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The relationship between the antioxidant system and the virulence in *Ustilago maydis*, a fungal pathogen

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ABSTRACT. Fungal cells deal with a wide variety of toxic environmental conditions during their life cycle. An important example of stress challenge to which fungi must cope is the high levels of ROS produced by the host during the oxidative burst. *Ustilago maydis* (D.C.) Corda is an important model system for plant pathogen smut and rust fungi. Genome wide analyses of this pathogen provide an insight into the cellular responses against reactive species in plant-microbe interactions. Little is known about the *U. maydis* antioxidant defense systems or whether constituents of these systems are essential for virulence. This review focuses on the search of sequences involved in oxidative stress response in the *U. maydis* genome. Finally, we compared different defense responses and how these might contribute to the virulence of fungal pathogen.

Key words: Antioxidant system, fungal pathogen, virulence, reactive oxygen and nitrogen species.

RESUMEN. Los hongos durante su ciclo de vida se encuentran continuamente expuestos a condiciones ambientales tóxicas. Un importante ejemplo de estrés es cuando el hongo debe contrarrestar las altas concentraciones de ROS producidas por su hospedero durante el estallido oxidativo. El hongo *Ustilago maydis* (D.C.) Corda es un modelo para el estudio de la interacción planta-patógeno, debido a que el análisis genómico provee un medio para conocer las posibles respuestas celulares del hongo contra las especies reactivas producidas durante su interacción con la planta. Sin embargo, muy poco se conoce acerca de sus sistemas antioxidantes y su relevancia durante la virulencia. Esta revisión se enfoca en la búsqueda de secuencias en el genoma de *U. maydis* involucradas en la respuesta al estrés oxidativo. Finalmente, se comparan diferentes respuestas de defensa y cómo éstas contribuyen a la virulencia de los hongos patógenos.

Palabras clave: Sistema antioxidante, hongo patógeno, virulencia, especies reactivas de oxígeno y de nitrógeno.

INTRODUCTION

Aerobic organisms are constantly exposed to reactive oxygen species (ROS) generated by normal cellular metabolism. The one-electron reduction of oxygen during respiration leads to the formation of superoxide anion radical ($O_2^{\cdot-}$), the main precursor of several ROS such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($^1O_2$). Reactions catalyzed by oxidases can also generate H_2O_2 (Halliwell and Gutteridge, 1989). ROS, and $OH^{\cdot}$ in particular, are highly reactive and cause rapid and deleterious oxidation of proteins, lipids and DNA (Vaughan, 1997). In addition, nitric oxide ($NO^{\cdot}$) can be oxidized into reactive nitrogen species (RNS), which may show a behavior similar to that of ROS. In particular, the combination of $NO^{\cdot}$ and $O_2^{\cdot-}$ yields a strong biological oxidant, the peroxynitrite ($ONOO^-$) (Murphy, 1998). Both oxidative and nitrosative stresses are common features of a variety of environmental conditions (Jones, 2006), that result from imbalance between ROS and RNS production and detoxification by antioxidant systems, leading to disruption of redox signaling and damage of biomolecules. To protect against ROS and RNS, aerobic cells have evolved enzymatic and non-enzymatic mechanisms to overcome these species.

In eukaryotic organisms, the energy required for growth, development, reproduction, and response to external stresses, comes mainly from the hydrolysis of ATP, which is produced to large extent during mitochondrial respiration. A supply of energy is also important for the pathogen's ability to produce disease inside the host environment. Because the production of ROS and RNS is increased during the stress imposed by host's defense system, pathogens are forced to respond with specific detoxifying enzymes to neutralize ROS-RNS (primary defense) and to repair or remove oxidized molecules (secondary defense). Thus, ROS-RNS

Abbreviations: AOX, alternative oxidase; Trx, thioredoxin; Trr, thioredoxin reductase; Prx, thioredoxin peroxidase; GSNO, S-nitrosoglutathione; GSNOr, GSNO reductase; Grx, glutaredoxin; Grr, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; Gpx, glutathione peroxidase; GST, glutathione S-transferase; PrxR, peroxiredoxin; Nar, nitrate reductase; Nir, nitrite reductase; Ccp, cytochrome c peroxidase; CAT-px, catalase-peroxidase; FeSOD and MnSOD, iron and manganese superoxide dismtase, respectively.

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detoxifying systems can be considered essential for the virulence of the fungi.

Cells contain multiple cytosolic ROS detoxifying enzymes, such as superoxide dismutases, catalases, cytochrome c peroxidases, glutathione peroxidases, glutaredoxins and peroxiredoxins (Collinson and Grant, 2003). In addition, some cellular compartments have mechanisms that protect the cell against the deleterious effects of ROS and RNS produced during the metabolism. It has been shown that filamentous fungi have supplementary mechanisms that decrease ROS production, such as the mitochondrial alternative oxidase. Moreover, SODs localized in the mitochondrial intermembrane space (CuZn SOD) and in the matrix (Mn SOD) form the first line of antioxidant defense against $O_2^{\cdot-}$ (Ito-kuma et al., 1999). It has not been fully elucidated how the resulting H_2O_2 is scavenged in mitochondria. Therefore, fungi evolved a dynamic network of antioxidant defense mechanisms that prevent the accumulation of ROS, detoxify ROS, and repairs oxidized molecules. Knowing how pathogenic fungi deal with ROS and RNS is important to understand how infection is established and how fungi survive within host.

Compared with other fungal pathogens of plants and animals, little is known about the oxidative stress response in *U. maydis*. This organism is a ubiquitous pathogen of maize and a well-established model organism for the study of plant-microbe interactions (Martínez-Espinoza et al., 2002). This basidiomycete does not use aggressive virulence strategies to kill its host. *U. maydis* belongs to the group of biotrophic parasites (the smuts) that depend on living tissue for proliferation and development (Mendgen and Hahn, 2002). Little is known about the genomic features responsible for the pathogenicity of this organism (Liu et al., 2000; Kämper et al., 2006), but it is tempting to speculate that detoxifying systems for ROS and RNS might be essential for the virulence of fungi. For this reason, the enzymes and metabolites involved in the control of these reactive species have been considered virulence factors in other pathogenic fungi. This review describes results of a bioinformatic search of the *U. maydis* defense mechanism in comparison with others fungal pathogen.

The Whitehead Institute/MITT Center for Genome Research (http://www.genome.wi.mit.edu/annotation/fungi/ustilago_maydis/index.html), the NCBI GenBank nr, and the ExPASy dbEST databases were screened for ORFs that have similarity to genes involved in antioxidant systems. For the initial identification of potential candidates, a BLAST search was made based on the enzymes that belong to the antioxidant systems in other pathogenic fungi. The subcellular localization of the proteins was predicted using TargetP program (Emanuelsson et al., 2000). The comparative analysis of the available *U. maydis* genome sequence data led to the proposal of the presence of different enzymes involved in the antioxidant pathways.

ROS GENERATION

Mitochondrial respiratory chain

Pathogenic fungi can produce ROS by different mechanisms. However, the principal sources of ROS are the respiratory chain and the NADPH oxidases. Mitochondrial electron transport consumes more than 90% of cellular oxygen and generates superoxide anions as minor by-products (Nohl, 1994). However, mitochondria are highly susceptible to oxidative damage, and ROS can inactivate enzymes and cause mutation in DNA. Several enzymes contribute to the antioxidant defense in mitochondria by preventing the accumulation of endogenous ROS in the matrix and intermembrane space. For example, there is a superoxide dismutase (SOD2) in yeast, which rapidly converts the superoxide radical to H_2O_2 (Moradas-Ferreira et al., 1996). H_2O_2 will diffuse out of mitochondria, preventing its dangerous building up within the matrix and the damage of mitochondrial DNA or proteins containing iron sulfur centers. Because catalase is absent in mitochondria of most eukaryotic cells, glutathione peroxidase and cytochrome c peroxidase, located in the matrix and inter membrane space, respectively, detoxify H_2O_2 to water and oxygen (Skulachev, 1997). Recently, it was proposed that one of the important physiological functions of the alternative respiratory pathway is to keep the ubiquinone pool sufficiently oxidized to prevent the autoxidation of semiubiquinone and the subsequent formation of ROS both in plants and fungi (Moore et al., 2002). A similar role in preventing the generation of ROS has been attributed to the uncoupling proteins in plants (Kono et al., 1995). Conversely, decreased mitochondrial activity under conditions of moderate oxidative stress limits further ROS release within the cell. This limitation of endogenous ROS production could be part of the adaptive response to oxidative stress.

OXIDASES

NADPH oxidases catalyze the reaction between NADPH and O_2 to generate $O_2^{\cdot-}$ anions, followed by the production of H_2O_2 by dismutation (Torres et al., 2002). In plants, animals, and fungi, the NADPH oxidases are implicated in the differentiation processes (Aguirre et al., 2005). Unlike many other fungi, NADPH oxidases are absent in *U. maydis* (Aguirre et al., 2005).

Although much attention has been given to NADPH oxidases and their possible role in cell signaling (Aguirre et al., 2005), other oxidases have been proposed to generate ROS.

Fungi usually contain a wide variety of sugar oxidases, including glucose oxidase, glyoxal oxidase and galactose oxidase.

Fungal extracellular glyoxal oxidases have so far been described only in the white rot fungus *Phanerochaete chrysosporium* (Kerten and Kirk, 1987). There, glyoxal oxidase is an essential component of the lignin degradation pathway, and provides extracellular hydrogen peroxide as a co-substrate for the lignin peroxidase and Mn-dependent peroxidase (Janse et al., 1998). Two substrates for this enzyme, glyoxal and methylglyoxal are found in the extracellular environment of the fungus. The *U. maydis* genome contains three ORFs that share a high degree of similarity to glyoxal oxidases (Table 1). Some of these enzymes are secreted and other is potentially a mitochondrial protein. Interestingly, it was suggested that H_2O_2 produced by glyoxal oxidase 1 is required for filamentous growth and pathogenicity in *U. maydis* (Leuthner et al., 2005). Although the specific functions of glyoxal oxidase 2 and 3 have not been determined, it seems that these two enzymes are not involved in pathogenicity.

The copper enzyme galactose oxidase catalyzes the oxidation of D-galactose and other primary alcohols by molecular oxygen to the corresponding aldehydes and H_2O_2 (Berkessel et al., 2005). Galactose oxidase is a 68 KDa monomeric enzyme that contains a single copper and an amino acid derived cofactor. The enzyme is produced by the filamentous fungus as an extracellular enzyme. Its biological function is unknown, but it may be involved in breaking down the plant cell wall prior to invasion. The *U. maydis* genome contains an ORF that encodes a putative galactose oxidase (Leuthner et al., 2005), with a signal peptide that directs the protein to the periplasm (Table 1).

Glucose oxidase, a fungal enzyme produced by *Aspergillus* and *Penicillium sp* (Bucke, 1983), is a glycoprotein that catalyzes the oxidation of β-D-glucose by molecular oxygen to ∂-gluconolactone, a compound that subsequently hydrolyzes spontaneously to gluconic acid and H_2O_2 (Bentley, 1963). The expression of glucose oxidase in *B. cinerea* and *P. chrysosporium* was induced by low glucose concentrations in the culture medium (Kelly and Reddy, 1986; Liu et al., 1998). A search of the *U. maydis* genome sequence identified four glucose oxidase homologues (Table 1), three possessing a signal sequence for extracellular location and one more that is cytoplasmatic. All of them contribute to the production of H_2O_2, suggesting their potential importance in fungal metabolism and the infection process (Mayer and Harel, 1979). Intracellular amine oxidase can also generate H_2O_2, which can diffuse out of cell to complement extracellular H_2O_2 generation.

RNS GENERATION

Nitric oxide ($NO^{\cdot}$) is a free radical produced by biological activities like the bacterial denitrification in soils (Ji and Hollocher, 1988). The main source of the free radical $NO^{\cdot}$ in mammalian cells is the enzymatic oxidation of L-arginine by $NO^{\cdot}$ synthases (NOS). Interestingly, NOS are also present in insects, mollusks, bacteria, parasites, and fungi (Muller, 1997; Klesing et al., 2000; Golderer et al., 2001; Adak et al., 2002).

In addition to the route involving the NOS-like enzyme, it has been shown that the production of $NO^{\cdot}$ in plants, fungi, and bacteria occurs as a side-reaction during NO_3^- assimilation via the NADPH dependent reduction of nitrite (NO_2^-) by nitrate reductase (NaR). During NO_3^- assimilation, NaR catalyzes the NADPH dependent reduction of NO_3^- to NO_2^- which is further reduced to ammonium by the enzyme nitrite reductase (NiR) (Takaya, 2002).

The biological consequences of $NO^{\cdot}$ formation in cellular systems are governed by a complex and, as yet, not completely elucidated network of competing reactions of the free radical $NO^{\cdot}$ with molecular O_2, ROS, transitions metals and thiols (Stamler et al., 2001). Such reactions yield various RNS, including nitrosyl-metal complexes, S-nitrosothiols, trioxide of dinitrogen (N_2O_3) and peroxynitrite

Table 1. Genes involved in ROS production in *U. maydis*.

U. maydis Accession number	Hypothetical protein	Number of aa and introns in sequencess	Organism/accession number/e-value	Predicting subcellular localization
UM00913.1	Glyoxal oxidase	625aa/with no introns	*Cryptococcus neoformans*/XP_571566/1e^{-142}	*Secreted 0.837 extracellular*
UM01149.1	Glyoxal oxidase	652aa/with no introns	*Cryptococcus neoformans*/XP_567162/1e^{-134}	*Secreted 0.744 endoplasmic reticulum*
UM02411.1	Glyoxal oxidase	862aa/with no introns	*Cryptococcus neoformans*/XP_571566/1e^{-107}	*Nuclear 0.62*
UM02809.1	Galactose oxidase	630aa/with no introns	*Hypomyces rosellus*/A38084/1e^{-99}	*Secreted 0.953 extracellular*
UM03551.1	Glucose oxidase	634aa/with no introns	*Botryotinia fuckeliana*/CAD88590.1/3e^{-82}	*Secreted 0.976 extracellular*
UM04957.1	Glucose oxidase	603aa/1 intron	*Coccidiodes immitis*/XP_001239189/1e^{-94}	*Secreted 0.934 extracellular*
UM03615.1	Glucose oxidase	693aa/with no introns	*Botryotinia fuckeliana*/CAD88590.1/7e^{-72}	*Secreted 0.836 extracellular*
UM01711.1	Glucose oxidase	612aa/with no introns	*Coccidiodes immitis*/XP_001239189/2e^{-95}	*Cytosolic 0.940*
UM05423.1	Amine oxidase	536 aa/with no introns	*Crassostrea gigas*/CAD89351/2e^{-64}	*Cytosolic 0.699*

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($ONOO^-$). Like ROS, RNS may damage and kill cells by mechanisms that include inactivation of respiratory chain (Brown, 1999) and metabolic enzymes (Beltran et al., 2000), and irreversible oxidative modification and degradation of DNA (Burney et al., 1999), proteins (Tien et al., 1999), and membrane lipids (Goss et al., 1999). Despite the many processes controlled and/or induced by $NO^{\cdot}$ in fungi, the molecular mechanisms responsible for the synthesis of this radical remain controversial. Nevertheless, a gene with homology to mammalian NOS enzyme was not found in *U. maydis*, but some genes involved in the formation of NO by dissimilatory nitrate reduction are present in the genome of this fungi (Table 2). Homologues of NaR and NiR were identified, suggesting their possible involvement in $NO^{\cdot}$ production. Both proteins are cytosolic and their presence in this organism supports their participation in the process of ammonification. In support of this physiologic role, *U. maydis* possess a nitrate transporter in the plasma membrane and can grow with nitrate as the sole nitrogen source. In addition, we identified the presence of NiR by mass spectroscopy in isolated mitochondria of *U. maydis* yeast cells, suggesting that this enzyme is probably involved in the generation of $NO^{\cdot}$ or RNS under our experimental conditions (unpublished results).

ANTIOXIDANT ENZYMATIC DEFENSES

Major ROS scavenging enzymes of fungi include superoxide dismutases (SOD), glutathione peroxidases (Gpx), thioredoxin peroxidases (Tpx), catalases (CAT), glutathione S-tansferases (GST), thiol peroxidases, flavohemoglobin and peroxiredoxins (PrxP). Together with the antioxidants glutathione and thioredoxin, these enzymes provide the cells with highly efficient machinery for detoxifying ROS and RNS.

ALTERNATIVE OXIDASE

The alternative oxidase (AOX) is a respiratory chain protein found in all higher plants, fungi, non-fermentative yeasts and trypanosomes (Affourtit et al., 2002). AOX is believed to have two roles, a metabolic one and as antioxidant, and both functions are potentially important for fungal pathogenesis (Joseph-Horne et al., 2001). Significantly $NO^{\cdot}$ is a potent reversible inhibitor of cytochrome c oxidase but not of AOX (Yamasaki et al., 2001). In addition, the maintenance of mitochondrial electron flow as well as the rapid consumption of oxygen by AOX at the inner mitochondrial membrane attenuates the production of ROS due to mitochondrial electron leak from ubisemiquinone directly to oxygen. *Candida albicans* produces both constitutive and inducible AOX isoforms in the presence of cyanide, antimycin A, hydrogen peroxide, paraquat, and hydrogen peroxide (Huh and Kang, 2001). In *C. albicans*, the MnSOD is involved in the expression of AOX (Hwang et al., 2003). A critical test of the role of AOX in fungal pathogenesis was accomplished in the study of aox1 null mutant of *Cryptococcus neoformans*, which displays increased sensitivity to peroxide stress and decreased virulence in mice (Akhter et al., 2003).

The *U. maydis* genome contains an AOX gene encoding a ~45 kDa protein (Table 3). We have characterized the AOX and found that in agreement with other pathogenic fungi, its activity and expression are influenced by different stress conditions. In addition, the enzyme is regulated, at the post-translational level, by AMP and other factors (Sierra-Campos, et al., 2009).

SUPEROXIDE DISMUTASES

Superoxide dismutase (SOD) plays a major role in the first line of antioxidant defense by catalyzing the dismutation of $O_2^{\cdot-}$ radicals to H_2O_2 and O_2; H_2O_2 is subsequently removed by catalase, catalase-peroxidase and GSH or Trx dependent peroxidase. Another function of SOD is to protect dehydratases (dihydroxy acid dehydratase, aconitase, 6-phosphogluconate dehydratase and fumarase) against inactivation by the $O_2^{\cdot-}$ (Benov and Fridovich, 1998). SODs are metallo-proteins classified as iron (Fe-SOD), manganese (Mn-SOD), nickel (Ni-SOD), and copper and zinc (CuZn-SOD) based on the metals in their active sites (Halliwell and Gutteridge, 1989). Of the four SODs, Fe and Mn SODs

Table 2. Genes involved in RNS production in *U. maydis*.

U. maydis Accession number	Hypothetical protein	Number of aa and introns in sequencess	Organism/accession number/e-value	Predicting subcellular localization
UM03849.1	Nitrate transporter	607 aa/1 introns	*Hebeloma cylindrosporum*/CAB60009/$5e^{-103}$	*Secreted 0.961 Plasma membrane*
UM03848.1	Nitrite reductase	602 aa/with no introns	*Hebeloma cylindrosporum*/CAB60008.2/0.0	*Cytosolic* 0.751
UM03847.1	Nitrate reductase (NADPH)	907 aa/with no introns	*Hebeloma cylindrosporum*/CAB60009/0.0	*Cytosolic* 0.862

belong to one family, whereas CuZn-SOD exhibits no sequence similarity to the Fe and Mn SODs (Gralla and Kosman, 1992). There are at least three forms of SOD in eukaryotes: a cytosolic CuZn-SOD, a mitochondrial Mn-SOD, and an extracellular CuZn-SOD (Halliwell and Gutteridge, 1989). With regard to fungi, different types of SOD have been found in various pathogens of plants and animals, including *Botrytis cinerea* (Choi et al., 1997; Ito-kuma et al., 1999). They have a Mn-SOD in the mitochondrial matrix and a CuZn-SOD that it is thought to reside in cytosol. Recently, CuZn-SOD has also been found in the mitochondrial intermembrane space of some fungi (Nedeva et al., 2004), and a cytoplasmatic Fe-SOD (Kono et al., 1995). While the contribution of CuZn-SOD to the virulence of pathogenic fungi has been extensively studied, the role of Mn-SOD is poorly understood (Hamilton and Holdom, 1999). In *C. neoformans* and *C. albicans*, Mn-SOD was involved in oxidative and high temperature stresses, two conditions encountered by pathogenic fungi in the mammalian host (Narasipura et al., 2005). A Fe-SOD is specifically expressed during chlamydospore formation in *Fusarium oxysporum* (Kono et al., 1995). *U. maydis* possess three genes corresponding to distinct SOD isoenzymes. Of these, two are Mn-SOD and the other is a Fe-SOD (Table 4). It is worth to mention that *U. maydis* possesses a Fe-SOD and a Mn-SOD in mitochondria, and a cytosolic Mn-SOD. The presence of two different mitochondrial SODs might be important in this fungus containing a highly active respiratory chain, which unavoidably leaks O_2^- . Notably this fungus does not have a CuZn-SOD, possibly implying that under its developmental stages, the generation of endogenous H_2O_2 by different oxidases could inactivate this isoform, while that Fe or the Mn-SOD are not inhibited by H_2O_2. Interestingly, we identified the presence of Fe-SOD by mass spectroscopy in isolated mitochondria of *U. maydis* yeast cells (Pardo and Flores, data unpublished).

CATALASES AND PEROXIDASES

Although catalase is a ubiquitous enzyme found in aerobic organisms, it is not an essential enzyme for decomposition of intracellular H_2O_2 to oxygen and water. Three general classes of catalases have been described in the literature: the typical or monofunctional catalases (Schomburg et al., 1994); the catalase-peroxidase with both activities; and the Mn-catalases or pseudocatalases (Kono and fridovich, 1965). Catalase is important in fungal pathogenicity and for the development of several human and plant pathogens, including *C. albicans*, *Aspergillus fumigatus*, and *Blumeria graminis* during host colonization (Wysong et al., 1998; Xu and Pan, 2000; Paris et al., 2003). However, in other phytopathogenic fungi like *Botrytis cinerea* (Schouten et al., 2002), *Cochliobolus heterostrophus* (Robbertse et al., 2003), and *Claviceps purpurea* (Garre et al., 1998), knock out mutants of the catalase have similar virulence as the wild-type strains. Unlike many other pathogenic fungi, *U. maydis* lacks the typical catalase, but possesses the catalase-peroxidase, potentially located in the cytoplasm. This is a key enzyme in many crucial cellular functions, including the disposition of H_2O_2 during sexual development of *Aspergillus nidulans* (Scherer et al., 2002). Another attractive possibility for a role of catalase-peroxidase during development comes from the observation that in *Neurospora crassa* a hyperoxidant state was detected at the start of different morphogenic transitions (Toledo et al., 1995).

Cytochrome c peroxidase (Ccp) is an antioxidant enzyme located in the mitochondrial intermembrane space, protecting aerobic organisms from the toxic effects of H_2O_2 (Yon-

Table 3. Alternative oxidase homolog in *U. maydis*.

U. maydis Accession number	Hypothetical protein	Number of aa and introns in sequences	Organism/accession number/e-value	Predicting subcellular localization
UM02774.1	Alternative oxidase	448aa/ with no introns	*Yarrowia lipolytica*/AAQ08896/$4e^{-58}$	*Mitochondrion 0.9740*

Table 4. Superoxide dismutase homologs in *U. maydis*.

U. maydis ccession number	Hypothetical protein	Number of aa and introns in sequences	Organism/accession number/e-value	Predicting subcellular localization
UM03085.1	Mn SOD	206 aa/1 intron	*Taiwanofungus camphoratus*/AAQ16628/$5e^{-71}$	*Cytosolic 0.946*
UM02453.1	Mn SOD	227 aa/with no introns	*Cryptococcus bacillisporus*/AAQ98967/$3e^{-74}$	*Mitochondrion 0.936*
UM06417.1	Fe SOD	305 aa/with no introns	*Neosartorya tischeri*/XP_001257839/$5e^{-16}$	*Mitochondrion 0.944*

etani and Ohnishi, 1996). Ccp detoxifies H_2O_2 by catalyzing its reduction to water using the reducing equivalents from two molecules of ferrocytochrome c. The presence of Ccp in pathogens that lack catalase indicates that this enzyme is the major H_2O_2 detoxificant in mitochondrion. Studies on the yeast *Saccharomyces cerevisiae* show that the expression of cytochrome c peroxidase gene increases when cells are under nitrosative stress. Therefore, this enzyme probably participates in the detoxification of peroxynitrite as well as H_2O_2 (Kwon et al., 2003). Loss of Ccp function increases the sensitivity of *C. neoformans* to exogenous oxidative stress, but does not diminish the virulence of fungi (Giles et al., 2005). *U. maydis* possesses two distinct genes of Ccp (Table 5), which belong to subfamily II, based on the overall sequence similarity (Zámocky and Dunand, 2006), and are potentially located both in cytoplasm and mitochondria. It can still be assumed that mitochondrial H_2O_2 is detoxified by Ccp in this fungus, while that H_2O_2 produced by other sources is detoxified by cytoplasmatic catalase-peroxidase. In addition, mitochondrial integrity has been shown to be important for pathogenicity (Bortfeld et al., 2004).

GLUTATHIONE SYSTEM

Sulfhydryl groups of glutathione (GSH) and thioredoxin (Trx) are important components of the cellular defense mechanisms against oxidative stress and for the maintenance of the redox homeostasis in cells (Grant, 2001).

The tripeptide GSH is a non-proteinous thiol compound abundant in almost all aerobic organisms. It is synthesized in two sequential reactions catalyzed by γ-glutamylcysteine synthetase (the GSH1 gene product) and glutathione synthase (GSH2 gene product) in the presence of ATP. The intracellular concentration of glutathione varies within the range 5-10 mM, depending on the cell type and cellular compartment. There are two major GSH pools in cells, the cytoplasmic and the mitochondrial pool; the later is important in detoxification of H_2O_2 produced by the electron transport chain. Mitochondrial GSH is considered vital for cell survival, because mitochondria in many eukaryotes do not contain catalase, implying that GSH in the mitochondrial matrix is the principal non-enzymatic defense against the potential toxic effects of H_2O_2 on the respiratory chain. GSH has many physiological functions in cells (Meister and Anderson, 1983). GSH acts as a radical scavenger with the redox active sulfhydryl group reacting with oxidants to produce glutathione disulphide (GSSG). Besides this, GSH can serve as an electron donor for a variety of proteins, including Gpx, GSTs and glutaredoxins. The Gpx, a member of the GSH system, reduces H_2O_2 in the presence of two molecules of GSH, forming one molecule of GSSG and water. The GSSG is reduced back to GSH by glutathione reductase, using NADPH as electron donor. The resulting $NADP^+$ is regenerated to NADPH through the reactions catalyzed by the enzymes glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the pentose phosphate pathway.

We identified the group of genes involved in the biosynthesis and recycling of glutathione within the *U. maydis* genome (Table 6). In addition, this organism contains various cytoplasmatic GSTs and a GST with a signal peptide for mitochondrial localization, a cytoplasmatic Gpx, and a cytoplasmatic GSH dependent formaldehyde dehydrogenase (GSNO reductase). Interestingly, the high number of GSTs possibly reflects that *U. maydis* is in contact with a large number of mutagens and by-products of oxidative stress. Although the GSH system has not been completely studied in any fungal pathogen to date, the GSH peroxidases have been linked to both virulence and viability of the fungal pathogen *C. neoformans* (Misall et al., 2005). In addition, the GSNO reductase is critical for S-nitrosothiols homeostasis and protects against nitrosative stress (Liu et al., 2001).

THIOREDOXIN SYSTEM

U. maydis possesses several proteins belonging to the thioredoxin system, including two cytoplasmatic thioredoxins (Trx), a potential mitochondrial thioredoxin reductase (Trr), and cytoplasmatic thiol (Tpx) (Table 7), suggesting

Table 5. Peroxidases homologs in *U. maydis*.

U. maydis Accession number	Hypothetical protein	Number of aa and introns in sequences	Organism/accession number/e-value	Predicting subcellular localization
UM03399.1	Catalase-peroxidase	702 aa/2 introns	*Aspergillus fumigatus*/XP_747039/0.0	*Cytosolic 0.781*
UM01947.1	Cytochrome c peroxidase	330 aa/with no introns	*Aspergillus terreus*/XP_001212584/$9e^{-124}$	*Cytosolic 0.756*
UM02377.1	Cytochrome c peroxidase	398 aa/with no introns	*Emericella nidulans*/POCOV3/$1e^{-109}$	*Mitochondrion 0.931*

that this organism possess both a cytosolic and mitochondrial thioredoxin cycle.

Trx are key proteins in many cellular functions, including oxidative stress management (Grant, 2001). Trr is an important component of the thioredoxin oxidative stress resistance pathway (Missall and Lodge, 2005). In addition to this role, the thioredoxin system of bacteria, yeast, and mammals is involved in DNA synthesis, gene transcription, cell growth, and apoptosis (Arner and Holmgren, 2000). Two different isoforms of Trr have been identified in prokaryotes and eukaryotes, the high molecular weight isoform, which is present in mammals and some parasites, and the low molecular weight isoform, found in most bacteria, plants and fungi (Hirt et al., 2002). The Trr is induced during oxidative and nitrosative stresses and it is necessary for viability in *C. neoformans* (Missall and Lodge, 2005).

Peroxiredoxins are a ubiquitous group of peroxidases that exert their reductive activity via active-site cysteine residues. These enzymes lack prosthetic groups and catalyze the reduction of H_2O_2, $ONOO^-$, and a wide range of organic hydroperoxides (ROOH) to their corresponding alcohols (Wood et al., 2003). They exist in all organisms and are highly abundant in cells, and proved to be important for resistance to H_2O_2 and for virulence in *C. neoformans* (Missall et al., 2004).

Table 6. Genes involved in glutathione system in *U. maydis*.

U. maydis ccession number	Hypothetical protein	Number of aa and introns in sequences	Organism/accession number/e-value	Predicting subcellular localisation
UM04004.1	Glutamate-cysteine ligase	705 aa/with no introns	*Cryptococcus neoformans*/XP_568556/0.0	*Cytosolic 0.836*
UM05504.1	GSH synthase	542 aa/with no introns	*Cryptococcus neoformans*/XP_567465.1/9e^{-107}	*Cytosolic 0.864*
UM04948.1	GSH reductase	102 aa/1 intron	*Aspergillus niger*/CAk43281/2e^{-17}	*Cytosolic 0.832*
UM05435.1	Glutaredoxin	345 aa/with no introns	*Candida albicans*/XP_721347/ 8e^{-11}	*Cytosolic 0.651*
UM03211.1	Thiol-peroxidase	721aa/with no introns	*Chlorobium chlorochromatii*/YP_379900/8e^{-20}	*Cytosolic 0.473*
UM04801.1	GSH S-transferase	233 aa/2 introns	*Neosartorya fischeri*/XP_001267449/6e^{-47}	*Cytosolic 0.603*
UM06325.1	GSH S-transferase	384 aa/3 introns	*Cryptococcus neoformans*/XP_572592/5e^{-42}	*Mitochondrion 0.683*
UM02241.1	GSH S-transferase	293 aa/2 introns	*Cryptococcus neoformans*/XP_572592/7e^{-47}	*Cytosolic 0.872*
UM01784.1	GSH peroxidase2	161 aa/2 introns	*Saccharomyces cerevisiae*/NP_009803/e^{-51}	*Cytosolic 0.892*
UM06244.1	GSH dependent formaldehyde dehydrogenase	313 aa/2 introns	*Coccidioides immitis*/XP_001246427/3e^{-122}	*Cytosolic 0.909*
UM02387.1	GSH oligopeptide transporter	985 aa/with no intron	*Candida albicans*/AAT95227/9e^{-146}	*Mitochondrion 0.627*
UM01140.1	GSH disulfide reductase	1,220 aa/1 introns	*Cryptococcus neoformans*/XP_ 570771/6e^{-145}	*Cytosolic 0.429*

Table 7. Genes involved in thioredoxin system in U. maydis.

U. maydis Accession number tion	Hypothetical protein	Number of aa and introns in sequences	Organism/accession number/e-value	Predicting subcellular localisa-
UM02947.1	Thioredoxin reductase	253 aa/1 intron	*Malassezia furfur*/P56577/3e^{-51}	*Mitochondrion 0.825*
UM03177.1	Thioredoxin peroxidase	172 aa/with no introns	*Candida albicans*/XP_715909/2e^{-26}	*Cytosolic 0.92*
UM06512.1	Thioredoxin	160 aa/2 introns	*Malassezia sympodialis*/CAI78451/$^{7e-35}$	*Cytosolic 0.954*
UM01370.1	Thioredoxin	178 aa/1 intron	*Xenopus tropicalis*/CAJ81685 /5e^{-19}	*Cytosolic 0.894*

Table 8. Genes involved in NADPH regeneration in *U. maydis*.

U. maydis Accession number	Hypothetical protein	Number of aa and introns in sequences	Organism/accession number/e-value	Predicting subcellula localis ation
UM04930.1	Glucose-6-phosphate dehydrogenase	502 aa/ 2 introns	*Cryptococcus neoformans*/XP_572045/0.0	*Cytosolic 0.79*
UM02577.1	6-phosphogluconate dehydrogenase	492 aa/ 3 introns	*Aspergillus nidulans*/XP_661558.1/0.0	Cytosolic 0.56

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It has been suggested that the thioredoxin and glutathione systems are maintained independently (Trotter and Grant, 2003), though compensation between these two systems has been observed under stress conditions (Inoue et al., 1999).

DEFENSES AGAINST RNS

Considerable evidence indicates that $NO^{\cdot}$ and its derivates, such as $ONOO^-$, are important reactive species in signal transduction and defense in animal and plants (Wendehenne et al., 2001). To evade the host oxidative attack, some microorganisms express flavohemoglobin denitrosylase, which converts $NO^{\cdot}$ to nitrate via a bound nitroxyl (NO^-) intermediate across a broad range of physiological oxygen concentrations. The enzyme is present in many fungi and bacterial species, but it is not found in higher eukaryotes (Liu et al., 2000). However, it might be conserved in plant and mammalian fungal pathogens (de Jesus-Berrios et al., 2003). In addition, flavohemoglobin denitrosylase is necessary for $NO^{\cdot}$ resistance in promoting infection.

In contrast, GSNO reductase is more widespread, conserved from bacteria to humans and it has been shown to reduce nitrosoglutathione (GSNO) to ammonia and GSSG (Liu et al., 2001). The thiol peroxidase, Tsa1, which is important for both oxidative and nitrosative stress resistance (Missall et al., 2004), contributes significantly to virulence in *C. neoformans*. At least in many fungi the resistance against the toxics effect of $NO^{\cdot}$ may be related to the activation of nitrosothiol metabolizing enzymes (Liu et al., 2001). In addition, NO reductase, which catalyzes the reduction of NO to the less toxic compound nitrous oxide (N_2O), plays a major role in protecting organisms from NO (Zumft, 1997).

We did not identify genes encoding flavohemoglobin denitrosylase and NO reductase in the *U. maydis* genome. However, like in other fungi, GSH peroxidase, GSNO reductase and thiol peroxidase are present in *U. maydis* (see table 6). These enzymes have been shown to be induced in response to nitrosylate stress (Missall et al., 2004).

NADPH PRODUCTION PATHWAY

The cellular pools of the antioxidants Trx and GSH are maintained in their reduced state by a set of enzymes that use NADPH to reduce GSSG or oxidized Trx (e.g. glutathione reductase and thioredoxin reductase; tables 5 and 6, respectively). Glucose 6 phosphate dehydrogenase is regarded as the major source of cellular NADPH because it decreases cellular oxidative stress by increasing the GSH concentration (Salvemini et al., 1999). A search of *U. maydis* genome sequence identified two genes showing high similarity to glucose-6-phosphate 1-dehydrogenase and 6-phosphogluconate dehydrogenase from the animal pathogens *C. neoformans* and *A. nidulans*, respectively (Table 8).

With this information at hand, we propose here a hypothetical model that integrates our data regarding the involvement of ROS and RNS production and detoxification systems of *U. maydis* (Fig. 1). We show that *U. maydis* antioxidant defense is multifaceted, but limited. Hence, although this organism contains some well studied proteins present in other pathogenic fungi, along with some unique mechanisms and novel physiological roles to combat oxidative stress, *U. maydis* is in a relative disadvantage, lacking a great number of isoforms of these enzymes. The antioxidant systems described above will be relevant for the pathogenic fungi to handle ROS and RNS in a regulated fashion.

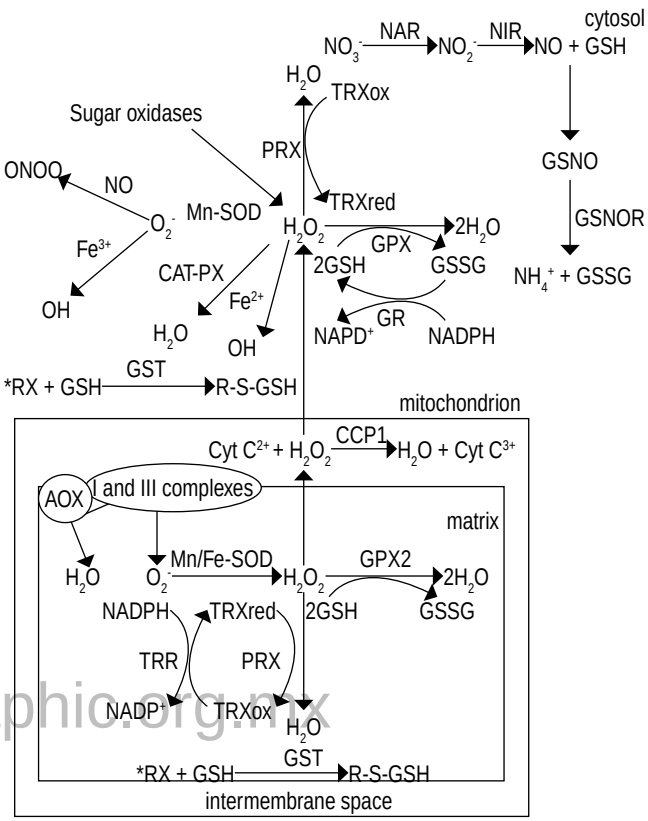

Figure 1. *Hypothetical model for the ROS and RNS detoxification systems in U. maydis. Sugar oxidases and mitochondrial respiratory complexes are the sources of ROS, while the enzymes of dissimilatory nitrate reduction are potential sources of NO in U. maydis. In addition, hydroxyl radicals are generated in the presence of free iron via the Fenton reaction. *R may be an aliphatic, aromatic or heterocyclic group; X may be a sulfate, nitrite or halide group. Cyt c, cytochrome c; NH4+, ammonium cation. ROS and RNS shown include the following: O2.-, superoxide anion; OH., hydroxyl radical; H2O2, hydrogen peroxide; NO., nitric oxide; ONOO-, peroxynitrite. See abbreviations to details.*

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FINAL CONCLUSION

The flexibility and multiplicity of the antioxidant defense mechanisms are particularly important for pathogenic microorganisms living freely in the environment or in association with a host expressing the defense responses, based on the production of reactive oxygen and nitrogen species. The *U. maydis* antioxidant system is multifaceted, but limited, lacking many of the isoforms found in other organisms. Although we hypothesized that the antioxidant system might contribute to the virulence of this fungus during plant-pathogen interaction, it is known that this biotrophic pathogen causes very little damage to its host until the final phases of infection. However, recently, Molina & Kahmann, 2007; identified and characterized an ortholog of *YAP1* (for Yeast *AP-1* like) from *Saccharomyces cerevisiae* that regulates the oxidative stress response in this organism. Thus, exposure of *U. maydis* cells to H_2O_2 leads to the accumulation of Yap1p in the nucleus and altered transcription of two peroxidase genes (*um01947* and *um10672*), which are considered likely to be involved ROS detoxification. Interestingly, single mutations in either gene showed reduced virulence, comparable to the Yap1 deletion strain. Therefore, further work is required to better understand the RSN and ROS defenses of this fungus.

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