An *Ustilago maydis* Gene Involved in H$_2$O$_2$ Detoxification Is Required for Virulence

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The fungus *Ustilago maydis* is a biotrophic pathogen of maize (*Zea mays*). In its genome we have identified an ortholog of *YAP1* (for Yeast AP-1-like) from *Saccharomyces cerevisiae* that regulates the oxidative stress response in this organism. *yap1* mutants of *U. maydis* displayed higher sensitivity to H$_2$O$_2$ than wild-type cells, and their virulence was significantly reduced. *U. maydis* *yap1* could partially complement the H$_2$O$_2$ sensitivity of a *yap1* deletion mutant of *S. cerevisiae*, and a Yap1-green fluorescent protein fusion protein showed nuclear localization after H$_2$O$_2$ treatment, suggesting that Yap1 in *U. maydis* functions as a redox sensor. Mutations in two Cys residues prevented accumulation in the nucleus, and the respective mutant strains showed the same virulence phenotype as *Δyap1* mutants. Diamino benzidine staining revealed an accumulation of H$_2$O$_2$ around *yap1* mutant hyphae, which was absent in the wild type. Inhibition of the plant NADPH oxidase prevented this accumulation and restored virulence. During the infection, Yap1 showed nuclear localization after penetration up to 2 to 3 d after infection. Through array analysis, a large set of Yap1-regulated genes were identified and these included two peroxidase genes. Deletion mutants of these genes were attenuated in virulence. These results suggest that *U. maydis* is using its Yap1-controlled H$_2$O$_2$ detoxification system for coping with early plant defense responses.

INTRODUCTION

To survive, plants have developed efficient defense systems against pathogenic microbes. One of the most rapid plant defense reactions after pathogen attack is the so-called oxidative burst, which constitutes the production of reactive oxygen species (ROS), primarily superoxide and H$_2$O$_2$, at the site of attempted invasion (Apostol et al., 1989). ROS is primarily generated by plasma membrane–localized NADPH oxidases (Doke et al., 1996). Apoplastic peroxidases bound to cell wall polymers use the generated H$_2$O$_2$ or phenolic substrates in a peroxidation cycle, leading to the synthesis of lignin and other phenolic polymers, which provide additional plant barriers against pathogen attack (Chen and Schopfer, 1999). The produced ROS activate plant defense responses, including programmed cell death, or function as second messenger in the induction of various plant defense-related genes (Torres and Dangl, 2005). Due to the toxicity of ROS molecules and their importance in plant defense responses, plants and plant pathogens have developed strategies for ROS detoxification (see Apel and Hirt, 2004). As one strategy, nonenzymatic antioxidants like ascorbate, GSH, tocopherol, flavonoids, alkaloids, and carotenoids are produced. The second strategy is enzymatic ROS scavenging through superoxide dismutase, ascorbate peroxidase, cytochrome C-peroxidase, glutathione peroxidase, and catalases, generally using NAD(P)H as reducing equivalents (Asada, 1999; Campos et al., 2005).

One of the central regulators whose action provides protection against oxidative stress in *Saccharomyces cerevisiae* is Yap1p (encoded by *YAP1*). This transcription factor is a basic domain/leucine zipper (bZIP) protein of the AP-1 family that was originally identified by its ability to recognize the mammalian AP-1 binding site (Moye-Rowley et al., 1989). Other functional domains include two Cys-rich domains (CRDs) (C-terminal domain designated c-CRD and the N-terminal domain termed n-CRD) (Delaunay et al., 2000; Toone et al., 2001), which are critical for the Yap1p-mediated resistance to oxidative stress and for the appropriate subcellular localization of Yap1p (Coleman et al., 1999; Kuge et al., 2001). In response to H$_2$O$_2$, Yap1p is oxidized by a glutathione peroxidase-like protein (Gpx3/Opr1) and changes its conformation by forming two intramolecular disulfide bonds that activate the protein and mask a nuclear export sequence (Delaunay et al., 2000). This compromises binding of the nuclear exportin Crm1p, leading to nuclear retention of the active form (Kuge et al., 1997; Wood et al., 2003). Proteins related to Yap1p from *S. cerevisiae* have been found in *Candida albicans* (Narco and Raymond, 1999), *Schizosacharomyces pombe* (Toone et al., 1998), *Kluveromyces lactis* (Billard et al., 1997), and *Cochliobolus heterostrophus* (Lev et al., 2005). In these microorganisms, Yap1p is involved in activating genes involved in oxidative stress tolerance, drug tolerance, and heavy metal resistance (Wu et al., 1993; Gounalaki and Thireos, 1994; Hirata et al., 1994; Lee et al., 1999; Dumond et al., 2000; Wysocki et al., 2004). Upon H$_2$O$_2$ stress, ~500 genes are upregulated in *S. cerevisiae* and many of them have Yap1p binding sites in their promoters (Harshman et al., 1988; Kuge and Jones, 1994; Wu and Moye-Rowley, 1994).
Among the Yap1p-activated genes, a significant number is directly involved in the detoxification of ROS, such as cytoplasmic catalase and superoxide dismutase isoenzymes, alkyl hydroxide reductases, peroxiredoxins, glutathione peroxidase, and cytochrome C peroxidase (Lee et al., 1999; Dumond et al., 2000; Gash et al., 2000).

*Ustilago maydis* is the causative agent of maize (*Zea mays*) smut disease. The disease cycle is initiated by fusion of compatible haploid cells. The resulting dikaryon switches to filamentous growth on the leaf surface, forms appressoria, and penetrates host cells in a process that is likely promoted by lytic enzymes. During penetration, the plasma membrane of the host invaginates and surrounds the hyphae. Plant cells stay alive, and there are no apparent defense responses triggered (Basse, 2005). After penetration, *U. maydis* extends into the deeper layers of the tissue. Massive fungal proliferation occurs within cells or in the apoplast, followed by hyphal fragmentation, karyogamy, and spore formation (Snetesilaar and Mims, 1994; Banuett and Herskowitz, 1996). These events take place in tumor tissue that develops in response to yet unknown fungal signals. Recent insights from the genome sequence have revealed that a number of *U. maydis* gene clusters coding for secreted proteins of unknown function play decisive roles in shaping the biotrophic interaction with the host (Kämper et al., 2006). However, at present, it is not yet clear at which stages these proteins are required and whether they shield fungal hyphae or interfere with host defense responses. Given the situation that many plant pathogens are recognized by their hosts through conserved pathogen-associated molecular patterns (Nünberger and Brunner, 2002) that elicit an oxidative burst, we reasoned that an H$_2$O$_2$ detoxification system of *U. maydis* might help to overcome this host response.

In this work, we characterized a Yap1-related protein of *U. maydis*. We show that it plays an important function for growth under oxidative stress conditions and is required for full virulence.

**RESULTS**

**Identification and Characterization of an AP-1 Homolog in *U. maydis***

A search for AP-1-related proteins in the *U. maydis* genome (Munich Information Center for Protein Sequences *Ustilago maydis* Database, http://mips.gsf.de/genom/proj/ustilago) revealed six predicted open reading frames that showed similarity to the bZIP motif (um00567, um01513, um02191, um04976, um10256, and um11176), but of these only um02191 also displayed similarity to the CRDs (Figures 1B to 1D). Additionally, in between the two CRDs, the *U. maydis* protein contains a hydropathic consensus nuclear export sequence that is characteristic for AP-1-like transcription factors and allows the binding of export substrates to Crm1p (Figure 1A) (Yan et al., 1998). In Yap1p of *S. cerevisiae*, such a motif colocalizes with the c-CRD domain (Figure 1A). Outside these conserved domains the AP-1-like protein from *U. maydis*, termed Yap1 (the respective gene is termed yap1), displays weak similarity to other Yap1 proteins (data not shown). The yap1 open reading frame is not expected to be interrupted by introns and encodes a protein of 758 amino acids that is predicted to be localized in the nucleus (http://psort.nibb.jp).

**Under Oxidative Stress Conditions, *U. maydis* yap1 Complements the Growth Defect of a yap1 Mutant of *S. cerevisiae***

To assess the function of *U. maydis* yap1, we analyzed whether yap1 can replace yeast YAP1. To this end, the full-length yap1 gene from *U. maydis* was expressed under control of the GAL4 promoter in an *S. cerevisiae* Δyap1 strain. As controls, the Δyap1 strain was transformed either with empty vector or with the full-length YAP1 gene from *S. cerevisiae* (see Methods). When serial dilutions of all strains were spotted on glucose- or galactose-containing medium to activate the expression of GAL4, no growth differences were observed (Figure 2). On glucose-containing medium to which 0.8 mM hydrogen peroxide was added, only the wild-type strain could grow (Figure 2). On medium containing galactose and hydrogen peroxide, the wild type and the Δyap1/pYESUst (expressing *U. maydis* yap1) and Δyap1/pYES2ac strains (expressing the *S. cerevisiae* YAP1 gene) formed colonies, while growth of the Δyap1 mutant and Δyap1/pYES2c (carrying the empty vector) was significantly inhibited (Figure 2). This indicates that the ability of *S. cerevisiae* to cope with H$_2$O$_2$ stress can be complemented by yap1 of *U. maydis*. However, as judged from the size of single colonies formed, the complementation by yap1 from *U. maydis* is less efficient than complementation by *S. cerevisiae* YAP1 (Figure 2, bottom right panel).

**yap1 Affects Survival of *U. maydis* under Oxidative Stress Conditions***

To analyze the cellular functions of yap1 in *U. maydis*, we constructed yap1 deletion derivatives of the compatible haploid strains FB1 and FB2. In these strains, the yap1 gene was replaced with a transcriptional fusion of *Pyap1*-enhanced green fluorescent protein (eGFP) and a hygromycin resistance cassette (see Methods for details). By DNA gel blot analysis, it was shown that in approximately one-third of the transformants an expected homologous recombination event had occurred (data not shown). To verify that the described phenotypes are caused by yap1 from *U. maydis* we analyzed whether the deletion mutants produced a brown pigment with unknown composition (Figure 3A). In liquid complete medium (CM) with glucose, the growth rate of FB2 and FB2Δyap1 was indistinguishable; however, when 10 mM H$_2$O$_2$ was added, FB2Δyap1 was unable to grow while FB2 still could proliferate (data not shown). To verify that the described phenotypes are caused by the deletion of yap1, one copy of the yap1 gene under the control of its own promoter was inserted in the carboxin locus (*cbx*) of the FB2Δyap1 and FB1Δyap1 strains. These strains no longer produced the brown pigment and showed resistance to hydrogen peroxide comparable to wild-type strains (FB2Δyap1/yap1; Figure 3; data not shown), illustrating that the phenotypes associated with deletion of the yap1 gene can be complemented by introducing a single copy of yap1.
Figure 1. Domain Organization of Yap1.

(A) Yap1p of S. cerevisiae and Yap1 of U. maydis are compared. The vertical gray lines indicate the position of Cys residues. NES (dark-gray bar), nuclear export sequence; aa, amino acids.

(B) Alignment of the bZIP domain of AP-1–like proteins from U. maydis, Neurospora crassa, S. cerevisiae, S. pombe, C. albicans, and C. heterostrophus. Accession numbers for these proteins are in Methods. Uppercase letters indicate identity in all proteins compared, and lowercase letters indicate that three or more proteins carry this residue.

(C) Alignment of the n-CRD domains of proteins listed in (A). Shading follows the scheme given in (A).

(D) Alignment of the c-CRD domains of proteins listed in (A). Numbers give amino acid positions in the respective proteins.
was exposed to different concentrations of H2O2. Fluorimetric
FB2. With respect to sensitivity to H2O2, these strains were
To study the regulation of
The Cellular Localization of Yap1 Is Modulated by H2O2
The growth of S. cerevisiae BY4742 (WT), S. cerevisiae BY4742-
mutant strains were used to infect maize seedlings, viru-
S. cerevisiae. YAP1 is activated during the early phases of biotrophic growth
The Deletion of yap1 Attenuates Virulence
When spotted on charcoal potato dextrose (PD) medium, mix-
Biotrophic Growth
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development of *U. maydis* suggests that the role of yap1 during pathogenesis might be confined to these stages.

**Cys-399 and Cys-407 Are Crucial for Functionality of Yap1**

In *S. cerevisiae*, nuclear export of Yap1p is blocked by the formation of two disulfide bridges, and this is required for full transcriptional activity in response to H$_2$O$_2$ (Kuge et al., 2001). To assess whether such a mode of action also applies to *U. maydis*, we generated a mutant yap1 allele in strains FB1 and FB2 in which two Cys residues in the n-CRD domain were substituted by Ala, yap1$^{C399A\ C407A\ :\ e3GFP}$. The respective Yap1$^{C399A\ C407A\ :\ e3GFP}$ fusion protein showed cytoplasmic localization, both in the absence and presence of 1 mM H$_2$O$_2$ (Figure 7A; data not shown). Protein gel blot analyses demonstrated that the respective mutant Yap1 protein is expressed under both conditions (Figure 7B). With respect to H$_2$O$_2$ sensitivity and production of the brown pigment, these mutant strains behaved like Δyap1 strains (Figures 3A and 3B). The behavior of the FB1yap1$^{C399A\ C407A\ :\ e3GFP}$ and FB2yap1$^{C399A\ C407A\ :\ e3GFP}$ strains in planta (Figure 7C) was comparable to the Δyap1 strains, and with respect to wild-type strains, a significant reduction in virulence was...
observed (Figure 7C). In the mutant strains, the fusion protein showed cytoplasmic localization throughout the infection (data not shown), indicating that there are no other means to activate Yap1p under these conditions. From these results, we infer a crucial role of the disulfide bridge between Cys-399 and Cys-407 for the function of Yap1 in *U. maydis*.

*U. maydis* yap1 Prevents the Accumulation of ROS

To gain insights into the mechanism that leads to the activation of Yap1 during the early plant colonization stages, we have analyzed the production of H$_2$O$_2$ in leaves of maize seedlings infected with *U. maydis*. To detect H$_2$O$_2$, we used the diamino benzidine (DAB) uptake technique (Thordal-Christensen et al., 1997; Fryer et al., 2002). In the presence of H$_2$O$_2$, DAB is converted to dark-brown polymers. In infections with wild-type strains, we have not seen an accumulation of DAB polymers, irrespective of the stage at which hyphae were analyzed (Figures 8A to 8C; data not shown). By contrast, an accumulation of H$_2$O$_2$ was observed in the vicinity of Δyap1 hyphal tips already during penetration (Figure 8A). H$_2$O$_2$ could be detected around the Δyap1 hyphae during the first 3 d after infection (Figures 8A to 8C, right panels; data not shown). During these stages, hyphal tips of Δyap1 strains often appeared swollen (Figures 8B and 8C, right panels), while hyphal tips from wild-type strains were straight (Figures 8B and 8C, left panels). DAB staining decreased at later time points and became undetectable during proliferation in tumor tissue and spore maturation (data not shown). These results suggest that in wild-type strains, yap1 is responsible for preventing the accumulation of H$_2$O$_2$ in the vicinity of hyphae during early stages of biotrophic growth.

The Inhibition of Plant NADPH Oxidase Restores Virulence of yap1 Mutant Strains

To determine whether the accumulation of H$_2$O$_2$ observed around yap1 mutant hyphae originates from the host, we applied diphenylene iodonium (DPI), an inhibitor of NADPH oxidase (Morre, 2002), together with the inoculum of *U. maydis* strains. Neither this compound nor its solvent DMSO affected the sensitivity of *U. maydis* wild-type strains or the Δyap1 mutant to H$_2$O$_2$ (data not shown). Plant NADPH oxidases are involved in the production of reactive oxygen intermediates in response to pathogens (Torres and Dangl, 2005). *U. maydis* lacks genes related to gp91phox (Aguirre et al., 2005), the transmembrane catalytic subunit of mammalian, plant, and fungal NADPH oxidases that catalyze the conversion of molecular oxygen to superoxide (Torres et al., 2002; Lambeth, 2004; Tanaka et al., 2006). Two days after maize seedling infection, H$_2$O$_2$ visualized by DAB staining was found in the vicinity of Δyap1 hyphal tips, while H$_2$O$_2$ production was no longer observed in the vicinity of Δyap1 hyphae when DPI was added to the inoculum. To exclude adverse effects of DPI, wild-type strains were also infected with DPI added (Figure 9A). In the infections with compatible wild-type strains, no H$_2$O$_2$ accumulation was detectable, fungal development visualized by microscopy was normal (Figure 9A), and disease symptoms were unaffected by DPI addition (Figure 9B). Interestingly, however, the presence of DPI in the inoculum of the compatible yap1 mutant strains prevented the appearance of bulbous hyphae and lead to an increase in virulence, reaching levels comparable to infections with wild-type strains (Figure 9B).

These results indicate that the H$_2$O$_2$ accumulated around yap1 mutant hyphae is produced by the host. The finding that inhibiting this accumulation restores virulence implies that Yap1 in...
Identification of Yap1 Target Genes by Microarray Analysis

To analyze in more detail the role of Yap1 in the detoxification of ROS, transcript profiles of strains FB1 and FB1Δyap1 were generated from cells grown in CM in the absence or presence of 5 mM H$_2$O$_2$ using the Affymetrix U. maydis DNA arrays. A set of 221 genes was downregulated in the comparison of FB1Δyap1/FB1 with a fold change of >1.5 (see Supplemental Table 1 online). The majority of these Yap1-dependent genes (203) was upregulated in FB1 when exposed to H$_2$O$_2$ (see Supplemental Table 1 online). Typical Yap1 binding sites TT/GAC/GT/CA/A (Harshman et al., 1988; Fernandes et al., 1997; Toone and Jones, 1999) were found in the promoter regions of 212 of these genes (see Supplemental Table 1 online). With respect to oxidative stress, the Yap1-regulated genes fall

U. maydis plays a prominent role in the detoxification of these molecules during plant colonization.
into three categories, namely, ROS detoxifying enzymes, such as peroxidases and catalases, genes involved in the biosynthesis of low molecular mass antioxidants (ascorbic acid, glutathione, tocopherols, NADH, and NADPH), and genes encoding enzymes regenerating the reduced forms of antioxidants (reviewed in Blokhina et al., 2003). Among the genes putatively involved in the detoxification of ROS were the haeme peroxidase um10672, the cytochrome C peroxidase um01947, and the alkyl hydroperoxide reductase um02153. Among the Yap1-regulated genes involved in antioxidant function, we detected um04930 and um02592 encoding glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, respectively, which are both involved in the generation of NADPH. Other Yap1-regulated genes are involved in the metabolism of antioxidants like glutathione (um05561, coding for a glutathione S-transferase), ascorbate (um04922, coding for a diketogulonate reductase), and folate (um02010, coding for

Figure 6. Subcellular Localization of Yap1 during the Life Cycle of U. maydis.

(A) Compatible strains FB1yap1:3XeGFP and FB2yap1:3XeGFP were inoculated in maize seedlings. Hyphae and appressoria on the leaf surface were visualized by calcofluor staining 16 h after infection.
(B) GFP fluorescence of the same leaf area shown in (A). Hyphae that have not yet penetrated do not show fluorescent nuclei (left panel). Hyphae that have penetrated show fluorescent nuclei (arrowheads), and these could be visualized in a different focus plane, indicating that the appressoria shown in (A) had already penetrated.
(C) Fluorescent nuclei (arrowheads) in intracellularly growing hyphae 2 d after infection.
(D) Intracellular hyphae with diffuse fluorescence 6 d after infection.
Bars = 10 μm.
a 2-keto-3-deoxy-D-arabino-heptulosonate 7-phosphate synthase). Among putative enzymes regenerating the reduced form of antioxidants, we detected um04578 coding for thioredoxin 2 as a Yap1-regulated gene. Besides these genes presumably directly involved in coping with oxidative stress, a large number of genes related to DNA stability and repair, protein folding and elimination of damaged proteins, transport and drug resistance, signaling, cell wall and membrane components, and lipid metabolism were upregulated after H$_2$O$_2$ treatment. The majority of these proved to be Yap1 dependent in their expression (see Supplemental Table 1 online).

**Yap1 Regulates the Expression of Two Peroxidase Genes**

Among the Yap1-regulated genes identified were genes encoding cytochrome C peroxidases precursor (um01947) and haeme peroxidase protein (um10672). In addition, *U. maydis* possesses one gene (um11067) related to catalase CTT2 of *S. cerevisiae* and one additional gene coding for a cytochrome C peroxidase precursor (um02377), neither of which was regulated by yap1 according to the array analysis. To verify the array data, RNA gel blot analyses were performed. These showed that the expression of um01947 and um10672 was upregulated in the *U. maydis* wild type after addition of H$_2$O$_2$, while the expression of um02377 and um11067 was not altered significantly (Figure 10). The identity of the transcripts seen with probes for um01947 and um02377 was verified using RNA from respective deletion strains (Figure 10). In the Δyap1 background without the addition of H$_2$O$_2$, genes um01947 and um10672 were barely expressed, while after H$_2$O$_2$ treatment, transcript levels increased but did not reach the levels found in the respective wild-type strain (Figure 10). This indicates that these genes might be targets of Yap1. However, to explain the induction seen after H$_2$O$_2$ treatment, both genes must be additionally regulated by a yap1-independent mechanism. The expression levels of um02377 and um11067 were not significantly altered under these conditions (Figure 10).

**A Fungal Peroxidase Gene Is Involved in the Detoxification of ROS**

To assess whether the putative yap1 target genes um01947 and um10672 play an active role in the detoxification of ROS, individual deletion mutants were generated in SG200. With respect to H$_2$O$_2$ sensitivity, SG200Δum10672 was even more sensitive than SG200Δyap1 (data not shown), indicating that the product of this gene assumes the main responsibility for detoxification of ROS in *U. maydis*. SG200Δum01947 showed higher sensitivity to H$_2$O$_2$ than SG200 but did not reach the sensitivity level of SG200Δyap1 (data not shown), indicating a minor role of this gene in ROS degradation.

To investigate the contribution of um01947 and um10672 to virulence, SG200Δum01947 and SG200Δum10672 were inoculated in maize seedlings. Twelve days after infection, both mutant strains showed a reduction in virulence relative to SG200 comparable to SG200Δyap1 (data not shown), indicating a minor role of this gene in ROS degradation.

**DISCUSSION**

In this work, we have characterized the *U. maydis* yap1 gene. Similar to Yap1p in *S. cerevisiae*, its product contains a bZIP domain and two CRDs. *U. maydis* yap1 complements the hydrogen peroxide–sensitive phenotype of a yeast yap1 mutant. The
finding that *U. maydis* yap1 mutants are more sensitive to H$_2$O$_2$ than the progenitor strains, the observed nuclear localization after H$_2$O$_2$ exposure, and the ability to complement the yeast yap1 mutant phenotype suggests that the *U. maydis* Yap1 protein also functions as a redox sensor that is activated by intramolecular disulfide bridge formation. This is additionally supported by the phenotype of a yap1 mutant in which two Cys residues, Cys-399 and Cys-407, likely to be involved in disulfide bridge formation, are substituted by Ala. This mutant protein fails to be retained in the nucleus after H$_2$O$_2$ treatment, and in its behavior, this mutant is indistinguishable from a strain where yap1 is deleted. In the *U. maydis* genome, genes related to *S. cerevisiae* ORP1/GPX3, encoding a glutathione peroxidase-like enzyme, and TPX1, encoding an upstream activator of the redox sensor Pap1 in *S. pombe* (Vivancos et al., 2005), are found. Thus, it is likely that Yap1 in *U. maydis* is posttranslationally activated by one of these activities.

Compatible Δyap1 strains were indistinguishable from wild-type strains in their ability to mate, to develop dikaryotic filaments, and to form appressoria. However, they showed increased sensitivity to H$_2$O$_2$ and were severely attenuated in virulence (i.e., they formed fewer and smaller tumors). This most likely reflects problems during the early post-penetration stages, since 2 d after infection, a significant reduction of fungal biomass was observed in plants infected with the Δyap1 mutant compared with infections with wild-type strains. Additionally, the swollen tips of yap1 mutant hyphae after penetration are likely to indicate some stress response, which could be related to the observed reduction in fungal biomass. A Yap1:3XeGFP fusion protein localized to the nucleus immediately after penetration, and this activation was maintained for 2 to 3 d and then declined and became undetectable at late stages when massive fungal proliferation takes place and spore development is initiated. This suggests that Yap1 is needed primarily during the early stages of biotrophic growth where its presence allows more efficient colonization. In line with this reasoning, H$_2$O$_2$ accumulation was detected around yap1 mutant hyphae only at those time points when Yap1 protein localized to the nucleus in wild-type strains.

The generation of ROS is a hallmark of successful recognition of plant pathogens by the host (Nürnberg et al., 2004). This plant response can be elicited by fungal, bacterial, and viral pathogens and serves to activate plant defense programs (Torres and Dangl, 2005). The absence of H$_2$O$_2$ accumulation

**Figure 8.** H$_2$O$_2$ Accumulation during the Early Stages of Biotrophic Growth of *U. maydis* Δyap1 Strains.

(A) Plant samples inoculated with either mixtures of FB1xFB2 (left panel) or FB1Δyap1xFB2Δyap1 strains (right panels) were stained with calcofluor and DAB 1 d after inoculation for visualization of hyphae and appressoria on the leaf surface by light microscopy using the 4',6'-diamidino-2-phenylindole filter. The closed arrowheads mark DAB precipitates.

(B) Samples were stained with DAB only, and intracellular hyphae were visualized by light microscopy 1 d after infection. DAB precipitates are marked with closed arrowheads. Intracellularly growing hyphae not surrounded by DAB precipitate are marked with open arrowheads.

(C) Samples were as in (B) 2 d after infection.

Bars = 10 μm.
infections with wild-type *U. maydis* strains and the strong accumulation of H$_2$O$_2$ around hyphae of yap1 deletion mutants indicate that Yap1 participates effectively in the detoxification of ROS. Since *U. maydis* does not code for genes related to NADPH oxidases (Aguirre et al., 2005), the enzymes responsible for ROS generation are likely to be of plant origin. The absence of NADPH oxidase genes in *U. maydis* is unusual, as most filamentous fungi have two or three NADPH oxidase isoforms (Lalucque and Silar, 2003). These proteins all have the structural core domains found in the animal gp91phox (Aguirre et al., 2005). *noxA* in *Podospora anserina* and *Aspergillus nidulans* is specifically induced and required during differentiation of sexual fruiting bodies (Lara-Ortíz...
et al., 2003; Malagnac et al., 2004). In the mutualistic *Epichloë festuca*/ryegrass (*Lolium perenne*) interaction, the deletion of noxA in the fungus changes the interaction of this biotrophic endophyte with its host from mutualistic to antagonistic (Tanaka et al., 2006). The noxA mutant accumulates significantly more biomass in infected hosts, and it has been speculated that ROS produced by NoxA during plant colonization negatively regulates hyphal tip growth, thereby preventing excessive colonization due to restricted growth of the fungus (Tanaka et al., 2006). The situation in the biotrophic interaction of *U. maydis* with its host is fundamentally different. *U. maydis* does not possess an NADPH oxidase but is likely to use its redox sensor Yap1 to detoxify H₂O₂ produced by the plant in response to being recognized. In the absence of yap1, H₂O₂ accumulates in the vicinity of fungal hyphae, and since the mutant is unable to detoxify it, this could negatively affect fungal proliferation in the infected tissue. We consider the finding that the inhibition of NADPH oxidase by DPI restores virulence to yap1 mutants as additional support for the assertion that it is the production of ROS by the plant that attenuates virulence of *U. maydis* yap1 mutants, although we cannot formally exclude that DPI modulates the activity of other enzymes. However, these are then not likely to be of fungal origin as the treatment of the Δyap1 mutant with DPI did not affect its sensitivity to H₂O₂. Thus, the Yap1-controlled ROS detoxification system serves an important function during the early *U. maydis* infection phase. In the *Claviceps purpurea*/rye interaction it has been demonstrated that the deletion of a CREB-like transcription factor, CPTF1, which positively controls catalase production, also induces an oxidative burst (Nathues et al., 2004) similar to what we have observed in yap1 mutants of *U. maydis*. However, in this case, it is speculated that CPTF1 negatively controls the activity of a fungal NADPH oxidase (i.e., when CPTF1 is deleted, the fungus would produce elevated levels of H₂O₂, which then in turn would trigger the oxidative burst) (Nathues et al., 2004).

The identification of Yap1-regulated genes by array analysis has revealed the same functional categories of genes that were found to be regulated through Yap1p in *S. cerevisiae* (Dumond et al., 2000). Among these genes were two Yap1-regulated peroxidase genes, um01947 and um10672, which we considered likely to be involved ROS detoxification. Interestingly, single mutations in either gene showed reduced virulence, comparable to the yap1 deletion strain. For the Δum10672 mutant, we could show that ROS accumulate around invading hyphae, while this was not observed for Δum01947 mutants. Since *U. maydis* lacks catalases like the *YAP1*-regulated CTT1 in *S. cerevisiae* (He and Fassler, 2005), we consider the Yap1-regulated heme peroxidase encoded by um10672 as prime activity for ROS detoxification in the *U. maydis*/maize system. The protein encoded by um01947 is predicted to have a mitochondrial targeting sequence; therefore, its function is likely to be confined to mitochondria. Mitochondrial integrity has already been show to be crucial for pathogenicity (Bortfeld et al., 2004).

The nuclear localization of Yap1 during the early stages of plant colonization in wild-type strains suggests that *U. maydis* may trigger a transient oxidative burst, which is sufficient to allow activation of Yap1 but may not be strong enough to allow its visualization by DAB staining. It is likely that at later time points there is no oxidative burst, as this should have resulted in continued nuclear localization of Yap1. Recent experiments...
indicate that secreted proteins of *U. maydis* are actively involved in suppressing plant defense responses (K. Schipper, T. Brefort, G. Doehlemann, K. Münch, and R. Kahmann, unpublished data). This situation in *U. maydis* contrasts with findings in the necrotrophic plant pathogen *C. heterostrophus* where a *YAP1*-related gene, *CHAP1*, has been studied (Lev et al., 2005). *CHAP1* deletion mutants were also more sensitive to hydrogen peroxide, but their virulence was unaffected. *CHAP1* showed nuclear localization already in conidal germ tubes on the leaf surface, and this localization persisted throughout the infection. Since nuclear localization of *CHAP1* was only observed at high H$_2$O$_2$ concentrations that were not detected in plant extracts and since plant extracts triggered nuclear localization even when H$_2$O$_2$ was eliminated, it has been speculated that *CHAP1* is induced by an as yet unidentified plant compound and serves to adapt the redox state of the cell to the plant environment (Lev et al., 2005). Our finding that strains carrying the mutant allele *yap1C399A C407A::e3GFP* do not show nuclear localization of the fusion protein and behave like the *yap1* deletion mutant with respect to reduced virulence suggests that activation of Yap1 in *U. maydis* requires H$_2$O$_2$ and this cannot be bypassed by plant compounds. For *C. heterostrophus*, one could hypothesize that the levels of ROS encountered by this necrotrophic pathogen during growth may be insufficient to damage the fungus, and this would explain why *CHAP1* is not needed for virulence. In this respect, it is of interest that induction of the hypersensitive response by necrotrophic fungi like *Botrytis cinerea* and *Sclerotinia sclerotiorum* actually facilitates infection (Govrin and Levine, 2000). It is thus an attractive possibility that such a situation also exists in *C. heterostrophus* (i.e., in this scenario, it would not be in the interest of the pathogen to detoxify ROS, as the fungus would require dead plant tissue for proliferation). Conversely, biotrophic pathogens, like *U. maydis*, might be more sensitive to the detrimental effects of ROS during infection and depend on the *yap1* system at least during the early infection stages. In the future it will be very interesting to analyze how ROS production and detoxification systems in different fungi that live in close association with plants determine the outcome of the respective interactions and whether specific strategies are used depending on the type of interaction.

**Figure 11.** The Two Yap1-Regulated Peroxidases Affect Virulence.

(A) Plants were infected with the strains indicated below each column. Disease rating followed the scheme described in Figure 5.
(B) DAB staining of infected leaves 2 d after infection with the same strains assayed in (A). Intracellular hyphae are visualized by light microscopy and are marked by open arrowheads. DAB precipitates in the vicinity of the hyphae are marked by closed arrowheads. Bars = 10 μm.
METHODS

Strains and Growth Conditions

The Escherichia coli K12 derivatives DH5α (Bethesda Research Laboratories) and Top10 (Invitrogen) were used for cloning purposes. Saccharomyces cerevisiae strains were grown in SD medium supplemented with the necessary amino acids (Yeast Protocols Handbook; Clontech) and the necessary amino acids (Yeast Protocols Handbook; Clontech) and Top10 (Invitrogen) were used for cloning purposes.

Escherichia coli Strains and Growth Conditions

 Yeast standard yields 30% [w/v] in liquid CM (Holliday, 1974), YEPSL (0.4% yeast extract, 0.4% peptone, and 2% sucrose), or PD (2% PD broth [Difco]) medium or solid PD agar.

inoculated in CM medium and cultured overnight, diluted 1:10 into fresh CM medium, and grown to an OD of 0.6. H2O2 was added in the concentrations indicated in each experiment. To assay H2O2 sensitivity, U. maydis strains were plated on PD or PD medium supplemented with 0.5 μM DPI. Filter disks of Whatman paper (5 mm) were soaked with 1 or 2 μL of H2O2 (30% [w/v]) and placed on the plates. The halo sizes were measured in four duplicates after 48 h of incubation.

Mating assays were performed by cospotting compatible strains onto PD plates containing 1% charcoal. Subsequent incubation was done at 21°C (Holliday, 1974).

Pathogenicity assays were performed as described (Kämper et al., 2006). For plant infections, the U. maydis strains were grown to an OD of 0.6 to 0.8 in YEPLS medium, centrifuged, and resuspended to an OD of 1 and injected into young maize (Zea mays) (Kämper et al., 2006), its derivatives were grown as indicated at 28°C in liquid CM (Holliday, 1974); YEPSL (0.4% yeast extract, 0.4% peptone, and 2% sucrose), or PD (2% PD broth [Difco]) medium or solid PD agar.

Deletion and fusion constructs were generated according to Kämper (2004). To generate a transcriptional fusion of the yap1 promoter to egfp, a 1.0-kb fragment comprising the 5′ flank and a 1.0-kb fragment comprising the 3′ flank of the yap1 open reading frame were generated by PCR on U. maydis FB1 genomic DNA with primer combinations 5′-GCAAGGTGTCTGGAACACTGAAG-3′/5′-GGCGGCGGGTTGCGCGCTGTAAAGATACCCAG-3′ and 5′-GGCGGGCTGAGTGCCCGAAATCAAACCGGAAGATG-3′/5′-CACCTCAGAATCAGTCCAGAG-3′, respectively. These fragments were then digested with SfiI and ligated to the 3.7-kb SfiI eGFP-hygromycin resistance cassette from pUMa229 (Brachmann et al., 2004). The resulting ligation product was cloned into PCR2.1-TOPO.

The flanks of plasmid, yap1-eGFP-hyg, were sequenced and shown to match the wild type. This plasmid was subsequently used as a template to amplify the yap1 deletion construct with primers 5′-CGAGAGGTTGCGGGTTAATCGAAG-3′ and 5′-CAACTCGTGAAATCGAAG-3′. This fragment was then used in transformation to generate the yap1 deletion mutants of strains FB1, FB2, and SG200. Deletion derivatives were identified by DNA gel blot analysis. To generate the transcriptional fusion between yap1 and three copies of the egfp gene, a 1.0-kb fragment comprising from 1285 to 2274 bp of the yap1 open reading frame and a 1.0-kb fragment comprising the 3′ flank of yap1 were generated by PCR on U. maydis FB1 DNA using primer combinations 5′-GTTGCACTGCAATCCACTC-3′/5′-CAGGTGGGTTGCCGGTGGTGTCGTCCCTTGCGGCA-3′ and primer combinations 5′-GGCGGGCTGAGTGCCCGAAATCAAACCGGAAGATG-3′/5′-CACCTCAGAATCAGTCCAGAG-3′, respectively. These fragments were then digested with SfiI and ligated to the 5.2-kb SfiI fragment from the plasmid pUMa647 (K. Zarnack, unpublished data) containing 3XeGF and the hygromycin resistance cassette. The resulting ligation product was cloned into PCR2.1-TOPO to yield yap1-3XeGFP. Border regions of this plasmid were sequenced and shown to match the wild type. This plasmid was subsequently used as a template to amplify the yap1-3XeGFP construct with primers 5′-GTTGCACTGCAATCCACTC-3′ and 5′-GACCTTACAGAATCAGTCCAGAG-3′, respectively. This fragment was transformed in strains FB1, FB2, and SG200. Homologous integration was verified by DNA gel blot analysis.

To generate the transcriptional fusion between yap1 and three copies of the egfp gene carrying the mutation C99A and C407A, a 0.8-kb fragment of the yap1 open reading frame was generated by PCR on U. maydis FB1 DNA using primer combinations 5′-AAGTGGGCATCTTCTGTCGGAGGAGGCTGTGGCTTT, respectively. These fragments were then digested with BglII and cloned into the yeast expression vector pYES2 (Invitrogen), and transformed into BY4742 by electroporation. Colonies were selected on synthetic CM lacking uracil. As a control, these strains were also transformed with empty pYES2 vector. Transformed yeast cells were grown on synthetic CM without uracil containing either glucose 3% (w/v) or 2% galactose and 2% raffinose, respectively. Five-microliter drops from serial dilutions from cultures with an OD of 0.5 were spotted on plates with and without 0.8 mM H2O2 and grown for 2 d at 30°C. This experiment was repeated three times.

Plasmids and Strain Construction

Plasmid pCR2.1TOPO (Invitrogen) was used for cloning and sequencing of fragments generated by PCR. Sequence analysis of genomic fragments and fragments generated by PCR was performed with an automated sequencer (ABI 377; Applied Biosystems) and standard bioinformatic tools.

Deletion and fusion constructs were generated according to Kämper (2004). To generate a transcriptional fusion of the yap1 promoter to egfp, a 1.0-kb fragment comprising the 5′ flank and a 1.0-kb fragment comprising the 3′ flank of the yap1 open reading frame were generated by PCR on U. maydis FB1 genomic DNA with primer combinations 5′-GCAAGGTGTCTGGAACACTGAAG-3′/5′-GGCGGCGGGTTGCGCGCTGTAAAGATACCCAG-3′ and 5′-GGCGGGCTGAGTGCCCGAAATCAAACCGGAAGATG-3′/5′-CACCTCAGAATCAGTCCAGAG-3′, respectively. These fragments were then digested with SfiI and ligated to the 3.7-kb SfiI eGFP-hygromycin resistance cassette from pUMa229 (Brachmann et al., 2004). The resulting ligation product was cloned into PCR2.1-TOPO.

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of the um10672 open reading frame were generated by PCR on *U. maydis* FB1 genomic DNA with primer combinations 5'-AAGTTGTGCGCCTG-CAGGC-3'/5'-GATCGGCTAGCTCAGGAACTCC-3' and 5'-GATCGGCTAGCTCCATTGACAGGACGG-3'/5'-ATG-TGGGCGAAGTCTACTCC-3', respectively. These fragments were then digested with SfiI and ligated to the 2.7-kb SfiI hygromycin resistance cassette from pBS-Hyg(+) (Keon et al., 1991). The ligation products were transformed into strain SG200. Homologous integration was verified by DNA gel blot analysis.

For amino acid comparisons with Yap1, the following proteins were used: *Neurospora crassa* (GenBank accession number NP_013707), *S. cerevisiae* (GenBank accession number NP_593662), *Candida albicans* (GenBank accession number EAL02784), and *Cochliobolus heterothal-asmus* (GenBank accession number AY486158).

**U. maydis yap1 Complementation**

A fragment of 3.7 kb, containing the yap1 gene from *U. maydis* and 1 kb of its promoter region, was amplified by PCR using FB1 genomic DNA as template and the primer combination 5'-AGGCGGAGTACTACTCC-3'/5'-GATCGGGCTAGCTCAGGAACTCC-3'. This fragment was subcloned into pCP2-1-TOPO giving the plasmid pYAP1st. The DNA fragment containing the yap1 gene and its promoter region were sequenced and subcloned as a KpnI-EcoRV fragment into plasmid pBS-Cbx(+)(Keon et al., 1991), yielding plasmid pBS-Cbx-yap1. This plasmid was transformed into *U. maydis* strains FB1 yap and FB2 yap. Carboxin-resistant clones were recovered, and single integration of the yap1 gene in the carboxin locus was confirmed by DNA gel blot analysis.

**Tagging of Nuclei with Triple RFP**

To visualize nuclei, the strains FB1 yap1-3XeGFP and FB2 yap1-3XeGFP were transformed with the integrative plasmid pANNE3090 (kindly provided by A. Straube and G. Steinberg) carrying a fusion between the strong Potef promoter, a nuclear localization sequence, a triple RFP gene (Potef-NLS-3RFP), and a carboxin resistance marker. These strains are designated FB1 yap1-3XeGFPcbx:pANNE3090 and FB2 yap1-3XeGFPcbx:pANNE3090. Single integrations in the carboxin locus were verified by DNA gel blot analysis.

**DNA and RNA Procedures**

Standard molecular techniques were used (Sambrook et al., 1989). Transformation of *U. maydis* was performed as published previously (Schulz et al., 1990). *U. maydis* DNA was isolated as described (Hoffman and Winston, 1987). RNA was isolated following the TRIZOL reagent protocol (Invitrogen). Probes for detecting transcripts from the peroxi-dase genes were generated by PCR and verified by diagnostic digestions. To generate the probe for the um01947 gene, a 0.9-kb fragment was generated using the primer combination 5'-CTCTCTTCTAAGTGTCGGAAG-3' and 5'-AACGTGATATGACGGGAAAG-3'. To generate the probe for the um02377 gene, a 0.9-kb fragment was generated using the primer combination 5'-AACGTGATATGACGGGAAAG-3' and 5'-ATGGGCGCCTG-AATAGTGCG-3'. To generate the probe for um02377 gene, a 0.9-kb fragment was generated using the primer combination 5'-AAGTTTTTCAGCTTGGAACCC-3' and 5'-ATGGGCGCCTG-AATAGTGCG-3'. To generate the probe for um10672 gene, a 1.0-kb fragment was generated using the primer combination 5'-CTTCTTGCTGACAGGCACCC-3' and 5'-ATGGGCGCCT-GATGTGGCC-3'. To generate the probe for um11067 gene, a 0.8-kb fragment was generated using the primer combination 5'-CTCTCTTCTAAGTGTCGGAAG-3' and 5'-AACGTGATATGACGGGAAAG-3'. Probes were labeled by the PCR DIG labeling kit following the manufacturer’s instructions (Roche).

**Immunodetection**

Immunoblot analysis was performed as described (Basse et al., 2000) using a monoclonal GFP IgG mouse antibody (Roche).

**Sample Preparation and Microarray Analysis**

Two independent overnight cultures of *U. maydis* FB1 and FB1 yap1 grown in CM-glucose (OD600 of 0.8) were diluted in 100 mL of the same medium (OD600 of 0.2) and grown at 28°C until an OD600 of 0.6. The cultures were divided, and one half was supplemented with 5 mM H2O2. After 1 h of exposition to H2O2, cells were harvested by centrifugation and frozen in liquid nitrogen. RNA extraction, purification, cDNA generation, purification, and labeling were performed according to standard protocols (Affymetrix). DNA array analysis was performed on two biological replicates each, using custom-designed Affymetrix chips (MPUstilagio). Data were analyzed using a GeneArray Scanner (Agilent/Affymetrix) and GeneChip Expression Analysis software (GCOS) Microarray Suite 5.0 (Affymetrix) as described (Eichhorn et al., 2006). Data analysis was performed using the Biconductor R package (http://www.biocductor.org) as described (Eichhorn et al., 2006). The P values for the coefficients/contrasts of interest were adjusted for multiple testing by the false discovery rate method (Benjamini and Hochberg, 1995). For the data set of Yap1-regulated genes, genes were filtered by applying the following criteria: genes should be at least 1.5-fold downregulated in the comparison of FB1 yap1+: 5 mM H2O2 with FB1 + 5 mM H2O2 and have a corrected P value of <0.05 in the biological duplicates analyzed.

**Calcofluor and Chlorazole Black E Staining**

Calcofluor staining using Fluorescent Brightener 28 (Sigma-Aldrich) for the microscopy of prepenetration stages of *U. maydis* and Chlorazole Black E staining for microscopy of post-penetration stages were performed as described (Brachmann et al., 2003).

**Histochemical Detection of H2O2**

H2O2 production was visually detected in infected plants using DAB as substrate (Orozco-Cárdenas and Ryan, 1999). Briefly, plants were decapatied at the base of the stem with a razor blade and placed in a 1-mg/mL solution of DAB for 16 h under darkness at room temperature. Leaves were decolorized by immersion in ethanol (96%) for 48 h. Brown polymerization products that result from the reaction of DAB with H2O2 were microscopically identified in regions at least 1 cm above the immersed plant parts.

**DPI Treatment**

DPI solved in DMSO was added at final concentrations of 0.5 μM directly to the mixtures of compatible *U. maydis* strains prior to inoculation. As control, DMSO alone was added to the mixtures of compatible *U. maydis* strains.

**Microscopy Observation**

For microscopy observation, a Zeiss Axiopt microscope with differential interference contrast optics was used. Calcofluor fluorescence was observed with a standard 4'6'-diamidino-2-phenylindole filter set. GFP fluorescence was detected with a specific filter set (band-pass 470/20, beam splitter 493, band-pass 505 to 530 nm; Zeiss). RFP fluorescence was detected with standard filters for rhodamine. DAB precipitates were visualized by differential interference contrast optics. Pictures were taken with a CCD camera (Hamamatsu). Image processing was done with Image Pro (Media Cybernetics), Adobe Photoshop 6.0, and Canvas 6.0 (Deneba Systems).

**Accession Numbers**

Sequence data for yap1 can be found in the GenBank/EMBL data libraries under accession number BN000987. The GenBank accession numbers
of the studied peroxidases are XP_758094 (um01947), XP_758524 (um02377), XP_760856 (um10672), and XP_759546 (um11067). The Gene Expression Omnibus number for array data included in this article is GSE7518.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Table 1. List of Genes Regulated by Yap1.

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**Supplemental Data**
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