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Review article

Reactive oxygen species metabolism and plant-fungal interactions

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Abstract

Fungal interactions with plants can involve specific morphogenetic developments to access host cells, the suppression of plant defenses, and the establishment of a feeding lifestyle that nourishes the colonizer often—but not always—at the expense of the host. Reactive oxygen species (ROS) metabolism is central to the infection process, and the stage-specific production and/or neutralization of ROS is critical to the success of the colonization process. ROS metabolism during infection is dynamic—sometimes seemingly contradictory—and involves endogenous and exogenous sources. Yet, intriguingly, molecular decision-making involved in the spatio-temporal control of ROS metabolism is largely unknown. When also considering that ROS demands are similar between pathogenic and beneficial fungal-plant interactions despite the different outcomes, the intention of our review is to synthesize what is known about ROS metabolism and highlight knowledge gaps that could be hindering the discovery of novel means to mediate beneficial plant-microbe interactions at the expense of harmful plant-microbe interactions.

Keywords: Reactive oxygen species, Fungi, Nox complex, Antioxidation, Plant-associated microbes

1. Introduction

Plant pathogens account for annual losses of about 15% of global crop production, and fungi have the potential to destroy enough food to feed up to around 60% of the world's population (Fisher et al., 2013; Oerke, 2006). At the same time, crop production needs to rise by 60–110% to meet food demands by 2050 (FAO, 2009; Ray et al., 2013). Understanding the basic biology of fungal plant pathogens, and the molecular and cellular underpinnings of plant-fungal interactions, are thus important components of future food security strategies. Plant pathogenic fungi are characterized by their infection strategies and lifestyles as either biotrophs, necrotrophs, or hemibiotrophs (Fernandez et al., 2014; Lo Presti et al., 2015). Biotrophs colonize and survive off living hosts. Hemibiotrophs first grow as biotrophs in living host tissue, before switching to a necrotrophic phase, feeding off of dead tissue. Necrotrophs kill their hosts first in order to feed. In contrast, root-associated ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, and root and leaf endophytes, provide direct benefits or incur no fitness cost to the host (Rodriguez et al., 2009; Smith and Smith, 2011), and attention has focused recently on how these groups of beneficial microbes could be used to boost plant productivity (Ceballos et al., 2013; Rodriguez and Sanders, 2015). During plant infection, both pathogenic and beneficial fungi experience a host-derived oxidative burst of reactive oxygen species (ROS) (Heller and Tudzynski, 2011). This burst is part of the plant innate immune response and must be subdued in order to avoid triggering more robust plant defenses. Besides overcoming plant-derived ROS, fungi must also overcome ROS produced as a byproduct of aerobic respiration. Common ROS includes singlet oxygen ($^1\text{O}_2^-$), superoxide ($\text{O}_2^{\bullet-}$), hydroxyl ($\text{OH}\bullet$) and hydrogen peroxide (H_2O_2). These ROS react readily with lipids, DNA, proteins and other macromolecules, leading to death and aging of the cell (Beckman and Ames, 1998). ROS can also react with oxides of nitrogen such as NO to generate damaging reactive nitrogen species (RNS) such as peroxyxynitrite (Marroquin-Guzman et al., 2017). To eliminate ROS (and RNS), fungal pathogens have developed robust antioxidation systems involving superoxide dismutases (SODs), catalases, peroxidases, glutathione and thioredoxin. Interestingly, although ROS was thought to act as a toxifying component in hosts against plant pathogens, it is not produced in amounts sufficient to

overcome these pathogens (Marroquin-Guzman et al., 2017; Samalova et al., 2014) and instead is emerging as a signaling component of the plant defense response.

Focused endogenous ROS production is also known to play important roles in key developmental processes in various phytopathogens, and the lack of fungal ROS producing systems can affect virulence and symbioses between fungi and plants (Kayano et al., 2013; Malagnac et al., 2004). Consequently, redox homeostasis is likely finely regulated during fungal-plant interactions. Furthermore, whereas in *M. oryzae*, for example, ROS-producing NADPH oxidase (Nox) enzymes are needed for the proper development of infection structures and host penetration (Egan et al., 2007; Ryder et al., 2013), NADPH also provides the reducing power for enzymes involved in antioxidation, specifically the glutathione (GSH) and thioredoxin (TR) systems, whose genes in *M. oryzae* are expressed in response to elevated NADPH levels (Fernandez and Wilson, 2014). Thus, NADPH production is central to ROS metabolism and provides a link between ROS production during infection-related development, and ROS neutralization during fungal-plant interactions.

Here, we focus on the known molecular details of ROS metabolism, and ROS metabolic control, during plant-fungal interactions. Our intention is this will act as a resource for researchers interested in this topic while also bringing attention to the following ROS conundrums: (i) Endogenous ROS produced as a by-product of metabolism is damaging to cells and must be neutralized, yet local ROS bursts are required for fungal differentiation, including the morphogenesis of specialized appressorial cells required to access host plant cells. What controls/ maintains this altered redox balance? (ii) Once inside the host, neutralization of exogenous ROS is important, as fungal invaders must overcome several lines of plant defense including ROS produced from a host oxidative burst which, left unchecked, triggers additional plant defenses, but internal ROS arising from fungal growth must also be dealt with. How? (iii) ROS metabolism involves NADPH and is thus energy intensive, but must be balanced with the energy requirements of growth, which also produces ROS. How are growth and ROS metabolism integrated? (iv) Pathogenic fungi and beneficial mycorrhizal or endophytic fungi—the former being characterized by short-lived plant associations with vigorous ramifications in host tissue, while the latter has long-lived associations and little *in planta*

growth—must both neutralize host ROS. Future gains in understanding plant-microbe interactions might come from molecular comparisons of plant pathogens versus endophytes with regards to redox balance, but molecular and genetic differences in growth and redox strategies between the two are unknown. The intention of our review is to stimulate thought in these areas and, where possible, both synthesize what is known about ROS metabolism and highlight knowledge gaps that could be hindering the discovery of novel means to mediate beneficial plant-microbe interactions at the expense of harmful plant-microbe interactions.

2. What role does ROS metabolism play in fungal growth and development?

2.1. NADPH oxidase complexes

Oxidative stress was thought to be a cost of metabolic processes in aerobic organisms and antioxidation systems arose to neutralize these toxic byproducts (Lambeth, 2004). However, it is now known that many multicellular organisms actively produce ROS via NADPH-dependent oxidase (Nox) enzyme complexes (Lambeth, 2004; Heller and Tudzynski, 2011) (Fig. 1A and B). Nox enzymes produce ROS by transferring electrons from NADPH to molecular oxygen to produce superoxide and other ROS. They are ubiquitous in filamentous fungi but have not yet been found in *Ustilago maydis* and *Cryptococcus neoformans* (reviewed in Aguirre et al., 2005; Bedard et al., 2007; Takemoto et al., 2007). Nox were first described in mammalian phagocytes, although they are now known to be present in many mammalian cells—the best studied is the mammalian Nox2 complex comprising the catalytic subunit gp91^{phox} (Nox2) and adaptor protein p22^{phox}. The Nox2 complex also comprises the cytosolic regulatory subunits p67^{phox} (NoxR), p47^{phox}, p40^{phox} and the GTPase RacA (Lambeth, 2004). Plants also possess Nox complexes known as respiratory burst oxidase homologs (Rboh), with roles in defense against pathogens, environmental stress and mediating symbiotic nodulation (Mittler et al., 2011; Suzuki et al., 2011). Fungi possess three Nox enzymes; NoxA (also known as Nox1) and NoxB (also known as Nox2) are homologous of the gp91^{phox} catalytic subunit while NoxC carries a Ca²⁺-binding EF hand and is similar

Fig. 1. Components and roles of NADPH oxidase (Nox) complexes. Model of Nox components that have been described in at least one fungal pathogen for **(A)** NoxA and **(B)** NoxB systems. Listed above each complex are the fungal developmental processes that require NoxA/B. Question marks refer to unknown components (???) or unverified functions (?) of the Nox complexes. See text for details.

to mammalian Nox5 (Aguirre et al., 2005; Heller and Tudzynski, 2011; Ryder et al., 2013). Nox requires adapter proteins for function, yet the homolog of the adaptor protein p22^{phox} (NoxD) has only recently been characterized in *Podospira anserina*, *Botrytis cinerea* and *Magnaporthe oryzae* (Lacaze et al., 2015; Scott, 2015; Siegmund et al., 2015; Galhano et al., 2017). NoxD acts with Nox1 in *P. anserina* and NoxA in *B. cinerea*, and NoxD deletion strains in these fungi are phenotypically identical to the respective Nox1/NoxA mutant, suggesting they act together (Lacaze et al., 2015; Scott, 2015; Siegmund et al., 2015). In *M. oryzae*, Nox1 and NoxD interact—but participate differently in septin-mediated cytoskeleton organization—indicating the fungal NADPH oxidase complex is dynamic (Galhano et al., 2017). Interestingly, *B. cinerea*, but not *M. oryzae* $\Delta noxD$ mutants, had growth defects in the presence of oxidative stress, suggesting a diversification of NoxD function and differences in ROS signaling between fungi (Galhano et al., 2017; Siegmund et al., 2015). In *M. oryzae*, *NOXD* gene expression is dependent on a Zn(II)₂Cys₆ family transcription factor that also interacts with machinery of the Map kinase signaling cascade (Galhano et al., 2017). In *B. cinerea*, the RasGAP protein homolog IQGAP interacts with NoxA, has a role in oxidative stress, and connects ROS signaling with MAP kinase and calcium signaling cascades (Marschall and Tudzynski, 2016c). Because the NoxD adapter protein only interacts with NoxA (Fig. 1A) and not NoxB (Fig. 1B), the tetraspanin Pls1 was proposed as an adaptor protein for NoxB (Scott, 2015). In *P. anserina* (Lambou et al., 2008) and *B. cinerea* (Siegmund et al., 2013) Pls1 mutants have the same phenotype as $\Delta noxB$ loss of function mutants. However, direct evidence of Pls1 as the adaptor protein for NoxB was lacking until recently when, in the root pathogen *Verticillium dahliae*, VdNoxB and VdPls1 co-localized during infection and were shown to interact *in vitro* and *in vivo* by yeast two-hybrid (Y2H) and bimolecular fluorescence complementation (BiFC) assays, respectively (Zhao et al., 2016). Loss of VdPls1 resembled loss of VdNoxB such that both Vd $\Delta noxb$ and Vd $\Delta pls1$ mutants were impaired for tip-high Ca²⁺ accumulation in hyphopodia, but not in vegetative hyphal tips. This impacted pathogenicity in the mutant strains by abrogating nuclear targeting of VdCrz1 and activation of calcineurin-Crz1 signaling required for penetration peg formation.

Other important components of the filamentous fungal NADPH oxidase enzyme complex include the regulatory subunit NoxR, the

small GTPase Rac—regulating both NoxA and NoxB—and the yeast polarity proteins BemA and Cdc24 (Takemoto et al., 2011). In the plant symbiotic fungus *Epichloë festucae*, BiFC showed NoxR interacted with BemA, BemA with Cdc24, and Cdc24 with NoxR at hyphal tips, and characterization of these genes suggested Cdc24 activates RacA, while BemA recruits and assembles NoxR and RacA at the plasma membrane to activate NoxA/NoxB (Takemoto et al., 2011). However, these components were not shown to have the same significance in *B. cinerea*, calling into question the conservation of BemA and Cdc24 in fungi (Fig. 1A and B) (Marschall et al., 2016b). Differences in NoxA and NoxB interactions with adapter proteins might account for the variation in roles for fungal development and differentiation between fungi (Lambou et al., 2008; reviewed in Scott, 2015).

2.2. Role of NOX complexes during plant infection

Fungal ROS generation mediated by NoxA (Nox1) and NoxB (Nox2) have important functions in fungal growth and development, including infection-related development. In *M. oryzae*, a fungal oxidative burst is necessary for infection-related development (Egan et al., 2007). Moreover, Nox1 and Nox2 complexes are vital for differentiation of the specialized rice infecting cell, the appressoria, and the penetration peg, thereby mediating virulence (Egan et al., 2007). Yet, there are distinct roles for the two homologs in plant infection. Nox2 and the regulatory subunit NoxR have a role in septin-mediated re-orientation of the F-actin cytoskeleton during hyphal polarization and remodeling of the penetration peg, while Nox1 is required for maintenance of the cortical F-actin network during plant infection (Fig. 2A) (Ryder et al., 2013). Tpc1 also controls the spatial and temporal regulation of cortical F-actin through the regulation of the NADPH oxidase complex during appressorium re-polarization (Galhano et al., 2017). Additionally, neither $\Delta noxA$ or $\Delta noxB$ mutants were reduced for ROS production in *M. oryzae* hyphae, raising the question of an alternative ROS producing system or a temporal control regulator of the Nox complex (Egan et al., 2007). In *B. cinerea*, a necrotrophic fungal pathogen that kills host cells before colonization, there is a strong *in planta* fungal oxidative burst at the hyphal tips and around the penetrated cell wall that correlates with virulence (Segmüller et al., 2008). Both NoxA and NoxB, and the NoxR regulator of both, are

Fig. 2. Redox metabolism in *Magnaporthe oryzae*. Model indicating the *Magnaporthe oryzae* gene products involved in ROS production or ROS detoxification during **(A)** appressoria and penetration peg formation and **(B)** the growth of invasive hyphae in the first infected rice cell. If known, the associated gene regulatory pathways are indicated in B, right panel. Arrows (→) indicate involvement in ROS production in (A), or in gene and protein activation in (B). T-lines (—|) indicate neutralization of ROS (B, left panel) or inhibition of protein function and gene expression (B, right panel). See text for details. Hpi is hours post inoculation. Question marks (??) refer to possible but unknown connections between regulators.

involved in development and pathogenicity in *B. cinerea*. However, NoxA and NoxB are not equivalent—NoxB is required for host penetration while NoxA is needed for post-infection hyphal growth (Segmüller et al., 2008). Moreover, NoxA interacts with NoxD, and loss of NoxD function in *B. cinerea* impair host colonization (Siegmond et al., 2015). In the endophyte *E. festucae*, NoxA mediates the symptomless symbiotic relationship between fungus and host (Takemoto et al., 2006; Tanaka et al., 2006) such that inactivation of the *NOXA* gene switched the interaction from mutualistic to antagonistic, resulting in

the development of disease symptoms (Tanaka et al., 2006). Normally, ROS accumulation was observed in the extracellular matrix of the endophyte and at the interface between extracellular matrix and host cell walls of meristematic tissue, but this was abolished in the $\Delta noxA$ mutants. NoxA function requires NoxR, but only *in planta* (Tanaka et al., 2006). In the root pathogen *V. dahliae*, NoxB and VdPls1 are indispensable for pathogenicity and co-localize in infection structures called hyphopodium where they mediate ROS production to regulate Ca^{2+} -dependent polarized penetration peg formation via the transcription factor VdCrz1 (Zhao et al., 2016). Together, these examples highlight how roles for Nox in plant infection are diverse, and might be tailored to specific lifestyles—compare the roles of ROS-generating Nox in the endophyte *E. festucae* with the hemibiotroph *M. oryzae*—but the molecular basis for these differences in function are largely unknown. Moreover, loss of Nox in some fungi such as *B. cinerea* and *Claviceps purpurea* results in sensitivity to oxidative stress, which was unexpected considering the enzymes are involved in ROS production (Segmüller et al., 2008). These observations might implicate Nox complexes in oxygen sensing and indicate the mechanisms of redox homeostasis are complex.

Although we are focused on host infection, other developmental roles for NoxA/1 and NoxB/2 that are likely relevant to the lifestyle of a fungal plant pathogen or symbiont include: sexual fruiting body formation (*P. anserina*; Malagnac et al., 2004), hyphal fusion (*E. festucae*; Kayano et al., 2013), conidial anastomosis 'CAT' tube formation (*B. cinerea*; Roca et al., 2012), fungicide resistance (*A. alternata*; Yang and Chung, 2013) and sclerotia formation (*C. purpurea*, *S. sclerotiorum*, *B. cinerea*; Giesbert et al., 2008; Kim et al., 2011; Segmüller et al., 2008).

3. How do plant-associated fungi neutralize host ROS?

3.1. Plant innate immune system

Fungal Nox complexes produce ROS for the differentiation of plant-infecting structures, but the host oxidative burst generates superoxide around host infection sites that must be neutralized. Plants have developed finely-tuned innate immune responses to detect the presence of pathogens via pathogen associated molecular patterns (PAMP) triggered Immunity (PTI) and effector triggered immunity (ETI) (Chisholm

et al., 2006; Jones and Dangl, 2006; Hillmer et al., 2017). Innate immune responses include callose deposition, increased pathogenesis related (PR) gene expression and oxidative bursts producing ROS. Plants recognize extracellular motifs of microbes (flagella, chitin, lipopolysaccharides, etc.) and this leads to the rapid release of ROS during PTI (Apostol et al., 1989; Chi et al., 2009). Plant pathogens have developed effector proteins to evade this first basal line of defense. Consequently, plants have developed resistance (R) proteins that detect effector targets or perturbations, triggering ETI which is a more robust version of PTI. ETI also results in an oxidative burst that accompanies a type of programmed cell death (PCD) known as the hypersensitive response (HR) (Baxter et al., 2014; Tanaka et al., 2003; Torres et al., 2005). Due to the unwanted effects of ETI—especially PCD which would hinder biotrophic survival—pathogens have evolved effector proteins to bypass detection from R proteins. This co-evolution between plant and pathogens serves as the basis of the zig-zag model of plant pathology (Hillmer et al., 2017; Jones and Dangl, 2006). However, oxidative bursts are not effective in preventing proliferation of the necrotrophic fungi *B. cinerea* and *Sclerotinia sclerotiorum*, and can be tolerated by the hemibiotrophs, *Septoria tritici* and *M. oryzae* (Govrin and Levine, 2000; Shetty et al., 2007; Samalova et al., 2014; Marroquin-Guzman et al., 2017). How do fungi deal with host ROS and maintain redox balance during host infection?

3.2. Transcriptional control of ROS catabolism

Fungi have robust antioxidation systems to mediate redox homeostasis and neutralize host ROS. The thioredoxin system contains thioredoxins (Trx), and the NADPH-dependent enzyme thioredoxin reductase (TrxR) (Lu and Holmgren, 2014; Fernandez and Wilson, 2014). Thioredoxins are ubiquitous redox proteins containing cysteine residues that are reversibly redox active, cycling between oxidized disulfide (Trx-S₂) and reduced dithiol [Trx-(SH)₂] in a TrxR dependent manner (Holmgren, 1989; Zhang et al., 2016). Small ubiquitous glutaredoxin proteins, part of the glutathione system, are also required for ROS scavenging. Glutaredoxins have overlapping functions with Trx but are non-enzymatically reduced by the essential antioxidant glutathione (GSH) (Holmgren, 1989), which is itself recycled to its reduced form by the action of NADPH-dependent glutathione reductase

(Fernandez and Wilson, 2014). Other enzymes involved in ROS detoxification include superoxide dismutase (SOD), peroxidases and catalases. SOD catalyzes the dismutation of the superoxide radical ($O_2^{\bullet-}$) to H_2O_2 , which can then be further converted to H_2O by catalases or neutralized by glutaredoxins. There are multiple SODs with CuZnSOD being the primary $O_2^{\bullet-}$ cellular detoxifier in the cytosol, nucleus, and mitochondrial inner membrane, compared to Fe-, Mn-, or Ni-SODs (Miller, 2004). Catalase B (CatB) has been studied in many phytopathogenic fungi with varying effects on pathogenicity.

The best-studied transcriptional regulator of antioxidation genes is Yap1 (yeast activating protein 1-like) in *Saccharomyces cerevisiae*. Yap1 is a basic leucine zipper (bZIP) transcription factor regulating enzymatic and non-enzymatic antioxidation genes and is required for the oxidative stress response (Kuge et al., 1997; Toone et al., 2001; reviewed in Toone and Jones (1999)). Yap1 activates genes from the glutathione and thioredoxin system by preferentially binding to a specific sequence in their promoter region (reviewed in Lushchak (2011), Toone and Jones (1999)). Under normal conditions Crm1, a nuclear export protein, recognizes the nuclear export signal (NES) in the cysteine residues of Yap1 which prevents nuclear accumulation (Kuge et al., 1998). During oxidative stress, conserved cysteine residues carried by Yap1, required for Crm1 recognition, are oxidized by the glutathione peroxidase GPx3 (Hyr1), which itself is oxidized by H_2O_2 , resulting in the formation an intramolecular disulfide bond and conformational change. This leads to Yap1 dissociation from Crm1 and allows its accumulation in the nucleus where it binds to promoters of oxidative stress response genes encoding most antioxidants and components of the cellular thiol-reducing pathways (Delaunay et al., 2002; Kuge et al., 1998; Toone et al., 1998). The importance of yeast AP1-like transcription factors in response to oxidative stress have led to the study in many fungal phytopathogens systems (Table 1), as detailed below.

3.3. Neutralizing the host oxidative burst

The role of Yap1 homologs and components of the oxidative stress response in plant infection has been studied in fungi of different lifestyles. Biotrophic fungi are considered as symptom-causing fungi that feed off of living hosts, whereas endophytes reside in intimate contact with living host cells without causing symptoms. Both must deal

with host ROS. In contrast, necrotrophic fungi feed after first killing their host cells and neutralizing host oxidative bursts—which otherwise lead to programmed cell death (PCD) in plants—may not be a beneficial infection strategy for these pathogens. Hemibiotrophs such as the rice and wheat blast fungus *M. oryzae* colonize living host cells and spread into neighboring, living cells, as an asymptomatic biotroph before switching to necrotrophy. At least the early, biotrophic growth phases of hemibiotrophs require host defense suppression and ROS neutralization. However, despite a host oxidative burst being common to all types of fungal-plant interactions, and, as detailed below, despite fungi sharing common regulatory systems and antioxidants, work has mainly focused on the host response and a clear understanding of antioxidation strategies utilized by these fungi is not realized. Some examples of progress made are shown below.

3.3.1. *Biotrophs and endophytes*

It is known that endophytes and other beneficial symbionts—such as mycorrhizal fungi—experience an oxidative burst upon colonization of their host. This oxidative burst is thought to be beneficial to the successful colonization in symbiotic relationships. Upon successful colonization of arbuscular mycorrhizae (AM) fungi in plant roots, upregulation of antioxidation genes has been reported (Kapoor and Singh, 2017). This leads to an increase in stress tolerance of plant hosts. Although antioxidation activity in plants has been measured during these symbiotic relationships, less attention has been placed on understanding the roles of antioxidation enzymes and genes in the fungal partners. In the ericoid mycorrhizal fungus *Oidiodendron maius* loss of *SOD1* activity in the fungus increased ROS sensitivity and, importantly, reduced the capacity for the fungus to colonize host roots, suggesting redox homeostasis is important for mycorrhization (Abbà et al., 2009). It is thus important to continue to tease apart the arsenal of genes required to successfully colonize plants through molecular analysis. Likewise, genes and enzymes required to overcome host oxidative bursts by biotrophic pathogens have not been well characterized. Although homologs of Yap1 have been studied across a range of fungal-host systems, its role in biotrophic and endophytic colonization cannot be generalized due to the lack of functional characterization in endophytes (Table 1). Nevertheless, the Yap1 homolog of the biotrophic maize pathogen *Ustilago maydis*, UmYap1, has been shown

to localize to the nucleus upon H_2O_2 exposure and is required for virulence, suggesting *U. maydis* virulence relies on ROS neutralization, although other roles for UmYap1 cannot be ruled out (Molina and Kahmann, 2007). A Yap1 homolog, YapA, has been characterized in one symbiotic fungi, *E. festucae*. *E. festucae* is an endophyte that intercellularly colonizes the aerial tissue of its perennial ryegrass host, *Lolium perenne*. In $\Delta yapA$ mutant strains of *E. festucae*, conidia were hypersensitive to H_2O_2 while hyphae were not (Cartwright and Scott, 2013). However, unlike UmAp1, YapA was not required for plant infection.

A well-studied ROS-scavenging enzyme, catalase, has also been investigated in a biotroph. One component of the catalase group, *CATB*, is not required for virulence in the biotrophic fungi *C. pupurea*, probably due to a functional overlap with other secreted catalases (Garre et al., 1998a, 1998b). However, absence of all catalase activity, via the knockout of *CpTF1* (a regulator of catalase activity in *C. purpurea*), did reduce virulence (Zhang et al., 2004). Clearly, elucidating molecular mechanisms required to combat host oxidative bursts across other biotrophic and endophytic fungi would help better understand the antioxidation strategies required to maintain beneficial relationships.

3.3.2. Hemibiotrophs: linking redox homeostasis with primary metabolism

The hemibiotroph, *M. oryzae*, possesses a large toolbox of anti-oxidation genes to combat its rice host (Fig. 2B). The transcriptional regulator, MoAp1, is required for biotrophic growth and proper conidiogenesis (Guo et al., 2011). Additionally, the thioredoxin and glutathione antioxidation systems play a crucial role in pathogenicity. Thioredoxin peroxidase (Tpx1), thioredoxin reductase (Trr1) and the thioredoxin Trx2 (but not Trx1), are required for *in planta* proliferation and intracellular ROS metabolism, but not for overcoming plant-derived ROS (Fernandez and Wilson, 2014; Zhang et al., 2016). Glutathione reductase (Gtr1) is required for both *in planta* proliferation of *M. oryzae* and neutralizing host-derived ROS accumulation (Fernandez and Wilson, 2014). The cell-intrinsic glucose-6-phosphate/NADPH-sensing Tps1 enzyme mediates activation of the thioredoxin and glutathione systems in *M. oryzae*. Tps1 controls the expression of NADPH-dependent enzymes—including those of the thioredoxin and glutathione antioxidation system—in response to NADPH availability while balancing the production of NADPH with glucose-6-phosphate

availability, thus providing molecular links between primary metabolism and antioxidation during host infection (Fernandez et al., 2012; Fernandez and Wilson, 2014; Wilson et al., 2010). Building on this concept, a more recent study in *M. oryzae* showed that the *NMO2* gene, encoding a nitronate monooxygenase enzyme catalyzing oxidative denitrification of nitroalkanes, is carbon and nitrogen responsive, regulated by Tps1, and required for axenic growth on nitrate media (Marroquin-Guzman et al., 2017). Nmo2 was subsequently discovered to protect fungal cells from reactive nitrogen species (RNS) as a component of a previously unknown stress pathway in eukaryotes that mediates nitrooxidative stress. During rice cell colonization, loss of *NMO2* elicited a strong host oxidative burst that triggered rice innate immune responses—effectively trapping but not killing the $\Delta nmo2$ mutant—in the first invaded cell. Importantly, the inability to suppress the oxidative burst perturbed the formation of the effector-secreting biotrophic interfacial complex (BIC). Suppressing the host oxidative burst restored single BIC development and $\Delta nmo2$ growth in rice cells. This work consequently demonstrated how a mutation in the fungus resulted in a response from the plant that in turn affected the development of the fungus (Marroquin-Guzman et al., 2017). Thus, investigations into basic fungal metabolism have uncovered molecular and metabolic decisions underlying fungal growth and plant defense suppression in rice cells. Results arising from this work shed light on the hierarchy of molecular events resulting in host immunity by demonstrating that even though $\Delta nmo2$ strains were secreting effectors in host cells, they were ineffective in the presence of an unsuppressed host oxidative burst. The Tps1 and Nmo2 studies also highlight how *M. oryzae* metabolism is dedicated to fueling antioxidation during host infection, and thus connect nutrient availability to ROS metabolism.

The Yap1 homolog, MoAp1, has been studied in *M. oryzae* (Guo et al., 2011), and is required for virulence, but its relationship to Tps1 sugar signaling, is not known (Fig. 2B, *right panel*). Other antioxidation enzymes not under Tps1 control are also required for *M. oryzae* virulence and neutralizing oxidative bursts. CatB is required for host infection due to its role in strengthening the cell wall and appressoria melanization, but it is not required for detoxifying host-derived ROS (Skamnioti et al., 2007). In contrast, catalase peroxidase (CpxB) is required for neutralizing host-derived ROS but does not affect virulence (Tanabe et al., 2011). It is likely that low catalase activity is

compensated by other antioxidation systems during infection. This makes sense as Gpx has an overlapping role with catalases in converting H_2O_2 to H_2O . Therefore, it would be interesting to compare the temporal expression and H_2O_2 affinity of catalases and Gpx when challenged with host-derived H_2O_2 . *M. oryzae* is able to detoxify higher concentrations of ROS than the concentration of ROS due to host oxidative bursts, suggesting that it has a robust ability to combat host defense (Samalova et al., 2014). This ability is further underscored by the role of *M. oryzae* MoSir2, which is an *in planta* specific regulator of antioxidation critical to growth within the host. Loss of MoSir2 function—which, among other effects, results in the loss of *SOD1* function—results in an inability to neutralize host ROS, leading to elevated defense responses that trap, but do not kill, the $\Delta sir2$ mutant (Fernandez et al., 2014). When considered together, these studies suggest that for *M. oryzae*, dealing with host ROS—even in compatible reactions—is the cardinal step in the infection process in order to avoid triggering host defenses.

3.3.3. *Necrotrophs*

In contrast to the rice-*M. oryzae* interaction, host ROS has been proposed to aide plant infection by necrotrophic fungi like *B. cinerea* and *S. sclerotiorum* (Govrin and Levine, 2000; Heller and Tudzynski, 2011). Rather than quenching host ROS, necrotrophic fungi might permit and even contribute to host ROS accumulation as a means to trigger HR and kill host cells. Consistent with this, the aggressiveness of *B. cinerea* isolates corresponds to the intensity of the oxidative burst it produces (Tiedemann, 1997). Moreover, the *B. cinerea* *YAP1* homolog (encoding Bap1) is needed for ROS detoxification in axenic culture, but is not required for *in planta* proliferation (Temme and Tudzynski, 2009). The gene encoding the Yap1 homolog (ChAP1) of the necrotrophic southern corn leaf blight causal agent, *Cochliobolus heterostrophus*, was deleted and found to be dispensable for virulence, although it was required for oxidative stress tolerance with nuclear localization being observed during H_2O_2 exposure (Lev et al., 2005). An exception, though, is *Alternaria alternata*, in which the *AaAP1* gene was required for virulence (Lin et al., 2009). The question remains why a loss of antioxidation transcription factor would be detrimental to *A. alternata* lifestyle while having no effect in other necrotrophs, although AaAp1 might have other roles in virulence beyond regulating

antioxidation. Interestingly, SOD is required for host infection by *B. cinerea*, although this could be due to roles either in ROS neutralization or H_2O_2 production (Rolke et al., 2004). Moreover, Viefhues et al. (2014) showed that the thioredoxin, but not the glutathione system, is essential for survival of *B. cinerea* in its host. Trx1 and Trx2 were required for infection without affecting the cytosolic glutathione pool. Conversely, the absence of glutathione reductase—responsible for recycling oxidized glutathione back to its reduced form—did not have a major effect on *B. cinerea* development or pathogenicity, placing it in a minor role compared to the thioredoxins and raising the question of how infection strategy affects the expression of particular antioxidation pathways (Marschall and Tudzynski, 2016d; Viefhues et al., 2014). Even within a single fungal-host system, it is difficult to form a clear understanding of the mechanisms employed by the fungus. How, for instance, is *B. cinerea* sensing and responding to ROS, if the central oxidative stress response gene regulator *BAP1* is not necessary for virulence, but other components of the antioxidation system are required for infection? The role of particular antioxidation genes and enzymes in the pathogenicity of *B. cinerea* could be due to a difference between their role(s) as a detoxifier of extracellular host-derived ROS versus intracellular ROS. Still, there is difficulty in deriving a comparison for the role of antioxidation genes and enzymes across fungal lifestyles due to a disagreement between the function of the components during these interactions. For example, in agreement with the role of SOD for *B. cinerea* infection, *S. sclerotiorum* also requires SOD for pathogenicity due to its role in oxalate production, a secreted metabolite required for pathogenicity (Veluchamy et al., 2012). However, in contrast to the role of CatB in the necrotrophs *B. cinerea* and *C. heterostrophus*, catalases were required for pathogenicity by *S. sclerotiorum* but not for scavenging extracellular host-derived ROS, which is similar to the role of CatB described for *M. oryzae* (Robbertse et al., 2003; Schouten et al., 2002; Yarden et al., 2014).

In an intriguing twist that might tie the host oxidative burst with the fungal Nox oxidative burst, it has been postulated that the oxidative stress encountered in the host induces the Nox-catalyzed differentiation processes in *B. cinerea* which are needed for penetration or colonization (Segmüller et al., 2008).

4. Tools for measuring fungal ROS

4.1. Rationale

Despite progress in the systems highlighted above, it is still unclear what role plant-derived ROS plays in fungal-plant interactions. Fluorescent redox sensors have the potential to elucidate this role (Hamilton et al., 2012; Donofrio and Wilson, 2013).

4.2. Traditional tools

Although there are several products available for detecting ROS, quantifying intracellular redox state has been difficult. The limitations of these commonly used products are their difficulty in tracking and quantifying intracellular ROS during infection. The difficulty of measuring ROS *in planta* or at the subcellular level comes from the short half-life characteristic of many ROS. However, H_2O_2 has a longer half-life than most, thus leading to the development of a number of tools for H_2O_2 detection (Cruz de Carvalho, 2008). 3,3'-diaminobenzidine (DAB) staining in conjunction with the NADPH oxidase inhibitor Diphenylene iodonium (DPI) have frequently been used to understand the dynamics of H_2O_2 during plant-pathogen interactions (Moore et al., 2002; Marschall and Tudzynski, 2014; Fernandez et al., 2014; Marroquin-Guzman et al., 2017). However, DAB is a slow-acting stain (8–12 h) (Thordal-Christensen et al., 1997). Another method used for H_2O_2 detection during plant-pathogen interactions is the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (H_2DCDA) (Huang et al., 2011; Mir et al., 2015). H_2DCDA is membrane permeable and fluoresces when oxidized but can leak over time or be easily saturated, thus skewing results (Tarpey, 2004).

4.3. HyPer redox sensors

ROS specific genetically-encoded redox sensors have the potential to allow scientists to measure the fine-tuned redox balance during plant-fungal interactions in real-time, whereby the fluorescence of a protein labeled with a fluorophore measures exposure to oxidative stress. A genetically encoded HyPer (hydrogen peroxide) sensor used

in filamentous fungi—originally developed to detect intracellular ROS in mammalian cells using a circularly permuted yellow fluorescent protein (cpYFP) bound to the regulatory domain of the H₂O₂-sensing OxyR protein in *Escherichia coli* (Belousov et al., 2006)—has been optimized for several fungal phytopathogens (Huang et al., 2016; Ronen et al., 2013). The HyPer sensor allows for intracellular measurement of ROS and is specific and sensitive to H₂O₂ (Belousov et al., 2006). The necrotrophic maize pathogen, *C. heterostrophus*, redox sensor pHyPer was initially used to measure the role of the transcription regulator *ChAP1* in mediating redox homeostasis (Ronen et al., 2013). Ronen et al. (2013) used the pHyPer probe to monitor the time it took to re-establish redox homeostasis after challenge with oxidative stress in a $\Delta chap1$ mutant by measuring fluorescent excitation ratios. Subsequently, a more sensitive HyPer2 sensor was developed to measure redox balance in mutants impaired for oxidative stress regulation in *F. graminearum* (Mentges and Bormann, 2015). This sensor has been further codon optimized from *Neurospora crassa* for use in *M. oryzae* and other fungi (Huang et al., 2016). The MoHyPer sensor tracks ROS during the development of *M. oryzae* on artificial hydrophobic surfaces and *in planta*. Although this sensor was optimized for use in *M. oryzae*, it has the potential for use in other ascomycete fungi. It will be interesting to see how these sensors will be utilized in future experiments to understand ROS dynamics in fungi both during development and plant infection.

Heller et al. (2012) studied the oxidative stress sensitive transcription factor *AP1* in necrotroph *B. cinerea* (*BAP1*). They used a fungal $\Delta bap1$ mutant strain expressing radiometric redox-sensitive green fluorescent protein (roGFP2), to measure the dynamics of the glutathione pool in *B. cinerea* *in vivo* and *in planta*. This sensor was later used to study the temporal changes to redox state in various other mutants (Marschall et al., 2016a). Also, the glutaredoxin-bound GFP (Grx1-roGFP2) vector has been expressed in *M. oryzae* to understand the antioxidant defense system of the rice blast fungus *in planta*, with this work concluding that plant oxidative bursts were not sufficient to prevent infection (Samalova et al., 2014).

5. Summary and future issues

Fungal Nox complexes are involved in fungal differentiation necessary for phytopathogenicity, while neutralizing host ROS is emerging as a cardinal event in the suppression of host defenses and the establishment of disease. Components of both processes are not functionally equivalent across fungal species and lifestyles. For example, some Yap1 homologs are important for infection and others are dispensable (**Table 1**), but these roles do not align with fungal lifestyles. More investigations of Nox complexes, Yap1 homologs, antioxidation systems, and their links to primary metabolism are required in plant-associated fungi with a range of lifestyles, including endophytes and mycorrhizal fungi, in order to form general rules about the role of these processes in plant-fungal interactions. Comparing closely related fungi with different lifestyles might also be informative here. For example, future gains in understanding plant-microbe interactions might come from molecular comparisons of plant pathogens versus endophytes with regards to redox balance. For example, functional comparisons of the endophyte, *Harpophora oryzae* (Xu et al., 2014; Yuan et al., 2010), with its pathogenic relative, *M. oryzae*, might unpack differences in how

Table 1. Functionally characterized Yap1 homologs in plant-associated fungi.

Pathogen name	Infection lifestyle	Yap1 homologue	Phenotype(s)	Required for virulence?	Reference
<i>Alternaria alternata</i>	Necrotroph	AaAp1	ROS detoxification	Yes	Lin et al. (2009)
<i>Botrytis cinerea</i>	Necrotroph	Bap1	ROS detoxification	No	Temme and Tudzynski (2009)
<i>Colletotricum gloeosporioides</i>	Hemibiotroph	CgAp1	ROS detoxification	Yes	Sun et al. (2016)
<i>Cochliococcus heterostrophus</i>	Necrotroph	ChAp1	ROS detoxification	No	Lev et al. (2005)
<i>Epichloe festucae</i>	Endophyte	YapA	ROS detoxification	No	Cartwright and Scott (2013)
<i>Fusarium graminearum</i>	Hemibiotroph	FgAp1	ROS detoxification/ Secondary metabolism/ Osmotic stress resistance	No	Montibus et al. (2013)
<i>Magnaporthe oryzae</i>	Hemibiotroph	MoAp1	ROS detoxification/ Conidiation/ Hyphal branching/ Peroxidase and laccase secretion	Yes	Guo et al. (2011)
<i>Monilinia fructicola</i>	Necrotroph	MfAp1	ROS detoxification	Yes	Yu et al. (2016)
<i>Ustilago maydis</i>	Biotroph	UmYap1	ROS detoxification	Yes	Molina and Kahmann (2007)

metabolism, nutrient signaling, growth, and antioxidation are connected in two fungi with opposing lifestyles.

The importance and interconnectivity of host and pathogen ROS metabolism is becoming evident, but molecular understanding of the underlying mechanisms are still lacking. How far have we gone in closing the knowledge gaps outlined in the introduction? (i) *Endogenous ROS produced as a by-product of metabolism is damaging to cells and must be neutralized, yet local ROS bursts are required for fungal differentiation, including the morphogenesis of specialized appressorial cells required to access host plant cells.* The control/maintenance of this altered redox balance might be due to the role of Nox complexes in delivering ROS in a precisely defined manner. Specific understanding of Nox complex localization, the regulatory components of the system, and regulation at the gene expression level will be useful here. (ii) *Once inside the host, neutralization of exogenous ROS is important, as fungal invaders must overcome several lines of plant defense, including ROS produced from a host oxidative burst which, left unchecked, triggers additional plant defenses, but internal ROS arising from fungal growth must also being dealt with.* Here, different antioxidation systems might be dedicated to different ROS regimes. For example, the thioredoxin system is required for maintaining internal ROS balance, but the glutathione system is necessary for exogenous ROS neutralization in *M. oryzae* during host infection. (iii) *ROS metabolism involves NADPH and is thus energy intensive, but must be balanced with the energy requirements of growth, which also produces ROS. How are growth and ROS metabolism integrated?* Tps1 functions to connect available nutrients to metabolism and antioxidation. Understanding how such regulators operate in other pathosystems will be valuable. (iv) *Pathogenic fungi and beneficial mycorrhizal or endophytic fungi—the former being characterized by short-lived plant associations with vigorous ramifications in host tissue, while the latter has long-lived associations and little in planta growth—must both neutralize host ROS.* As mentioned above, more comparisons among and between fungi with different lifestyles is warranted. When considered together, this topic is likely to remain an important and fertile field of study for the foreseeable future.

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