Suppression of plant-generated reactive oxygen species is required for successful infection by the rice blast fungus

Kun Huang,1 Kirk J. Czymmek,2 Jeffrey L. Caplan,3 James A. Sweigard4 and Nicole M. Donofrio1,*
1Plant and Soil Sciences; 2Department of Biological Sciences; University of Delaware; 3Delaware Biotechnology Institute; 4Stine-Haskell Lab; DuPont; Newark, DE USA

Magnaporthe oryzae is a filamentous ascomycete that continuously threatens global rice production. The infection cycle of this pathogen commences with the attachment of conidia to rice plants, followed by the formation and maturation of a specialized infection structure—the appressorium. Melanized appressoria generate immense turgor pressure, which allows the fungus to break through the plant cuticle and cell wall by means of a penetration peg. These stages occur within the first 24 h after which time the penetration peg gives rise to and subsequent primary and secondary infection hyphae. Upon infection, the plant recognizes the pathogen, triggering a series of defense responses and signaling events including the secretion of reactive oxygen species (ROS). In a recent paper, we showed that barley plants generate ROS and cell wall appositions (CWAs) around infection sites and that a fungal gene we termed MoHYR1 is necessary for ameliorating these defense reactions and ensuring successful infection and colonization. When this gene is deleted from the M. oryzae genome, the plant oxidative responses are stronger and disease is reduced.

The rice blast fungus, *Magnaporthe oryzae* (*M. oryzae*), causes serious and recurrent problems in rice-growing regions worldwide.1 It not only infects rice, the world’s most important food crop, but also other economically important members of the Poaceae, including wheat, barley and turf grass.2 Every year, the amount of rice destroyed by *M. oryzae* is enough to feed about 60 million people.1 In recent years, 5.7 million hectares of rice fields were destroyed in China, Korea, Japan, Vietnam and the US.3 The infection cycle of *M. oryzae* begins when asexual spores called conidia germinate and develop single-celled appressoria after landing and attaching themselves to leaves. The appressorium consists of a melanin layer and a non-melanized pore ring; this dome-shaped structure generates enormous turgor pressure, which allows the penetration peg to break through the plant cuticle and cell wall. The penetration peg soon gives rise to bulbous primary infection hyphae, and colonization by secondary hyphae begins within 48 h in a susceptible host.3

During the evolutionary arms races between plants and pathogens, plants have evolved different mechanisms to detect and defend against pathogen invasion. One of the first lines of defense is the generation of reactive oxygen species (ROS), including superoxide (O2−), hydrogen peroxide (H2O2), hydroxyl radical (OH·), nitric oxide (NO) and singlet oxygen (1O2). Oxidative bursts have been detected when plant cells are inoculated with biotrophic pathogens,5 necrotrophic pathogens 6 and pathogen elicitors.7 A study performed on tobacco suspension cells demonstrated that there are two phases of oxidative burst when inoculated with the bacterial pathogen *Pseudomonas syringae*.8 The first burst of ROS is observed with both susceptible and resistant interactions9 and thought to be related with basal immunity, which is a wide-spectrum defense associated with pathogen or pathogen-induced elicitors, such as MAMPs (microbes associated...
molecular patterns. Expressing the bacterial EF-Tu MAMP receptor in tobacco leaves stimulated the production of ROS and made it resistant to a wide range of phytopathogens. The second burst of ROS is termed “recognition response” because it is specific to avirulent pathogens. During this process, more ROS is generated, and less ROS is degraded, due to a decrease in catalase activity.

We are keenly interested in understanding how fungi manage stressful environments for successful infection and colonization. In our recent study on ROS detoxification mechanisms in *M. oryzae*, we observed that both wild-type and ΔMohyr1 mutant strain (deficient in the virulence factor MoHYR1 that is involved in plant-generated ROS sensing and detoxification), are capable of triggering ROS generation in a susceptible host 1 h after inoculation. This indicates that the plant, even without a cognate resistance gene, recognizes invasion, which immediately triggers defenses. Differences in the plant ROS pattern were seen later (24 h post-inoculation) when either wild-type or ΔMohyr1 were applied to barley leaf surface; ROS “halos” are observed around the wild-type and the mutant appressoria but were seen much more frequently and in greater abundance with the former (Fig. 1). The halos were localized inside the plant cell wall and around the fungal penetration peg, and perfectly overlaid with another defense-related structure, Cell Wall Appositions (CWAs). CWAs are formed by cross-linking of phenolics and are believed to physically block pathogen penetration, which are seen in both susceptible and resistant interactions. Callose and ROS are known to be involved in cell wall appositions; specifically, H2O2 plays an important role in this process and enzymatic removal of H2O2 by catalase significantly reduces the frequency of CWAs. Peroxidases, a calcium influx and K+ Cl- efflux, extracellular alkalization and post-Golgi vesicles were also reported to be essential for the oxidative burst that leads to the formation of CWAs.

In our study, we observed that ROS and callose deposits were positionally-related during attempted penetration by both wild-type and ΔMohyr1 mutants, immediately underneath the appressorium, but were more strongly expressed during mutant infection. Based on these data, we hypothesize that plants (barley in this case) respond defensively to the virulent fungus by generating ROS, which in turn activates CWA formation. These two defensive compounds work together to block pathogen penetration. In support of our data, Wang et al. observed that both superoxide (O2−) and hydrogen peroxide (H2O2) were mostly seen in the cell wall, plasma membrane and tonoplast of mesophyll cells in contact with cells killed off by the plant during the hypersensitive response (HR) and mesophyll cells around the infection hyphae themselves.

Many plant pathogenic fungi must be capable of inhibiting the plant ROS burst and negotiating through a potentially hostile environment in order to colonize and proliferate. Indeed, a transcriptome analysis of *M. oryzae* invasive plant infection vs. growth in vitro determined that 407 genes were commonly differentially regulated when *M. oryzae* was inoculated in rice, barley and paraquat-amended medium, which mimics oxidative stress, demonstrating that the infected plant cell is indeed an

![Figure 1](image-url)
environment rich in ROS. Fungi contain a suite of ROS-detoxification enzymes including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR), and cytochrome c peroxidases (CcP).17,18 These enzymes function in different sub-cellular locations and regulate ROS equilibrium. We found that the virulence factor MoHYR1 was involved in disrupting plant-generated ROS and managing ROS levels during early infection stages.12 Deletion of this gene caused a virulence defect in M. oryzae, allowing the plant (both rice and barley) to effectively fend off the mutant strain. The glutathione pathway-related gene GLR1 (glutathione reductase), GTO1 (omega class glutathione transferase 1) and GSH1 (γ-glutamylcysteine synthetase) all increased in the fungus during H$_2$O$_2$ challenge; in the wild-type, however, had extremely decreased expression in the mutant line. This suggested that the regulation of ROS detoxification is a complicated yet important network in M. oryzae, and the disruption of a major regulatory component such as MoHYR1 might affect how well the fungus can adapt to the host environment, which will in turn lead to a reduction in virulence. A gene recently reported in M. oryzae called DES1 (Defense Suppressor 1)19 also has a demonstrated role in virulence. Deletion of this gene triggered a stronger plant response, manifested by both an increase of the oxidative burst, as well as expression of two plant defense genes. Plant cells are strongly stained with DAB (3,3’-diaminobenzidine) when infected with Δdes1 mutant strains compared with wild-type, indicating a higher level of H$_2$O$_2$. Equally intriguing is a transmembrane protein called TmpL,20 identified in human and plant fungal pathogens, Aspergillus fumigatus and Alternaria brassicicola, respectively. Deletion of TmpL caused severe hypersensitivity to exogenous ROS and a defect in virulence. Egan et al. discovered that M. oryzae undergoes an oxidative burst of its own during plant infection, and moreover, this oxidative burst is important for development of the appressorium. Our data showed minimal differences between the ΔmoHYR1 mutant and wild-type when examining internal ROS generation during spore germination and appressorium formation on an artificial surface,12 indicating that mechanisms other than HYR1 likely play a role in internal ROS generation and/or maintenance.

Together, these data strongly imply that loss of a ROS detoxification gene is highly likely to cause a virulent defect in a fungal pathogen.

Plants possess numerous pathways for generating ROS,22 and fungi have the ability to manipulate ROS levels by dynamically re-programming host metabolic reactions.23 A metabolite fingerprint study of three rice cultivars infected by the rice blast fungus proves that the activities for ROS generation via certain pathways are suppressed in a susceptible interaction;5 an increase in ornithine and putrescine levels suggests that the cell wall-localized diamine oxidases-related defense, H$_2$O$_2$ production, may be inhibited in susceptible interactions. Further, NADP-ME (NADP- malic enzyme, substrate for NADPH-oxidase) activity is likely to be suppressed in cells adjacent to penetration sites.5 Interestingly, broad-spectrum suppression of innate immunity by ROS detoxification mechanisms has also been shown for plants and endophytic fungi. Zhang et al.24 reported that while an increase in the expression level of PR-10 (pathogenesis-related class 10) was important for ryegrass defense its expression level remained the same after inoculation with the endophytic fungus Neotyphodium lolii. At the same time, a high level of the fungal Cu/Zn superoxide dismutase was detected in the infected tissue, indicating that the endophyte was breaking down ROS. Inoculation of Piriformospora indica, which is a beneficial root-colonizing basidio- mycete fungus, suppressed the expression of flg22-induced transcript for MTI (MAMPs-triggered immunity). Together, these studies suggest that microbial colonizers suppress various plant ROS pathways in order to colonize/infect their hosts. In our study, while several ROS pathways might be suppressed by M. oryzae,5 the barley plant was clearly utilizing other means to generate ROS, which the fungus then had to directly combat (Fig. 1).

Moreover, ROS species are not only the products of plant defense, but are also found to be long-distance, cell-to-cell signaling compounds. A study from University of Nevada reported a high level of ROS traveling at a rate of 8.4 cm/min, which were triggered by wounding, heat, cold, high-intensity light and salinity stresses.25 As a self-amplification system, ROS are not only the trigger, but are also the consequences of activation of MAPK signaling pathways.26 Upon activation, MAP kinases function in the nucleus and induce expression of defense genes.27 Transcript analysis in multiple ROS-inducing conditions suggests that transcription factors activated during this process are predominantly via the MEKK1-MKK1/2-MPK4 pathway in both feed-forward and feed-back loops.26 SA, GA, ABA and other hormones are also found to be involved in ROS regulation. For example, SA is reported to inhibit catalase activity, thus, amplification of SA increases H$_2$O$_2$ concentration in plants.28 In this scenario, the inhibition of the initial ROS levels might suppress the higher and sustained accumulation of ROS, preventing hypersensitive cell death that often accompanies resistant interactions.18

When a pathogen lands on plant surface, in our case the rice blast fungus on barley, the plant will rapidly trigger an oxidative burst as part of its basal defenses. Plant can generate ROS via organelles with high electron-flow rates, such as mitochondria, chloroplasts and peroxisomes (Fig. 1). Plant ROS can also be generated via enzymatic sources, such as membrane-associated NADPH oxidases, cell wall peroxidases and oxalate oxidases.13,29 A wild-type pathogen, harboring the requisite ROS detoxification mechanisms, will ameliorate host ROS production and regulate the ROS level within the host cell. As described earlier, NADPH oxidase activity, diamine oxidase activity and lignin-based cell wall strengthening are impaired in susceptible rice cultivars after infection by M. oryzae.23 In this case this initial weakened ROS burst might not be enough to trigger later stronger defense reactions. On the other hand, if the pathogen is lacking important genes for ROS detoxification such as MoHYR1, this initial ROS burst may be sufficient to either block penetration, kill the pathogen or activate downstream defense responses (as shown in the model presented in Fig. 1).
When considered with the studies described above, our work on the MoHYR1 gene demonstrates that the rice blast fungus likely uses immunosuppression strategies in order to cope with early ROS bursts upon initial infection, and subsequent successful infection and colonization. An alternative yet related hypothesis is that when *M. oryzae* lacks the HYR1 gene, it is unable to directly detoxify the plant-produced ROS compounds. Further scrutiny of the interaction between HYR1 minus mutants and other genetic components of this ROS management pathway are necessary to test the delicate balanced interplay between ROS generation and breakdown in this plant-pathogen interaction.

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