 an XbaI/EcoRI fragment containing the 1-kb region upstream of the *catA* start codon and a 0.7-kb XbaI/EcoRI fragment containing the EGFP coding sequence. The P*catA* fragment was generated by PCR using the pcatA3-pcatA4 primer pair and ligation into pGC10, and the P*catA-eGFP* XbaI/XhoI fragment was subcloned into pSF17.8. A destabilized version of the P*catA-eGFP* reporter construct, P*catA-eGFP-CL1* (pGC14), was generated by adding a 16-amino-acid degron sequence (CL1 [ACKNWFSSLSHFVIHL]) to the C terminus of EGFP. Two-step PCR using the pCatA-CL1 R1 and pCatA-CL1 R2 primer pairs was used to generate the 1.2-kb SacII/NdeI fragment, which was then used to replace the corresponding region in the original P*catA-eGFP* vector.

Fungal transformation. *S. cerevisiae* transformation was carried out using the lithium acetate/single-stranded carrier DNA/polyethylene glycol method (50). *E. festucae* protoplasts were prepared as previously described (51). Protoplasts were transformed with 3 to 5 μg of linear PCR-amplified or circular plasmid DNA by a previously described method (52). Transformants were selected on RG medium (regeneration medium, which is PD medium with 0.8 M sucrose) containing 150 μg/ml hygromycin or 200 μg/ml Geneticin and nuclear purified by three rounds of subculturing on hygromycin- or Geneticin- containing PD medium (51).

Putative *yapA* replacement mutants were screened by PCR using primers that flank the *hph* cassette (yap22 and yap23; 1 kb of wild-type sequence and 1.6 kb of replacement sequence) and the 5′ (yap9 and yap10; 2.6 kb)- and 3′ (yap11 and yap21; 2.4 kb)-flanking regions of the replacement. Putative *gpxC* replacement mutants were screened by PCR using primers that flank the *nptII* cassette (gpx15 and gpx16; 1 kb of wild-type sequence and 1.9 kb of replacement sequence) and the 5′ (gpx9 and gpx10; 2.3 kb)- and 3′ (gpx11 and gpx12; 2.9 kb)-flanking regions of the replacement. Putative *tpxA* replacement mutants were screened by PCR using primers that flank the *hph* cassette (tpx13 and tpx14; 1 kb of wild-type sequence and 1.6 kb of replacement sequence) and the 5′ (tpx15 and tpx16; 2.5 kb)- and 3′ (tpx17 and tpx18; 2.5 kb)-flanking regions of the replacement. For complementation of Δ*yapA* mutants, Δ*yapA* protoplasts were transformed with pGC11 and transformants selected on medium containing 200 μg/ml Geneticin. Reintroduction of the *yapA* gene was confirmed by PCR using the yap5-yap6 primer pair.

Transformants expressing EGFP or DsRed fusion proteins were selected using an Olympus BX51 stereomicroscope with Olympus U-MWIBA2 and U-MWIG2 filters to detect EGFP and DsRed, respectively.

Microscopy. Cultures to be analyzed by microscopy were inoculated onto a thin layer of PD agarose (2% [wt/vol]) layered on top of the base layer of PD agar (1.5% [wt/vol]) and grown for 5 days. For examination of *E. festucae* spores, spore suspensions were spread onto the PD agarose and examined after 16 h. Square blocks were cut from the agarose and placed in an imaging chamber (CoverWell; 20-mm diameter by 0.5-mm depth) (Molecular Probes) filled with 500 μl of PD broth and sealed with a 22- by 60-mm glass coverslip.

Localization of YapA-EGFP and DsRed-StuA(NLS) in hyphae was analyzed by confocal laser scanning microscopy using a Leica SP5 DM6000B confocal microscope (488-nm argon laser, ×40 oil immersion objective, numerical aperture [NA] = 1.3). Differential interference contrast (DIC) imaging was used in conjunction with confocal microscopy, with the DIC image overlaid on the confocal fluorescence images.

Bioinformatic analysis. *E. festucae* genes were identified by tBLASTn analysis of the *E. festucae* Fl1 (E894) genome (http://csbio-l.csr.uky.edu/ef894-2011) with *S. cerevisiae* and *S. pombe* protein sequences obtained from the SGC (http://www.yeastgenome.org/) and GeneDB (http://old.genedb.org/genedb/pombe/) databases, respectively. *C. albicans* and *Yarrowia lipolytica* protein sequences were obtained from the Candida (http://www.candidagenome.org/) and Genolevures (http://www.genolevures.org/yali.html) genome databases.

Other fungal sequences used in the phylogenetic analysis were retrieved

from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/) or the Broad Institute website (http://www.broad.mit.edu). Identity and similarity scores were calculated after ClustalW pairwise alignments of sequences by use of MacVector, version 12.0.5. Multiple-sequence alignments were edited using Jalview (http://www.jalview.org/). For phylogenetic relationships, Mega5.05 was used to generate maximum likelihood trees from ClustalW multiple-sequence alignments, using the default parameters in the program. Gene annotation and naming are given in accordance with the Broad Institute *Aspergillus* Comparative Database. The *E. festucae* genome sequence data, curated by C. L. Schardl at the University of Kentucky, are available at http://csbio-l.csr.uky.edu/ef894-2011/.

Nucleotide sequence accession numbers. The *yapA*, *gpxC*, and *tpxA* gene sequences of *E. festucae* strain Fl1 (894) are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html) under accession numbers KC121577, KC121578, and KC244374, respectively.

RESULTS

Identification and characterization of an AP-1-like transcription factor. A tBLASTn analysis of the *E. festucae* genome with the *S. cerevisiae* Yap1 protein sequence identified a gene, designated *yapA* (accession number KC121577; *E. festucae* Fl1 gene model EfM2.092760), encoding a bZIP transcription factor that shares 57% identity with FgAP1 from *Fusarium graminearum* and 12 to 16% identity with AP-1 proteins from the more distantly related basidiomycete fungus *Ustilago maydis* and the yeast species *S. cerevisiae*, *Kluyveromyces lactis*, *S. pombe*, and *C. albicans*. The *yapA* coding sequence, comprising three exons separated by 72- and 64-bp introns, is predicted to encode a polypeptide of 580 amino acids that shares several conserved features with *S. cerevisiae* Yap1, including an NLS, an NES, a bZIP domain, and two cysteine-rich domains, n-CRD and c-CRD, that are distal and proximal, respectively, to the N and C termini of the protein (Fig. 1A). Both the bZIP and c-CRD motifs are highly conserved among all of the fungal AP-1 transcription factors analyzed (Fig. 1B and D). A multiple-sequence alignment of the n-CRDs from AP-1 homologs of various yeasts and filamentous fungi identified a major difference in the amino acid sequence of this domain between yeast and filamentous fungal species (Fig. 1C). While there are two conserved cysteine residues in the n-CRDs of yeast species, just one of these (corresponding to residue 310 in *S. cerevisiae*) is conserved in the filamentous fungi. The two cysteine residues in the c-CRD of the *S. cerevisiae* protein that form disulfide bonds with partners in the n-CRD upon oxidation are conserved in the filamentous fungi.

Complementation of a yeast *yap1* mutant. To test whether YapA is a functional homolog of *S. cerevisiae* Yap1, a full-length cDNA encoding *E. festucae* YapA was cloned into the pYES2 yeast expression vector and transformed into the *S. cerevisiae* Δ*yap1* strain. In addition, the Δ*yap1* strain was transformed with pYES2 vector alone or pYES2 vector containing *S. cerevisiae YAP1* (Fig. 2). Cells were grown on medium containing galactose and raffinose to activate the *GAL1* promoter in pYES2, which was used to control expression of these genes in *S. cerevisiae*. All strains grew equally well on medium to which no H_2O_2 had been added. On medium containing 0.8 mM hydrogen peroxide, growth of the Δ*yap1* strain and the Δ*yap1*/pYES2 strain was significantly inhibited in comparison to that of the wild-type strain. Expression of *E. festucae* YapA (Δ*yap1*/Ef*yapA*) or *S. cerevisiae* Yap1 (Δ*yap1*/Sc*YAP1*) in the *S. cerevisiae* Δ*yap1* background was able to restore growth of this mutant on H_2O_2 to levels comparable to those of the wild type (Fig. 2). These results demonstrate that *E. festucae yapA* is able to functionally complement the oxidative stress sensitivity defect of the *S. cerevisiae* Δ*yap1* mutant. However, it is evident from the number of colonies formed that complementation by *yapA* from *E. festucae* is less efficient than that achieved by *S. cerevisiae YAP1* (Fig. 2).

Oxidative stress phenotype of mycelia from *E. festucae yapA* deletion strains. To investigate the role of the *E. festucae* YapA protein in conferring resistance to various oxidative stress-inducing compounds, *yapA* was deleted by targeted gene replacement (see Fig. S1 in the supplemental material). Two independent knockouts of *yapA* were generated, with each containing multiple copies of the deletion construct. Mycelial plugs of the wild-type and Δ*yapA* strains were inoculated onto PD agar supplemented with various stress-inducing compounds, including the thiol-oxidizing agents menadione and diamide as well as the peroxides H_2O_2 and *t*-BOOH and superoxide-generating KO_2 (Fig. 3; see Fig. S2 in the supplemental material). Colony radial growth of two independently isolated Δ*yapA* mutants was severely reduced on 40 μM menadione and reduced to a lesser extent on 1 mM diamide in comparison to that of the wild type. Introduction of a wild-type allele of *yapA* into each of the Δ*yapA* strains restored growth on menadione and diamide to wild-type levels, confirming that loss of functional YapA confers sensitivity to these oxidizing agents. In contrast, addition of H_2O_2, *t*-BOOH, or KO_2 had no effect on colony radial growth of Δ*yapA* strains (Fig. 3). At higher concentrations of these compounds, wild-type growth was reduced to the same extent as that of mutants (see Fig. S2 in the supplemental material). The lack of sensitivity of vegetative tissue to H_2O_2 was surprising given that, to date, all published studies of fungal AP-1 mutants report sensitivity to H_2O_2.

Conidial germination sensitivity of *E. festucae* Δ*yapA* mutants to H_2O_2. To investigate the possibility of a developmental stage-specific role for YapA in providing resistance against peroxides, *E. festucae* spores were spread onto PD medium containing H_2O_2. Spores derived from the wild-type strain were able to germinate on PD medium containing 0.5 mM H_2O_2, whereas spores of the Δ*yapA* strain were highly sensitive to H_2O_2 and were unable to germinate, as confirmed by light microscopy (Fig. 4). Reintroduction of *yapA* to the Δ*yapA* mutant restored H_2O_2 resistance of the spores back to wild-type levels. These results suggest that YapA is indispensable for conidial resistance to peroxide stress.

Expression of *catA* in the Δ*yapA* mutant. To explain the elevated sensitivity of Δ*yapA* spore germination to H_2O_2, we tested whether YapA was required for activation of a spore-specific catalase, an enzyme responsible for H_2O_2 detoxification. A homolog of the *Aspergillus nidulans* spore-specific catalase gene, *catA* (53), was identified in *E. festucae* (EfM2.069120). The predicted protein shares 74.1% similarity with *A. nidulans* CatA. The promoter of *E. festucae catA* was fused to the EGFP gene to monitor its expression. EGFP fluorescence was detected in wild-type spores but not vegetative mycelia, confirming that expression of *E. festucae catA* is spore specific (Fig. 5A). To enhance the sensitivity of the spore expression analysis, a second construct was prepared, in which the CL1 degron sequence was fused to the C terminus of the *catA*-EGFP construct. Addition of the CL1 degron sequence, specific for ubiquitination and degradation by the proteasome, is reported to confer a 20- to 30-min half-life on EGFP (54). Spores of the Δ*yapA* mutant strains showed a significant reduction ($P \ll 0.001$) in EGFP fluorescence, indicating reduced *catA* promoter activity

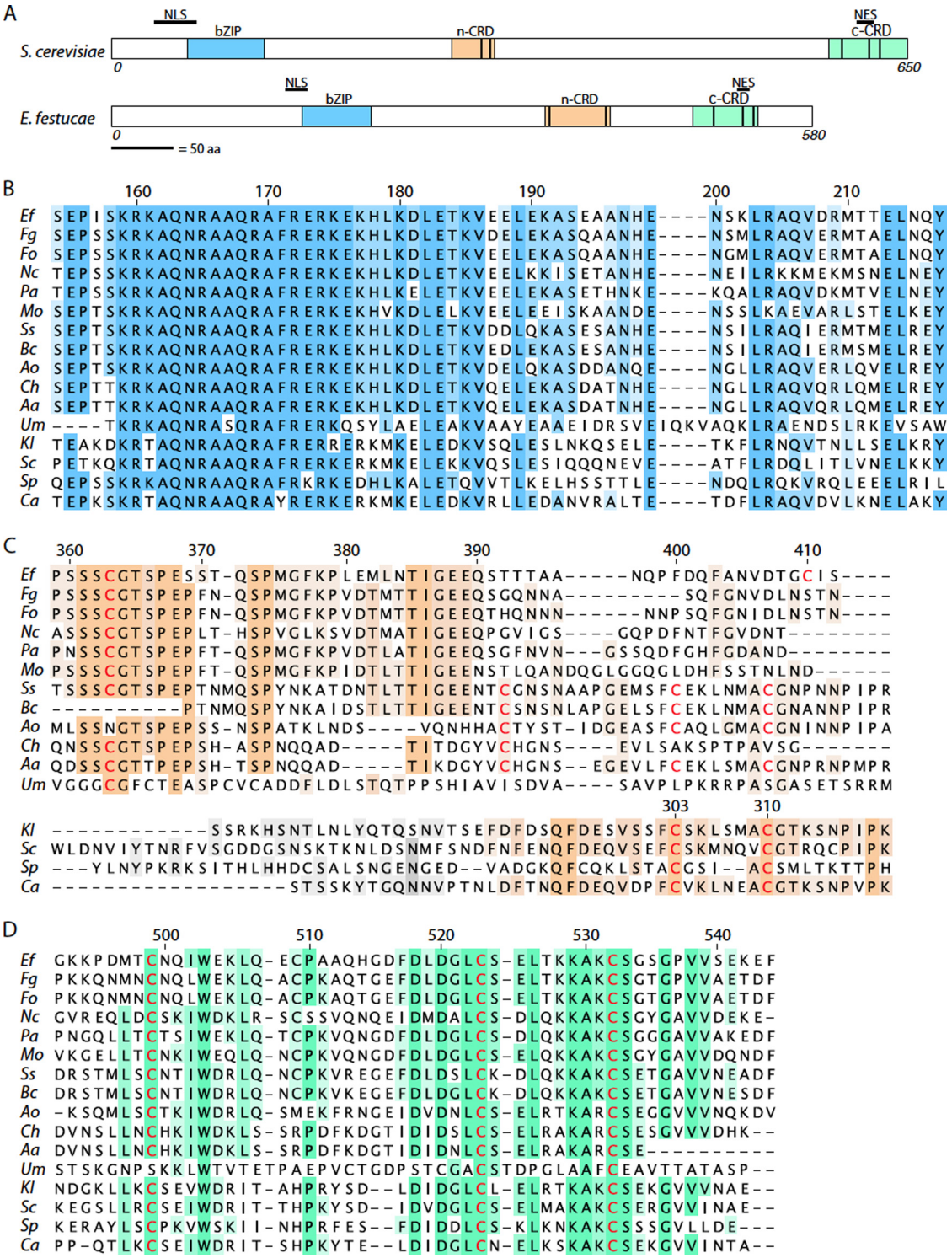


FIG 1 Functional domains of *E. festucae* YapA. (A) Characteristic motifs of *S. cerevisiae* Yap1 and *E. festucae* YapA proteins. bZIP, basic leucine zipper DNA binding domain; n-CRD and c-CRD, N- and C-terminal cysteine-rich domains; NLS, nuclear localization signal; NES, nuclear export sequence. The length of each protein (in amino acids [aa]) is indicated. The vertical black bars within the proteins identify positions of conserved Cys residues. (B to D) Multiple-sequence alignments of bZIP domains, n-CRDs, and c-CRDs of fungal AP-1 proteins. Conserved cysteine residues are highlighted in red. Numbering indicates amino acid residue positions with respect to the *E. festucae* protein (bZIP [B], n-CRD [upper part of panel C], and c-CRD [D] sequences) or the *S. cerevisiae* protein (n-CRD [lower part of panel C] sequences). *Ef*, *Epichloë festucae* YapA (EfM2.092760; accession no. KC121577); *Fg*, *Fusarium graminearum* FGSG_08800.3 (accession no. XP_388976.1); *Fo*, *Fusarium oxysporum* FoAP1 (accession no. EGU84635.1); *Nc*, *Neurospora crassa* NcAp-1 (NCU03905.5; accession no. XP_957544.2); *Pa*, *Podospora anserina* PaAP1 (accession no. XP_001905945.1); *Mo*, *Magnaporthe oryzae* MoAP1 (MGG_12814.7; accession no. XP_001408783.1); *Ss*, *Sclerotinia sclerotiorum* SSAP1 (SS1G_09561.3; accession no. XP_001589839.1); *Bc*, *Botrytis cinerea* Bap1 (BC1G_14094.1; accession no. XP_001547321.1); *Ao*, *Aspergillus oryzae* AorAP1 (AO090001000627; accession no. XP_001819128.1); *Ch*, *Cochliobolus heterostrophus* CHAP1 (accession no. AAS64313); *Aa*, *Alternaria alternata* AaAP1 (accession no. ACM50933.1); *Um*, *Ustilago maydis* Yap1 (UM02191.1; accession no. XP_758338.1); *Kl*, *Kluyveromyces lactis* KlYap1 (accession no. XP_451077.1); *Sc*, *Saccharomyces cerevisiae* Yap1 (YML007W; accession no. CAA41536.1); *Sp*, *Schizosaccharomyces pombe* Pap1 (SPAC1783.07c; accession no. NP_593662.1); *Ca*, *Candida albicans* Cap1 (CaO19.1623; accession no. XP_721702.1).

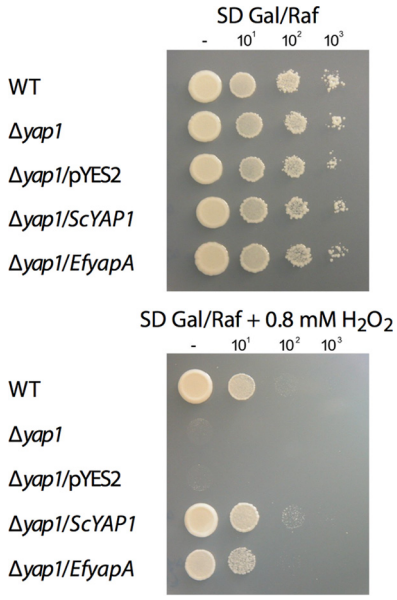


FIG 2 *S. cerevisiae* complementation by *E. festucae yapA*. The growth of *S. cerevisiae* BY4741 (PN2735) (wild type [WT]), *S. cerevisiae* BY4741-ΔYML007W (Δ*yap1*; PN2736), and derivatives of this strain transformed with the empty vector pYES2 (PN2847), pYES2*ScYAP1* (PN2845), or pYES2*EfyapA* (PN2846) was tested on SD plates containing galactose and raffinose (SD Gal/Raf) with and without 0.8 mM H_2O_2. Serial 10-fold dilutions of the cultures indicated on the left were spotted onto plates.

compared to that in wild-type spores (Fig. 5B). EGFP fluorescence was restored in the *yapA*-complemented Δ*yapA* strains, indicating that the reduction in EGFP reporter gene expression was due to a lack of *yapA* expression. This result suggests that YapA is required for activation of *catA* expression and that the sensitivity of Δ*yapA* conidial germination to H_2O_2 may be due to decreased expression of *catA*.

Activation of YapA by H_2O_2 in axenic culture. In *S. cerevisiae*, oxidation of the Yap1 protein by H_2O_2 correlates with its activation and nuclear accumulation. To monitor the cellular localization and inferred activation of H_2O_2-oxidized YapA, protoplasts of the wild type were transformed with a YapA-EGFP fusion construct, pGC9, together with a DsRed-StuA(NLS) construct, pJW19, to confirm nuclear localization. In the absence of oxidative stress, YapA-EGFP localized to the cytoplasm, but upon treatment with H_2O_2, YapA-EGFP was redistributed to the nucleus, where it colocalized with the nuclear marker DsRed-StuA(NLS) (Fig. 6).

Given that *S. cerevisiae* Gpx3 (glutathione peroxidase 3) and *S. pombe* Tpx1 (thioredoxin peroxidase 1) are responsible for the initial sensing of the H_2O_2 signal and activation of their respective AP-1 transcription factors (21, 24, 25), we tested whether homologs of these thiol peroxidases (peroxiredoxins) were required for activation of *E. festucae* YapA under conditions of oxidative stress. Homologs of *S. cerevisiae GPX3* and *S. pombe TPX1* in the *E. festucae* genome were identified by tBLASTn and reciprocal BLASTp analyses and designated *gpxC* (KC121578; EfM2.018640) and *tpxA* (KC244374; EfM2.113210), respectively. Multiple-sequence alignment and phylogenetic analyses confirmed that

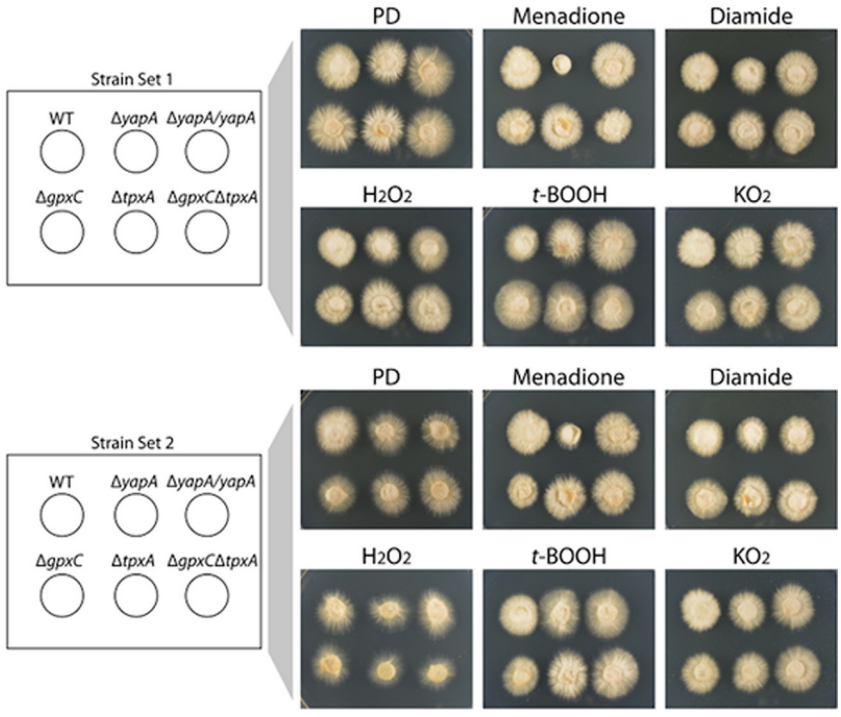


FIG 3 Oxidative stress sensitivity of *E. festucae* deletion strains. Five-millimeter-diameter agar plugs of the indicated strains were inoculated onto PD medium and PD medium containing 40 μM menadione, 1 mM diamide, 4 mM H_2O_2, 0.25 mM *t*-BOOH, or 7 mM KO_2 and cultured at 22°C for 7 days. (A) Strain set 1 included the wild-type (WT; PN2278), Δ*yapA*#145 (PN2740), Δ*yapA*#145/*yapA* (PN2788), Δ*gpxC*#10 (PN2741), Δ*tpxA*#105 (PN2821), and Δ*gpxC* Δ*tpxA*#22 (PN2831) strains. (B) Strain set 2 included the wild-type (WT), Δ*yapA*#243 (PN2739), Δ*yapA*#243/*yapA* (PN2787), Δ*gpxC*#34 (PN2742), Δ*tpxA*#157 (PN2822), and Δ*gpxC* Δ*tpxA*#168 (PN2830) strains (numbers in strain names indicate independent transformants).

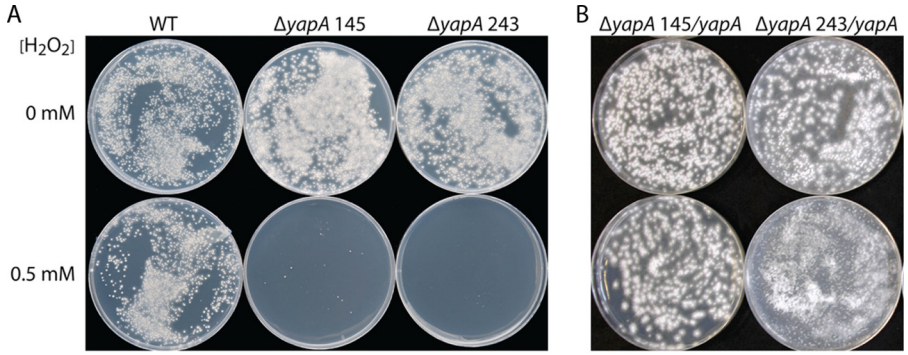


FIG 4 H_2O_2 sensitivity of *E. festucae* Δ*yapA* conidia. Spore suspensions (250 μl) of wild-type (WT), Δ*yapA*#145, and Δ*yapA*#243 strains (A) and complemented Δ*yapA*#145/*yapA* and Δ*yapA*#243/*yapA* strains (B) were spread over the surfaces of plates of PD medium and PD medium containing H_2O_2, and the spores were allowed to germinate at 22°C for 12 and 11 days, respectively.

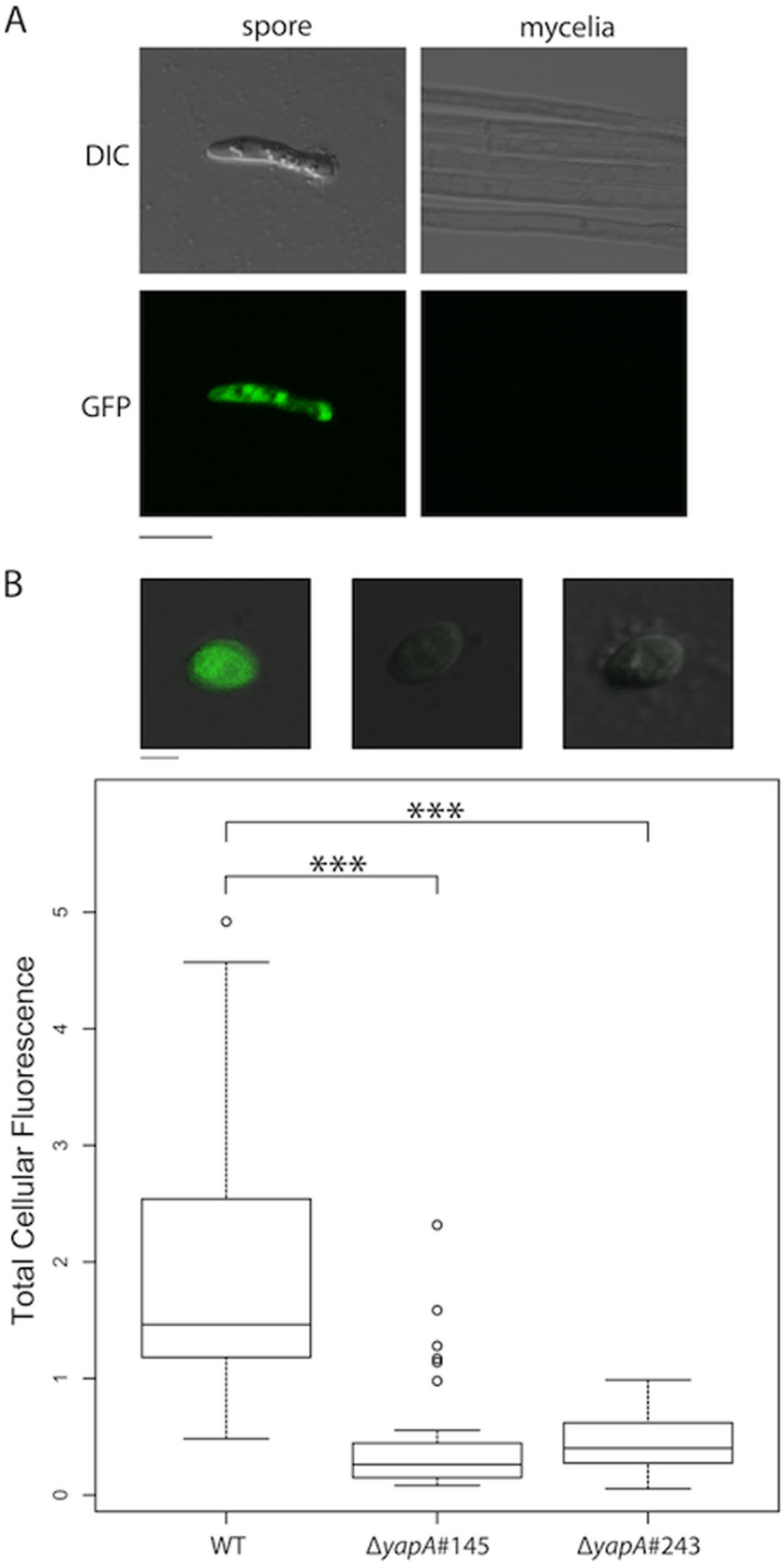
GpxC and TpxA are the most closely related proteins to *S. cerevisiae* Gpx3 and *S. pombe* Tpx1, respectively (see Fig. S3 and S4 in the supplemental material). However, in the case of the latter, filamentous fungal proteins are more closely related to *S. cerevisiae* Prx1, a 1-Cys peroxiredoxin, whereas *S. pombe* Tpx1 groups with *S. cerevisiae* Tsa1 and Tsa2, which are typical 2-Cys peroxiredoxins (see Fig. S4 in the supplemental material). Typical 2-Cys peroxiredoxins have both peroxidatic (C48 in *S. pombe*) and resolving (C169 in *S. pombe*) Cys residues and, in addition, contain the signature consensus sequence FTFVCPTEI for the first Cys (see Fig. S4 in the supplemental material). In contrast, the Tpxs of filamentous fungi have a single cysteine residue that forms part of the signature consensus sequence FTPVCTTEL. Two additional peroxiredoxins were identified in filamentous fungal genomes, but these appear to be homologs of *S. pombe* Pmp20 (EfM2.064230) and Bcp (EfM2.115510). These are atypical 2-Cys peroxiredoxins, indicating that typical 2-Cys peroxiredoxins appear to be absent from filamentous fungi.

Given that there are three different but closely related Gpxs in *S. cerevisiae*, i.e., Gpx1, Gpx2, and Gpx3, cDNA of *E. festucae gpxC* was expressed in an *S. cerevisiae* Δ*gpx3* strain to test if it could complement this mutant under conditions of oxidative stress. All strains grew equally well on medium to which no H_2O_2 had been added. On medium containing 1.25 mM hydrogen peroxide, growth of the Δ*gpx3* strain and the Δ*gpx3*/pYES2 strain was significantly inhibited in comparison to that of the wild-type strain. Expression of *E. festucae* GpxC (Δ*gpx3*/Ef*gpxC*) or *S. cerevisiae* Gpx3 (Δ*gpx3*/Sc*GPX3*) in the *S. cerevisiae* Δ*gpx3* background was able to restore growth of this mutant on H_2O_2 to levels comparable to those of the wild type (Fig. 7). The slightly higher concentration of H_2O_2 used for these experiments (1.25 mM) than for the *YAP1* complementation tests (0.8 mM) (Fig. 2) reflects the slightly greater tolerance of the Δ*gpx3* strain to oxidative stress, because of an alternative pathway for Yap1 activation (21). These results demonstrate that *E. festucae gpxC* is able to functionally complement the oxidative stress sensitivity defect of the *S. cerevisiae GPX3* mutant.

To test whether either GpxC or TpxA serves as a redox sensor for YapA activation in *E. festucae*, single (Δ*gpxC* or Δ*tpxA*) and double (Δ*gpxC* Δ*tpxA*) mutants of *gpxC* and *tpxA* were generated, and localization of YapA-EGFP was monitored in hyphae of these different mutant backgrounds (Fig. 6; see Fig. S1 in the supplemental material). In the Δ*gpxC*, Δ*tpxA*, and Δ*gpxC* Δ*tpxA* backgrounds, YapA-EGFP still translocated from the cytoplasm to the nucleus in response to H_2O_2 treatment, where it colocalized with the nuclear marker DsRed-StuA(NLS). These results demonstrate that neither GpxC nor TpxA is essential for YapA nuclear localization and that these proteins do not have overlapping functions (Fig. 6).

To assess whether *E. festucae* possesses a redox sensor capable of activating *S. cerevisiae* Yap1, a Yap1-EGFP fusion construct (pGC19) was expressed in *E. festucae* wild-type cells. However, this fusion failed to localize to the nucleus when cells were treated with H_2O_2 or diamide (Fig. 8). In a control experiment, the same Yap1-EGFP construct under the control of the *S. cerevisiae GAL1* promoter localized to the nuclei of *S. cerevisiae* wild-type and Δ*yap1* cells, but not Δ*gpx3* cells, in response to treatment of the cells with H_2O_2 (21) (see Fig. S5 in the supplemental material), demonstrating the functional fidelity of the construct. These results suggest that either *E. festucae* lacks an H_2O_2 redox sensor or the sensor is unable to interact with the Yap1-EGFP fusion.

Plant phenotypes of *E. festucae yapA*, *gpxC*, and *tpxA* mutants. To test whether disruption of *yapA*, *gpxC*, *tpxA*, or both *gpxC* and *tpxA* would affect the symbiotic interaction phenotype of *E. festucae* with *L. perenne*, seedlings were inoculated with each of these mutants, and whole-plant and *in planta* phenotypes were analyzed at 8 weeks postinfection. All mutants had a wild-type interaction phenotype at the whole-plant level (see Fig. S6 in the supplemental material) and subcellular level (see Fig. S7 in the supplemental material). Hyphae of all mutants were aligned parallel to the axes of the leaves and were similar to the wild type in morphology and growth habit. Given that inoculation of the plants required wounding of the seedlings by making a 2- to 3-mm longitudinal slit with a scalpel above the meristem of the hypocotyl, followed by placement of the inoculum on the wound site (55), we tested whether YapA was activated in the wild-type strain in response to the intense burst of ROS (H_2O_2) generated by wounding (56, 57). Consistent with the rapid generation of H_2O_2 in response to wounding, YapA-EGFP localized to the nuclei of wild-type hyphae within 30 min of inoculation (Fig. 9). After a period of about 6 h, YapA-EGFP was found predominantly in the cytoplasm, consistent with a return to homeostasis and recovery of the cells from the oxidative burst. This burst of ROS was insufficient to disrupt infection by the wild-type and Δ*yapA* mutant strains, despite the latter lacking a functional YapA oxidative stress signal-


A
spore
mycelia
DIC
GFP
B
***
***
Total Cellular Fluorescence
0
1
2
3
4
5
WT
ΔyapA#145
ΔyapA#243

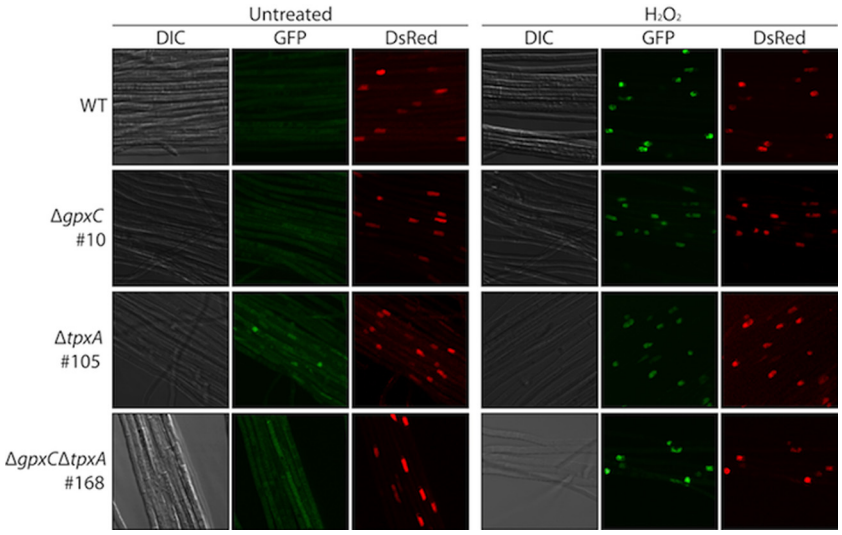


FIG 6 H_2O_2 activation of YapA-EGFP in axenic culture. *E. festucae* wild-type (WT; PN2790), Δ*gpxC*#10 (PN2789), Δ*tpxA*#105 (PN2823), and Δ*gpxC* Δ*tpxA*#168 (PN2851) cultures expressing YapA-EGFP and the nuclear marker DsRed-StuA(NLS) were examined using confocal laser scanning and DIC microscopy before (untreated) and 30 min after treatment with 16 mM H_2O_2. EGFP images were generated by maximum-intensity projection of confocal *z* stacks taken at 1-μm intervals from the top to the bottom of the section. Each box represents a 50-μm by 50-μm area.

ing pathway. These results suggest that *E. festucae* has a highly redundant system for protection from oxidative stress.

DISCUSSION

The results described here demonstrate that *E. festucae* YapA is a *bona fide* AP-1-like bZIP transcription factor involved in the oxidative stress response. *E. festucae* YapA functionally complemented the H_2O_2 stress sensitivity defect of an *S. cerevisiae* Δ*yap1* mutant and translocated to the nuclei of *E. festucae* cells in response to H_2O_2, properties that demonstrate that YapA is capable of activating genes required for an H_2O_2-induced oxidative stress response in *S. cerevisiae*. However, unlike other filamentous fungi (8, 32, 33, 58), the *E. festucae* Δ*yapA* mutants did not show increased hyphal sensitivity to H_2O_2 compared to the wild type. Hyphae of the *E. festucae* Δ*yapA* mutants were also resistant to KO_2 and *t*-BOOH but very sensitive to menadione and slightly sensitive to diamide. Thus, *E. festucae* is the exception among the filamentous fungi and yeast studied to date in that its AP-1-like transcription factor is not essential for hyphal resistance to oxidative stress induced by hydroperoxides. One possible explanation is the presence of functionally overlapping signaling pathways in *E. festucae* to protect against H_2O_2-induced oxidative stress.

The other well-studied pathway involved in fungal oxidative stress signaling is the Spc1/SakA (*S. pombe*/*A. nidulans*) MAPK pathway (59, 60). In this pathway, a multistep phosphorelay sensor-responder system transmits the H_2O_2 oxidative stress signal from the response regulator Mcs4/SskA to the Spc1/SakA MAPK, which phosphorylates and activates the bZIP transcription factor, Atf1/AtfA. Disruption of *spc1* and *atf1* in *S. pombe* (61, 62) or their homologs, *sakA* and *atfA*, in *A. nidulans* (58, 63, 64) leads to increased sensitivity to oxidative (H_2O_2) stress. Once again, *E. festucae* appears to be the exception among the filamentous fungi studied to date.

In contrast to the lack of sensitivity of vegetative mycelia to oxidative stress, conidia of the *E. festucae* Δ*yapA* mutant were very sensitive to H_2O_2 oxidative stress and failed to germinate. Introduction of the wild-type *yapA* gene into the mutant restored H_2O_2 tolerance of the germinating spores to wild-type levels, confirming a role for YapA signaling in spore adaptation to H_2O_2 oxidative stress. In *A. nidulans*, both SakA, through the transcription factor AtfA, and YapA pathways have been shown to be important for conferring spore resistance to H_2O_2 stress, as both *atfA* and *napA* (equivalent to *yapA*) mutants are sensitive to H_2O_2 stress (58, 64, 65). For the SakA pathway, tolerance to oxidative stress is mediated through a spore-specific catalase, CatA, whose expression is regulated by AtfA. Levels of *catA* mRNA are dramatically lower in the Δ*atfA* mutant than in the wild-type strain, suggesting that transcription of the *catA* gene is regulated in a spore-specific manner through AtfA (58, 64). The expression of *E. festucae catA* is also spore specific, but in this fungal species, as in *Aspergillus*

FIG 5 Spore-specific expression of P*catA-eGFP*. (A) Confocal and DIC microscopy images confirming the spore-specific expression of the P*catA-eGFP* reporter, pGC13. Bar = 10 μm. (B) Spore-specific expression of the P*catA-eGFP-CL1* reporter, pGC14. The box plot shows the total amount of fluorescence per spore, indicating a significant decrease in EGFP fluorescence in Δ*yapA* spores relative to wild-type (WT) spores. Significance between samples was determined using Student's two-tailed *t* test: for the WT versus Δ*yapA*#145 strains, $t_{100} = 9.6$ and the *P* value was $\ll 0.0001$ (***); for the WT versus Δ*yapA*#243 strains, $t_{89} = 12.6$ and the *P* value was $\ll 0.0001$ (***); and for the Δ*yapA*#145 versus Δ*yapA*#243 strains, $t_{34} = 0.3$, and the difference was not significant. The total cellular fluorescence of each spore was quantified using ImageJ software. The image analysis was performed on maximum-intensity projection images that were generated from 5- by 1-μm confocal *z* stacks. Representative merged DIC and confocal fluorescence images showing EGFP expression in wild-type (WT), Δ*yapA*#145 (PN2740), and Δ*yapA*#243 (PN2739) spores are shown above the box plot. Bar = 2 μm. Multiple transformants, including Fl1::P*catA-eGFP-Cl1* (PN2838, PN2839, PN2840, and PN2841), Δ*yapA*#243::P*catA-eGFP-CL1* (PN2836, PN2837, and PN2844), and Δ*yapA*#145::P*catA-eGFP-CL1* (PN2842 and PN2843) transformants, were analyzed.

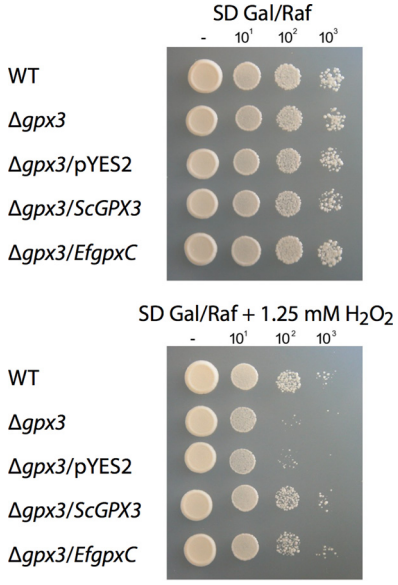


FIG 7 *S. cerevisiae* complementation by *E. festucae gpxC*. The growth of *S. cerevisiae* BY4741 (WT; PN2735), *S. cerevisiae* BY4741-ΔYIR037W (Δ*gpxC*; PN2737), and derivatives of this strain transformed with the empty vector pYES2 (PN2850), pYES2*ScGPX3* (PN2848), or pYES2*EfgpxC* (PN2849) was tested on SD plates containing galactose and raffinose with and without 1.25 mM H_2O_2. Serial 10-fold dilutions of the cultures indicated on the left were spotted onto plates.

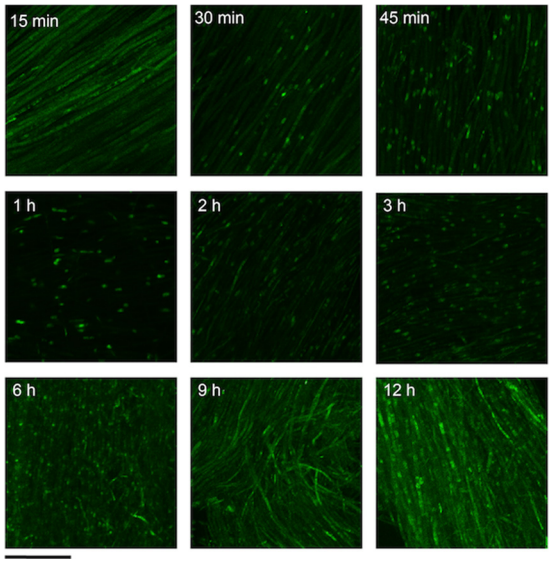


FIG 9 Time course of YapA-EGFP localization. An *E. festucae* wild-type strain expressing YapA-EGFP (PN2790) was inoculated into the meristematic region of grass seedlings, and the subcellular localization of YapA-EGFP was examined using confocal laser scanning microscopy at the indicated time points. EGFP images were generated by maximum-intensity projection of confocal *z* stacks taken at 1-μm intervals from the top to the bottom of the section. Bar = 10 μm.

ochraceus (66), *catA* appears to be regulated by YapA, as deletion of *yapA* confers a spore germination sensitivity phenotype under conditions of oxidative stress. How YapA regulates *catA* in *E. festucae* spores remains to be determined.

While infection of *L. perenne* with the *E. festucae* Δ*sakA* mutant results in a defective symbiotic interaction phenotype (42), *yapA* mutants are able to infect and colonize *L. perenne* seedlings as effectively as the wild type and give rise to mature plants with a phenotype similar to that with the wild type. These results contrast with the reduced virulence observed for AP-1 mutants of the biotrophic fungal pathogens *U. maydis* and *M. oryzae*, because of their sensitivity to the host oxidative burst (7, 8). However, an important lifestyle difference between these biotrophic fungal pathogens and *E. festucae* is the apparent absence of a leaf penetration mechanism for the latter. To infect endophyte-free seedlings of *L. perenne* with *E. festucae* in the laboratory, it is necessary to mechanically wound the seedlings to allow the endophyte to

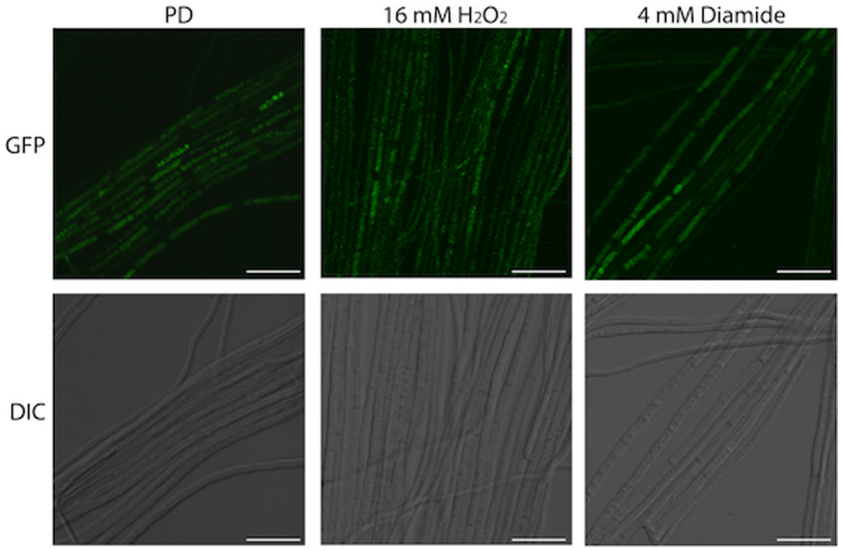


FIG 8 H_2O_2 activation of Yap1-EGFP in axenic culture. An *E. festucae* wild-type strain (PN2874) expressing *S. cerevisiae* Yap1-EGFP was examined by confocal laser scanning and DIC microscopy in the absence of oxidative stress (PD) or 30 min after treatment with 16 mM H_2O_2 or 4 mM diamide. EGFP images were generated by maximum-intensity projection of confocal *z* stacks taken at 1-μm intervals from the top to the bottom of the section. Bars = 20 μm.

colonize the grass host (55), as the natural route of infection through the stigmata and styles of the flowers is a process that is difficult to reproduce under laboratory conditions (67). However, wounding of seedlings should generate a burst of ROS and a state of oxidative stress for hyphae inoculated at the wound site (56, 57). Wounding may also result in a release of phenolics that have been shown to induce relocalization of GFP-ChAP1 to the nucleus in *Cochliobolus heterostrophus* (68). Using YapA-EGFP as a redox sensor, we were able to show that YapA-EGFP hyphae in direct contact with the wound, as well as in cells more distant from the wound site, relocalized to the nucleus within 30 min of wounding. The localization of YapA-EGFP in the nucleus was transient, peaking at 1 h postinoculation, followed by a gradual redistribution to the cytoplasm over a 6- to 9-h period, indicating restoration of redox homeostasis. If this short period of oxidative stress does impair hyphal growth at the infection site, it is not sufficient to prevent subsequent host colonization and establishment of a mutualistic symbiotic interaction.

Although AP-1 transcription factors have been characterized in a range of filamentous fungi, the mechanism by which oxidative stress is sensed and transduced to the AP-1 protein is still not known (8, 30–34). The assumption has been that the immediate sensor of increased levels of H_2O_2 would be a thiol peroxidase (7, 33), as has been established for the well-characterized Yap1-Gpx3 and Pap1-Tpx1 redox relay systems that operate in the yeast fungi *S. cerevisiae* and *S. pombe*, respectively (21, 24, 25). The abundance of these proteins in the cell and their favorable kinetic properties compared to those of alternative thiol-reactive proteins support this hypothesis (69). In addition, signaling through a thiol peroxidase/peroxiredoxin (Prdx1) was recently demonstrated in a mammalian system (70). However, our results show that YapA fused to EGFP still localizes to the nucleus in mutants defective in either GpxC or TpxA, to the same extent as it does in the wild type, when mycelia are subjected to H_2O_2-induced oxidative stress. Furthermore, YapA-EGFP still localizes to the nucleus when both genes are deleted, ruling out the possibility of functional redundancy between GpxC and TpxA. These results were surprising given the high degree of primary amino acid sequence conservation between GpxC and Gpx3 and the ability of GpxC to functionally complement the Δ*gpx3* mutant of *S. cerevisiae*, as does the *M. oryzae* homolog Hyr1 (35).

While most fungi have a single Gpx protein, *S. cerevisiae* has three isoforms: Gpx1, Gpx2, and Gpx3 (71). All are classified as atypical 2-Cys peroxiredoxins because they form, as a consequence of the peroxidase reaction, an intramolecular (as opposed to intermolecular) disulfide bond (72) which is cleaved by glutathione or thioredoxin. *GPX1* and *GPX3* are paralogs that have arisen from whole-genome duplication (WGD) (http://wolfe.gen.tcd.ie/ygob/). Although Gpx1 and Gpx2 show a high degree of conservation with Gpx3 and have known peroxidase activity *in vitro*, Δ*gpx1* and Δ*gpx2* mutants have no oxidative stress phenotype in culture and no known involvement in Yap1 activation (71, 73). The preferential localization of Gpx3 to the cytoplasm (74), compared to peroxisomal matrix and mitochondrial localizations for Gpx1 and Gpx2, respectively, may be one explanation for why Gpx3 can promote oxidation of Yap1 (73, 75). Alternatively, subtle structural changes in Gpx3 may promote the interaction with Yap1 (76).

In *S. pombe*, Tpx1 rather than Gpx1 has been shown to be the crucial peroxiredoxin (Prx) required for redox sensing and signaling (24, 25). Phylogenetic analysis identified *E. festucae* TpxA as the closest homolog of Tpx1. However, Tpx1 is a typical 2-Cys Prx, whereas *E. festucae* TpxA and homologs from other filamentous fungi are all 1-Cys Prxs. Another important biochemical difference between the typical 2-Cys and 1-Cys Prxs is the ability of the former to undergo hyperoxidation, a property that was recently shown to be crucial for oxidative stress signaling (77). A sulfiredoxin (Srx1) has been identified in the yeast group of fungi that reduces the cysteine-sulfinic acid in Prx (Tpx1/Tsa) back to a cysteine-sulfenic acid (78), but interestingly, this protein appears to be absent from the filamentous fungi (79). The absence of typical 2-Cys peroxiredoxins in the filamentous fungi and the corresponding absence of a sulfiredoxin are particularly interesting given the recent hypothesis that oxidation-reduction cycles of 2-Cys peroxiredoxins constitute universal markers for circadian rhythms (80).

While several studies have established a key role for thiol peroxidases (peroxiredoxins) in protection of yeast to oxidative stress, very few studies have been carried out in filamentous fungi. Like the Δ*yapA* mutant, growth of the *E. festucae* Δ*gpxC*, Δ*tpxA*, and Δ*gpxC* Δ*tpxA* strains in culture was not impaired by addition of H_2O_2, once again emphasizing that this fungal symbiont has a robust oxidative stress protection system. In contrast, growth of the *M. oryzae* Δ*hyr1* mutant in culture was impaired by H_2O_2 (35). In *S. cerevisiae*, there are at least eight thiol peroxidases that appear to have overlapping functions for protection from a range of different types of oxidative stress: five of the atypical 2-Cys family, including Gpx1 to -3, Dot5 (Bcp in *S. pombe*), and AHP1 (Pmp20); two of the typical 2-Cys family, including Tsa1 and Tsa2 (Tpx1); and one from the 1-Cys family (Prx1). The numbers of thiol peroxidase family members in *S. pombe* and *E. festucae* are, by comparison, reduced. *S. pombe* has a single Gpx homolog, Gpx1, and three peroxiredoxin homologs, Tpx1, Bcp, and Pmp20 (81). Similarly, *E. festucae* has a single Gpx1 homolog, a Tpx1 protein which groups more closely with *S. cerevisiae* Prx1 than with *S. pombe* Tpx1, and Bcp and Pmp20 homologs. The *E. festucae* thiol peroxidases may also constitute a cellular signaling network, but this remains to be tested.

Like the Δ*yapA* mutant, the *E. festucae* Δ*gpxC*, Δ*tpxA*, and Δ*gpxC* Δ*tpxA* mutants were able to infect and colonize *L. perenne* seedlings as effectively as the wild type and gave rise to mature plants with the same phenotypes as those of the wild type, demonstrating that the GpxC and TpxA thiol reductases are not crucial for establishment and maintenance of this fungus-grass symbiosis. As discussed above, the need for mechanical wounding to introduce the wild type and these mutants into the host grass limits our screen to postinfection and host colonization phenotypes. The only other filamentous fungal thiol peroxidase that has been analyzed functionally is the Gpx3 homolog Hyr1 from *M. oryzae*. The Δ*hyr1* mutant had reduced virulence on both susceptible barley and rice leaves and was sensitive to ROS produced at the infection sites (35), but the host phenotype observed was not nearly as severe as that with the Δ*yap1* mutant, which is completely blocked in host leaf infection (8).

Given that neither GpxC nor TpxA is required for YapA localization to the nucleus, we conclude that H_2O_2 oxidative stress signaling in *E. festucae* occurs by a mechanism distinct from the classic Gpx3 and Tpx1 redox relay systems that occur in *S. cerevisiae* and *S. pombe*. One possibility is that YapA is directly oxidized by H_2O_2, as has been shown for OxyR, a positive regulator of

hydrogen peroxide-inducible genes in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (82, 83). Interestingly, nearly all the Yap1 homologs found in filamentous fungi have just a single conserved Cys in the n-CRD, suggesting that formation of just a single disulfide bond may be sufficient to induce the necessary conformation change required for Yap to relocalize to the nucleus. Alternatively, oxidation of the conserved cysteines in the c-CRD alone may suffice. Whether there is an accessory protein similar to Ybp1 in *S. cerevisiae* that promotes Yap1 nuclear localization remains to be determined. A second possibility is that YapA is activated by a thiol peroxidase other than GpxC or TpxA. Although the genes for two additional atypical 2-Cys peroxiredoxins, homologs of yeast Dot5/Bcp and AHP1/Pmp20, were found in the genomes of *E. festucae* and other filamentous fungi, neither has been shown to function in redox signaling in yeast (81), and inactivation of Tpx1 alone is sufficient to completely abolish the transcriptional response to H_2O_2 stress in *S. pombe* (84). Given that peroxiredoxins have the most favorable kinetic and cell abundance properties (69), a mechanism that involves an alternative redox-active protein to activate YapA seems unlikely.

While our results do not support the hypothesis that a homolog of either *S. cerevisiae* Gpx3 or *S. pombe* Tpx1 is required for activation of *E. festucae* YapA in response to oxidative stress, we have shown that YapA readily relocalizes from the cytoplasm to the nucleus and is important in providing protection from oxidative stress in culture and *in planta*. Identifying the specific structural changes that occur upon oxidation of YapA to bring about the conformational change necessary to promote nuclear retention will be crucial for understanding how YapA is activated in filamentous fungi.

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G.M.C. and B.S. designed the experiments, and G.M.C. performed the experiments. G.M.C. and B.S. jointly analyzed the results and wrote the paper.

REFERENCES

1. **Simon-Plas F, Elmayan T, Blein J-P.** 2002. The plasma membrane oxidase NtrbohD is responsible for AOS production in elicited tobacco cells. Plant J. **31:**137–147.
2. **Torres MA, Jones JDG, Dangl JL.** 2005. Pathogen-induced, NADPH oxidase-derived reactive oxygen intermediates suppress spread of cell death in *Arabidopsis thaliana*. Nat. Genet. **37:**1130–1134.
3. **Yoshioka H, Numata N, Nakajima K, Katou S, Kawakita K, Rowland O, Jones JDG, Doke N.** 2003. *Nicotiana benthamiana* gp91*phox* homologs *NbrbohA* and *NbrbohB* participate in H_2O_2 accumulation and resistance to *Phytophthora infestans*. Plant Cell **15:**706–718.
4. **Doke N.** 1983. Generation of superoxide anion by potato tuber protoplasts during the hypersensitive response to hyphal wall components of *Phytophthora infestans* and specific inhibition of the reaction by suppressors of hypersensitivity. Physiol. Plant Pathol. **23:**359–367.
5. **Apostol I, Heinstein PF, Low PS.** 1989. Rapid stimulation of an oxidative burst during elicitation of cultured plant cells: role in defense and signal transduction. Plant Physiol. **90:**109–116.
6. **Torres MA, Dangl JL.** 2005. Functions of the respiratory burst oxidase in biotic interactions, abiotic stress and development. Curr. Opin. Plant Biol. **8:**397–403.
7. **Molina L, Kahmann R.** 2007. An *Ustilago maydis* gene involved in H_2O_2 detoxification is required for virulence. Plant Cell **19:**2293–2309.
8. **Guo M, Chen Y, Du Y, Dong Y, Guo W, Zhai S, Zhang H, Dong S, Zhang Z, Wang Y, Wang P, Zheng X.** 2011. The bZIP transcription factor MoAP1 mediates the oxidative stress response and is critical for pathogenicity of the rice blast fungus *Magnaporthe oryzae*. PLoS Pathog. **7:**e1001302. doi:10.1371/journal.ppat.1001302.
9. **Apel K, Hirt H.** 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. **55:**373–399.
10. **Foyer CH, Noctor G.** 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. Plant Cell **17:**1866–1875.
11. **Foreman J, Demidchik V, Bothwell JH, Mylona P, Miedema H, Torres MA, Linstead P, Costa S, Brownlee C, Jones JD, Davies JM, Dolan L.** 2003. Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. Nature **422:**442–446.
12. **Lara-Ortíz T, Riveros-Rosas H, Aguirre J.** 2003. Reactive oxygen species generated by microbial NADPH oxidase NoxA regulate sexual development in *Aspergillus nidulans*. Mol. Microbiol. **50:**1241–1255.
13. **Malagnac F, Lalucque H, Lepère G, Silar P.** 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.
14. **Egan M, Wang Z-Y, Jones MA, Smirnoff N, Talbot NJ.** 2007. Generation of reactive oxygen species by fungal NADPH oxidases is required for rice blast disease. Proc. Natl. Acad. Sci. U. S. A. **104:**11772–11777.
15. **Tanaka A, Christensen MJ, Takemoto D, Park P, Scott B.** 2006. Reactive oxygen species play a role in regulating a fungus-perennial ryegrass mutualistic association. Plant Cell **18:**1052–1066.
16. **Takeda S, Gapper C, Kaya H, Bell E, Kuchitsu K, Dolan L.** 2008. Local positive feedback regulation determines cell shape in root hair cells. Science **319:**1241–1244.
17. **Yan C, Lee LH, Davis LI.** 1998. Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. EMBO J. **17:**7416–7429.
18. **Wood MJ, Andrade EC, Storz G.** 2003. The redox domain of the Yap1p transcription factor contains two disulfide bonds. Biochemistry **42:**11982–11991.
19. **Wood MJ, Storz G, Tjandra N.** 2004. Structural basis for redox regulation of Yap1 transcription factor localization. Nature **430:**917–921.
20. **Lee J, Godon C, Lagniel G, Spector D, Garin J, Labarre J, Toledano MB.** 1999. Yap1 and Skn7 control two specialized oxidative stress response regulons in yeast. J. Biol. Chem. **274:**16040–16046.
21. **Delaunay A, Pflieger D, Barrault MB, Vinh J, Toledano MB.** 2002. A thiol peroxidase is an H_2O_2 receptor and redox-transducer in gene activation. Cell **111:**471–481.
22. **Okazaki S, Tachibana T, Naganuma A, Mano N, Kuge S.** 2007. Multistep disulfide bond formation in Yap1 is required for sensing and transduction of H_2O_2 stress signal. Mol. Cell **27:**675–688.
23. **Veal EA, Ross SJ, Malakasi P, Peacock E, Morgan BA.** 2003. Ybp1 is required for the hydrogen peroxide-induced oxidation of the Yap1 transcription factor. J. Biol. Chem. **278:**30896–30904.
24. **Vivancos AP, Castillo EA, Biteau B, Nicot C, Ayte J, Toledano MB, Hidalgo E.** 2005. A cysteine-sulfinic acid in peroxiredoxin regulates H_2O_2-sensing by the antioxidant Pap1 pathway. Proc. Natl. Acad. Sci. U. S. A. **102:**8875–8880.
25. **Bozonet SM, Findlay VJ, Day AM, Cameron J, Veal EA, Morgan BA.** 2005. Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. J. Biol. Chem. **280:**23319–23327.
26. **Kuge S, Arita M, Murayama A, Maeta K, Izawa S, Inoue Y, Nomoto A.** 2001. Regulation of the yeast Yap1p nuclear export signal is mediated by redox signal-induced reversible disulfide bond formation. Mol. Cell. Biol. **21:**6139–6150.
27. **Castillo EA, Ayte J, Chiva C, Moldon A, Carrascal M, Abian J, Jones N,**

Hidalgo E. 2002. Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. Mol. Microbiol. **45:** 243–254.
28. **Azevedo D, Tacnet F, Delaunay A, Rodrigues-Pousada C, Toledano MB.** 2003. Two redox centers within yap1 for H_2O_2 and thiol-reactive chemical signaling. Free Radic. Biol. Med. **35:**889–900.
29. **Alarco AM, Raymond M.** 1999. The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in *Candida albicans*. J. Bacteriol. **181:**700–708.
30. **Qiao J, Kontoyiannis DP, Calderone R, Li D, Ma Y, Wan Z, Li R, Liu W.** 2008. Afyap1, encoding a bZip transcriptional factor of *Aspergillus fumigatus*, contributes to oxidative stress response but is not essential to the virulence of this pathogen in mice immunosuppressed by cyclophosphamide and triamcinolone. Med. Mycol. **46:**773–782.
31. **Lev S, Hadar R, Amedeo P, Baker SE, Yoder OC, Horwitz BA.** 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.
32. **Lin CH, Yang SL, Chung KR.** 2009. The YAP1 homolog-mediated oxidative stress tolerance is crucial for pathogenicity of the necrotrophic fungus *Alternaria alternata* in citrus. Mol. Plant Microbe Interact. **22:**942–952.
33. **Temme N, Tudzynski P.** 2009. Does *Botrytis cinerea* ignore H_2O_2-induced oxidative stress during infection? Characterization of botrytis activator protein 1. Mol. Plant Microbe Interact. **22:**987–998.
34. **Tian C, Li J, Glass NL.** 2011. Exploring the bZIP transcription factor regulatory network in *Neurospora crassa*. Microbiology **157:**747–759.
35. **Huang K, Czymmek KJ, Caplan JL, Sweigard JA, Donofrio NM.** 2011. *HYR1*-mediated detoxification of reactive oxygen species is required for full virulence in the rice blast fungus. PLoS Pathog. **7:**e1001335. doi:10.1371/journal.ppat.1001335.
36. **Scott B, Becker Y, Becker M, Cartwright G.** 2012. Morphogenesis, growth and development of the grass symbiont *Epichloë festucae*, p 243–264. *In* Martin JP, Di Pietro A (ed), Morphogenesis and pathogenicity in fungi. Springer-Verlag, Heidelberg, Germany.
37. **Eaton C, Mitic M, Scott B.** 2011. Signalling in the *Epichloë festucae*-perennial ryegrass mutualistic symbiotic interaction, p 143–181. *In* Perotto S, Baluska F (ed), Signaling and communication in plant symbiosis, vol 11. Springer-Verlag, Heidelberg, Germany.
38. **Christensen MJ, Bennett RJ, Ansari HA, Koga H, Johnson RD, Bryan GT, Simpson WR, Koolaard JP, Nickless EM, Voisey CR.** 2008. *Epichloë* endophytes grow by intercalary hyphal extension in elongating grass leaves. Fungal Genet. Biol. **45:**84–93.
39. **Takemoto D, Tanaka A, Scott B.** 2006. A $p67^{Phox}$-like regulator is recruited to control hyphal branching in a fungal-grass mutualistic symbiosis. Plant Cell **18:**2807–2821.
40. **Takemoto D, Kamakura S, Saikia S, Becker Y, Wrenn R, Tanaka A, Sumimoto H, Scott B.** 2011. Polarity proteins Bem1 and Cdc24 are components of the filamentous fungal NADPH oxidase complex. Proc. Natl. Acad. Sci. U. S. A. **108:**2861–2866.
41. **Tanaka A, Takemoto D, Hyon GS, Park P, Scott B.** 2008. NoxA activation by the small GTPase RacA is required to maintain a mutualistic symbiotic association between *Epichloë festucae* and perennial ryegrass. Mol. Microbiol. **68:**1165–1178.
42. **Eaton CJ, Cox MP, Ambrose B, Becker M, Hesse U, Schardl CL, Scott B.** 2010. Disruption of signaling in a fungal-grass symbiosis leads to pathogenesis. Plant Physiol. **153:**1780–1794.
43. **Miller JH.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
44. **Sanderson KE, Srb AM.** 1965. Heterokaryosis and parasexuality in the fungus *Ascochta imperfecta*. Am. J. Bot. **52:**72–81.
45. **Moon CD, Tapper BA, Scott B.** 1999. Identification of *Epichloë* endophytes *in planta* by a microsatellite-based PCR fingerprinting assay with automated analysis. Appl. Environ. Microbiol. **65:**1268–1279.
46. **Moon CD, Scott B, Schardl CL, Christensen MJ.** 2000. The evolutionary origins of *Epichloë* endophytes from annual ryegrasses. Mycologia **92:** 1103–1118.
47. **Byrd AD, Schardl CL, Songlin PJ, Mogen KL, Siegel MR.** 1990. The β-tubulin gene of *Epichloë typhina* from perennial ryegrass (*Lolium perenne*). Curr. Genet. **18:**347–354.
48. **Southern EM.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
49. **Tanaka A, Tapper BA, Popay A, Parker EJ, Scott B.** 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. Mol. Microbiol. **57:**1036–1050.
50. **Gietz RD, Woods RA.** 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. **350:**87–96.
51. **Young CA, Bryant MK, Christensen MJ, Tapper BA, Bryan GT, Scott B.** 2005. Molecular cloning and genetic analysis of a symbiosis-expressed gene cluster for lolitrem biosynthesis from a mutualistic endophyte of perennial ryegrass. Mol. Gen. Genomics **274:**13–29.
52. **Itoh Y, Johnson R, Scott B.** 1994. Integrative transformation of the mycotoxin-producing fungus, *Penicillium paxilli*. Curr. Genet. **25:**508–513.
53. **Navarro RE, Stringer MA, Hansberg W, Timberlake WE, Aguirre J.** 1996. *catA*, a new *Aspergillus nidulans* gene encoding a developmentally regulated catalase. Curr. Genet. **29:**352–359.
54. **Bence NF, Sampat RM, Kopito RR.** 2001. Impairment of the ubiquitin-proteasome system by protein aggregation. Science **292:**1552–1555.
55. **Latch GCM, Christensen MJ.** 1985. Artificial infection of grasses with endophytes. Ann. Appl. Biol. **107:**17–24.
56. **Orozco-Cardenas M, Ryan CA.** 1999. Hydrogen peroxide is generated systemically in plant leaves by wounding and systemin via the octadecanoid pathway. Proc. Natl. Acad. Sci. U. S. A. **96:**6553–6557.
57. **Le Deunff E, Davoine C, Le Dantec C, Billard JP, Huault C.** 2004. Oxidative burst and expression of germin/oxo genes during wounding of ryegrass leaf blades: comparison with senescence of leaf sheaths. Plant J. **38:**421–431.
58. **Hagiwara D, Asano Y, Yamashino T, Mizuno T.** 2008. Characterization of bZip-type transcription factor AtfA with reference to stress responses of conidia of *Aspergillus nidulans*. Biosci. Biotechnol. Biochem. **72:**2756–2760.
59. **Aguirre J, Ríos-Momberg M, Hewitt D, Hansberg W.** 2005. Reactive oxygen species and development in microbial eukaryotes. Trends Microbiol. **13:**111–118.
60. **Quinn J, Malakasi P, Smith DA, Cheetham J, Buck V, Millar JB, Morgan BA.** 2011. Two-component mediated peroxide sensing and signal transduction in fission yeast. Antioxid. Redox Signal. **15:**153–165.
61. **Degols G, Shiozaki K, Russell P.** 1996. Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. Mol. Cell. Biol. **16:**2870–2877.
62. **Wilkinson MG, Samuels M, Takeda T, Toone WM, Shieh JC, Toda T, Millar JB, Jones N.** 1996. The Atf1 transcription factor is a target for the StyI stress-activated MAP kinase pathway in fission yeast. Genes Dev. **10:** 2289–2301.
63. **Kawasaki L, Sánchez O, Shiozaki K, Aguirre J.** 2002. SakA MAP kinase is involved in stress signal transduction, sexual development and spore viability in *Aspergillus nidulans*. Mol. Microbiol. **45:**1153–1163.
64. **Lara-Rojas F, Sánchez O, Kawasaki L, Aguirre J.** 2011. *Aspergillus nidulans* transcription factor AtfA interacts with the MAPK SakA to regulate general stress responses, development and spore functions. Mol. Microbiol. **80:**436–454.
65. **Asano Y, Hagiwara D, Yamashino T, Mizuno T.** 2007. Characterization of the bZip-type transcription factor NapA with reference to oxidative stress response in *Aspergillus nidulans*. Biosci. Biotechnol. Biochem. **71:** 1800–1803.
66. **Reverberi M, Gazzetti K, Punelli F, Scarpari M, Zjalic S, Ricelli A, Fabbri AA, Fanelli C.** 2012. A*oyap1* regulates OTA synthesis by controlling cell redox balance in *Aspergillus ochraceus*. Appl. Microbiol. Biotechnol. **95:**1293–1304.
67. **Chung K-R, Schardl CL.** 1997. Sexual cycle and horizontal transmission of the grass symbiont, Epichloë typina. Mycol. Res. **101:**295–301.
68. **Shanmugam V, Ronen M, Shalaby S, Larkov O, Rachamim Y, Hadar R, Rose MS, Carmeli S, Horwitz BA, Lev S.** 2010. The fungal pathogen *Cochliobolus heterostrophus* responds to maize phenolics: novel small molecule signals in a plant-fungal interaction. Cell. Microbiol. **12:**1421–1434.
69. **Winterbourn CC, Hampton MB.** 2008. Thiol chemistry and specificity in redox signaling. Free Radic. Biol. Med. **45:**549–561.
70. **Jarvis RM, Hughes SM, Ledgerwood EC.** 2012. Peroxiredoxin 1 functions as a signal peroxidase to receive, transduce, and transmit peroxide signals in mammalian cells. Free Radic. Biol. Med. **53:**1522–1530.
71. **Inoue Y, Matsuda T, Sugiyama K, Izawa S, Kimura A.** 1999. Genetic analysis of glutathione peroxidase in oxidative stress response of *Saccharomyces cerevisiae*. J. Biol. Chem. **274:**27002–27009.

72. **Tanaka T, Izawa S, Inoue Y.** 2005. GPX2, encoding a phospholipid hydroperoxide glutathione peroxidase homologue, codes for an atypical 2-Cys peroxiredoxin in *Saccharomyces cerevisiae*. J. Biol. Chem. **280:** 42078–42087.
73. **Ukai Y, Kishimoto T, Ohdate T, Izawa S, Inoue Y.** 2011. Glutathione peroxidase 2 in *Saccharomyces cerevisiae* is distributed in mitochondria and involved in sporulation. Biochem. Biophys. Res. Commun. **411:**580–585.
74. **Huh WK, Falvo JV, Gerke LC, Carroll AS, Howson RW, Weissman JS, O'Shea EK.** 2003. Global analysis of protein localization in budding yeast. Nature **425:**686–691.
75. **Ohdate T, Inoue Y.** 2012. Involvement of glutathione peroxidase 1 in growth and peroxisome formation in *Saccharomyces cerevisiae* in oleic acid medium. Biochim. Biophys. Acta **1821:**1295–1305.
76. **Zhang WJ, He YX, Yang Z, Yu J, Chen Y, Zhou CZ.** 2008. Crystal structure of glutathione-dependent phospholipid peroxidase Hyr1 from the yeast *Saccharomyces cerevisiae*. Proteins **73:**1058–1062.
77. **Day AM, Brown JD, Taylor SR, Rand JD, Morgan BA, Veal EA.** 2012. Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. Mol. Cell **45:**398–408.
78. **Biteau B, Labarre J, Toledano MB.** 2003. ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. Nature **425:**980–984.
79. **Morel M, Kohler A, Martin F, Gelhaye E, Rouhier N.** 2008. Comparison of the thiol-dependent antioxidant systems in the ectomycorrhizal *Laccaria bicolor* and the saprotrophic *Phanerochaete chrysosporium*. New Phytol. **180:**391–407.
80. **Edgar RS, Green EW, Zhao Y, van Ooijen G, Olmedo M, Qin X, Xu Y, Pan M, Valekunja UK, Feeney KA, Maywood ES, Hastings MH, Baliga NS, Merrow M, Millar AJ, Johnson CH, Kyriacou CP, O'Neill JS, Reddy AB.** 2012. Peroxiredoxins are conserved markers of circadian rhythms. Nature **485:**459–464.
81. **Kim JS, Bang MA, Lee S, Chae HZ, Kim K.** 2010. Distinct functional roles of peroxiredoxin isozymes and glutathione peroxidase from fission yeast, *Schizosaccharomyces pombe*. BMB Rep. **43:**170–175.
82. **Storz G, Tartaglia LA.** 1992. OxyR: a regulator of antioxidant genes. J. Nutr. **122:**627–630.
83. **Storz G, Tartaglia LA, Ames BN.** 1990. The OxyR regulon. Antonie Van Leeuwenhoek **58:**157–161.
84. **Veal EA, Findlay VJ, Day AM, Bozonet SM, Evans JM, Quinn J, Morgan BA.** 2004. A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. Mol. Cell **15:**129–139.