NADPH oxidases regulate septin-mediated cytoskeletal remodeling during plant infection by the rice blast fungus


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The rice blast fungus Magnaporthe oryzae infects plants with a specialized cell called an appressorium, which uses turgor to drive a rigid penetration peg through the rice leaf cuticle. Here, we show that NADPH oxidases (Nox) are necessary for septin-mediated reorganization of the F-actin cytoskeleton to facilitate cuticle rupture and plant cell invasion. We report that the Nox2–NoxR complex spatially organizes a heterologeric septin ring at the appressorium pore, required for assembly of a toroidal F-actin network at the point of penetration peg emergence. Maintenance of the cortical F-actin network during plant infection independently requires Nox1, a second NADPH oxidase, which is necessary for penetration hypha elongation. Organization of F-actin in appressoria is disrupted by application of antioxidants, whereas latrunculin-mediated depolymerization of appressorial F-actin is competitively inhibited by reactive oxygen species, providing evidence that regulated synthesis of reactive oxygen species by fungal NADPH oxidases directly controls septin and F-actin dynamics.

Results

M. oryzae NADPH Oxidase Mutants Are Impaired in F-Actin Organization During Appressorium Development. To understand the role of NADPH oxidases during infection-associated development, we identified M. oryzae NOX(R) (MGG 05280.6), which shares 38% amino acid sequence similarity with Homo sapiens p67phox (Fig. S1) and generated a ΔnoxR null mutant (Fig. S2). We then introduced a LifeAct-RFP gene fusion (14) into isogenic M. oryzae Δnox1, Δnox2, Δnox1Δnox2 (15), and ΔnoxR mutants to observe organization of F-actin by live-cell imaging (13, 14). In the wild-type M. oryzae strain, Guy11, a large toroidal-shaped F-actin network (~5.9 μm in diameter) formed around the appressorium (13), which we visualized by live-cell imaging and topologically characterized using transverse line scan analysis of LifeAct-RFP fluorescence intensity in the appressorium (Fig. L4 and Movie S1). In Δnox1 mutants, the F-actin network was partially disorganized (Fig. 1 and Fig. S3 and Movie S2). Individual actin cables were not observed and a dense, unorganized patch of actin instead accumulated at the appressorium pore (2.5 ± 0.4 μm in diameter, n = 20). In the Δnox2 mutant, F-actin accumulated at the appressorium pore in a dense, unorganized patch (3.8 ± 0.3 μm in diameter, n = 20), shown in Fig. L4 and Movie S3. A similar pattern was observed in both the Δnox1Δnox2 double mutant and in a ΔnoxR mutant (4.2 ± 0.4 μm in diameter, 


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PLANT BIOLOGY

NADPH oxidases (Nox) are flavoenzymes that function by transferring electrons across biological membranes to catalyze reduction of molecular oxygen to superoxide (1, 2). In animal cells, Nox enzymes are implicated in cell proliferation, cell signaling, and apoptosis (1–3), whereas in plants Nox are necessary for programmed cell death (4), the response to environmental stresses (4), pathogen infection (5), and polarized growth of root hairs (6). In filamentous fungi, Nox are necessary for cellular differentiation during sexual reproduction and for developmental processes that involve transitions from nonpolarized to polarized cell growth, such as tissue invasion by mutualistic and pathogenic fungi, and fungal virulence (7–9). The underlying function of Nox enzymes in these diverse developmental processes remains unclear.

In this study we set out to investigate the role of reactive oxygen species (ROS) synthesis by Nox during plant infection by the devastating rice blast pathogen Magnaporthe oryzae (10). Rice blast disease is considered a major threat to global food security and remains difficult to control (11). Nox1 and Nox2 are necessary for an oxidative burst that is required for plant infection by the fungus (12), but why this is necessary for rice blast disease is not known. To infect rice plants, M. oryzae forms a specialized infection structure called an appressorium (10). This dome-shaped cell generates enormous turgor of up to 8.0 MPa (12), which is translated into physical force at its base to rupture the rice leaf cuticle using a narrow, rigid, penetration peg that invades plant tissue. Recently, it has been reported that septin GTPases scaffold cortical F-actin in the appressorium at the site of plant infection, acting via the ezrin-radixin-moesin (ERM) protein, Teal, and specific phosphatidylinositol interactions at the fungal plasma membrane (13). M. oryzae septins assemble into a hetero-oligomeric ring that surrounds the appressorium pore, a region at the base of the infection cell from which the penetration peg emerges. This acts to scaffold and reorganize the F-actin cytoskeleton and as a partitioning diffusion barrier to localize Bin-Amphiphysin-Rvs (BAR)-domain proteins, implicated in membrane curvature and protrusion of the penetration peg through the rice leaf surface (13).

In this report, we show that NADPH oxidases play distinct, but essential, roles in F-actin remodeling during plant infection. The Nox2 complex, which includes the p67phox-like regulator NoxR, is necessary for assembly of septin GTPases at the appressorium pore, which is required for reorientation of the F-actin cytoskeleton at the point of plant infection. Consistent with this, Δnox2 mutants are unable to initiate penetration peg formation and cannot infect rice plants. We also show that Nox1 is required for correct organization of the toroidal F-actin network, acting after septum assembly. Δnox1 mutants therefore initiate penetration peg development but fail to elongate invasive hyphae to rupture the rice cuticle. These functions seem to require the regulated synthesis of ROS and are likely to be a direct consequence of the enzymatic activity of NADPH oxidases. Our results provide evidence that Nox complexes play critical roles in both initiation and maintenance of polarized growth by the rice blast fungus during plant infection.
Fig. 1. *M. oryzae* NADPH oxidase mutants show aberrant F-actin organization during appressorium development. (A) Micrographs of F-actin organization visualized by expression of LifeAct-RFP in Guy11, Δnox1, Δnox2, Δnox1:nox2, and ΔnoxR mutants. Conidial suspensions at $5 \times 10^7$ mL$^{-1}$ were inoculated onto glass coverslips for 24 h. Linescan graphs show LifeAct-RFP fluorescence in a transverse section of individual appressoria. (Scale bar, 10 μm.) (B) Bar chart to show percentage of appressoria containing intact F-actin rings after 16 h (black bars) and 24 h (white bars). Values are mean ±2 SE for three repetitions of the experiment, $n = 100$. (C) Recovery of LifeAct-RFP ring after partial photobleaching, with 75% recovery in fluorescence after 3.5 min. Values are means ±2 SE, $n = 24$. (Scale bar, 5 μm.)

Fig. 2. *M. oryzae* Nox2 is essential for septin ring formation at the appressorium pore. (A) Micrographs to show cellular localization of Sep5-GFP in Guy11, Δnox1, Δnox2, Δnox1:nox2, and ΔnoxR during appressorium development after 24 h. Linescan graphs represent Sep5-GFP fluorescence in a transverse section of individual appressoria. (B) Micrographs to show localization of the Chm1 septin kinase in appressoria. Chm1-GFP was expressed in Guy11, Δnox1, Δnox2, and ΔnoxR mutants. Linescan graphs showing Chm1-GFP fluorescence. (C) Micrographs to show localization of LifeAct-RFP, Sep5-GFP, and Chm1-GFP in the tetraspanin mutant Δspf1. (Scale bars, 10 μm.)
ΔnoxR mutants, aberrant septin rings formed, which were 19% smaller in diameter (mean = 4.76 μm, n = 20) than Sep5-GFP rings in Guy11 (Fig. 2). We also expressed a Chm1-GFP fusion in each of the Nox mutants. Chm1 is a protein kinase that in yeast is known to phosphorylate septins (16) and in M. oryzae is distributed in a ring conformation around the appressorium pore (14) (Fig. 2B). A Chm1-GFP ring was present in Guy11 and Δnox1 mutants but disorganized in Δnox2 and ΔnoxR mutants, consistent with a role for Nox2 in the regulation of septin assembly and Chm1 kinase localization (Fig. 2B). Quantitative analysis confirmed that Nox2 is essential for septin and Chm1 ring assembly, showing more severe disruption of septin ring assembly than Δnox1 or ΔnoxR mutants (Fig. 2A and B). The N-WASP protein Las17, a component of the actin-polymerizing Arp2/3 complex, was observed by expression of a Las17-GFP fusion and localized as puncta in the appressorium pore in Guy11 (13) and the Δnox1 mutant, whereas in Δnox2 and ΔnoxR mutants Las17-GFP localization was disrupted, with the protein distributed diffusely in the cytoplasm and plasma membrane (Fig. S7). The ERM domain fusion protein Tea1-GFP, which localises as a ring of puncta at the appressorium pore in both Guy11 (13) and Δnox1 mutants, also showed abnormal localization in both Δnox2 and ΔnoxR mutants. The localization of Rvs167-GFP, a BAR-domain protein (13), was also affected in Δnox2 and ΔnoxR mutants, with puncta present in a misshapen appressorium pore (Fig. S7). We conclude that ERM–actin interactions at the appressorium pore, which are essential for linking cortical F-actin to the membrane to facilitate penetration peg emergence, require the Nox2/NoxR complex. Furthermore, the role of septins in spatially localizing the WASP/Arp2/3 complex component Las17 and BAR-domain protein Rvs167 to the appressorium pore—which mediate penetration peg emergence—is compromised in the absence of Nox2/NoxR.

Given the distinct function of Nox2 in regulating septin-mediated F-actin reorganization, we were interested in identifying other potential components of the Nox2 complex that might be involved in its recruitment to the appressorium pore. Studies in M. oryzae, Podospora anserina, and Botrytis cinerea have highlighted a connection between Nox2 (NoxB) and the transmembrane tetraspanin protein, Psl1 (17–19). Mutants lacking PLS1 in these fungi strongly resemble nox2 (noxB) mutants, and there is a very close phylogenetic association between fungi possessing both Nox2 and Psl1 homologs (20). It has been proposed that Psl1 might therefore be associated with Nox2 activity or specific recruitment to its site of action (21). To test this idea, we therefore expressed LifeAct-RFP, Sep5-GFP, and Chm1-GFP in a M. oryzae Δpssl1 mutant (Fig. 2C). Strikingly, we observed mislocalization of F-actin, septin, and Chm1 in the absence of the Psl1 tetraspanin in the same manner as Δnox2 mutants.

**Reactive Oxygen Species-Dependent Organization of the F-Actin Regulator Gelsolin in M. oryzae.** We were interested in determining whether Nox-dependent cytoskeletal remodeling was due to regulated synthesis of ROS or a more indirect role in cell signaling. In mammalian systems, an increasing body of evidence suggests that ROS generation may affect F-actin dynamics during cell shape changes, migration, and cell motility (22), for example, in human sperm (23), low-density lipoprotein-induced stress fiber formation (24), endothelial cell mitogenesis, and during apoptosis (25). Antioxidants have been reported to exert a direct effect on F-actin cable elongation (26), and components of Nox complexes are also known to associate with F-actin (27) or actin-binding proteins (28). The actin-binding protein gelsolin, which regulates F-actin dynamics by uncapping free barbed ends to promote actin polymerization and by severing existing actin, septin, and Chm1 in the absence of the Psl1 tetraspanin in the same manner as Δnox2 mutants.

**Fig. 3.** Reactive oxygen species-dependent organization of the F-actin regulator, gelsolin, in M. oryzae. (A) Micrographs and linescan graph showing colocalization of LifeAct-RFP and gelsolin-GFP in the appressorium of the M. oryzae wild-type strain Guy11. (B) Micrographs and corresponding linescan graphs to show organization of gelsolin-GFP expressed in appressoria of Guy11, Δnox1, Δnox2, and ΔnoxR mutants after 24 h. (C) Recovery of gelsolin-GFP ring after partial photobleaching, with 83% recovery after 8 min. Values are means ±2 SE, n = 23. (Scale bar, 5 μm.) (D) Micrographs to show effect of exposure to the NADPH oxidase inhibitor diphenylene iodonium (DPI) or antioxidant ascorbate on gelsolin-GFP and Sep5-GFP organization. Conidia expressing gelsolin-GFP and Sep5-GFP were independently exposed to 5 μM DPI or 1 mM ascorbic acid after 16 h and observed by epifluorescence microscopy after 24 h. (E) Micrographs to show effect of latrunculin A treatment on gelsolin-GFP organization in the presence or absence of ROS. Gelsolin-GFP was organized as a ring in M. oryzae in the presence or absence of 20 mM H2O2. Treatment with 0.3 μM latrunculin A led to disorganization of gelsolin-GFP, which was partially remediated by simultaneous addition of 20 mM H2O2. (F) Bar chart showing percentage of appressoria containing intact gelsolin-GFP rings after 24 h. Values are means ±2 SE for three repetitions of the experiment, n = 50. (Scale bars, 10 μm.)
actin regulator. In a Δnox1 mutant, gelsolin-GFP still organized into a ring structure, but in Δnox2 or ΔnoxR mutants gelsolin-GFP formed a disorganized patch at the appressorium base (Fig. 3B). To understand the nature of the gelsolin ring, we investigated fluorescence recovery after partial photobleaching. We found 83% recovery of fluorescence after 8 min, consistent with gelsolin turnover’s being associated with F-actin dynamics (Fig. 3C and Movie S7). To determine whether F-actin and septin dynamics in appressoria were directly influenced by ROS synthesis, we applied the flavoenzyme (Nox) inhibitor diphenylenedioxonium chloride (15) and the antioxidant ascorbate, both of which prevented formation of gelsolin-GFP and septin rings, resulting instead in aberrant smaller ring structures at the appressorium pore (Fig. 3D). This is consistent with ROS generation’s being required for both F-actin and septin ring assembly. We therefore applied the actin de-polymerizing agent latrunculin A to M. oryzae appressoria, which (as expected) led to disruption of gelsolin-GFP organization. However, when we applied hydrogen peroxide (H₂O₂) in the presence of latrunculin A, this partially restored gelsolin-GFP ring formation (Fig. 3 E and F). The depolymerizing activity of latrunculin A can therefore be partially prevented by the presence of high concentrations of H₂O₂.

Latrunculin A treatment of mature appressoria (at 16 h) also inhibited appressorium-mediated cuticle penetration on rice epicotyl epidermal strips (Fig. S8), but this was partially remediated by simultaneous addition of H₂O₂ (Fig. S9). We conclude that regulated synthesis of ROS by M. oryzae Nox proteins can directly affect F-actin dynamics.

Nox1 and Nox2 Play Distinct Roles During Plant Infection. Given the distinct phenotypes of Δnox1 and Δnox2 mutants with respect to F-actin and septin dynamics, we were interested to see how they differed in their ability to cause rice blast disease. Previously, Δnox1 and Δnox2 mutants have been shown to be nonpathogenic (15), and we confirmed that ΔnoxR and Δnox1Δnox2 mutants were also unable to cause rice blast disease (Fig. 4 A–D). Transmission electron microscopy of appressoria, however, showed that Δnox1 mutants still initiated penetration peg formation from the base of the infection cell (Fig. 4C). These short projections were unable to extend through the plant cell wall (Fig. 4B) and Δnox1 mutants were therefore unable to colonize rice tissue and cause disease. By contrast, appressoria of Δnox2, Δnox1Δnox2, and Δnox1ΔnoxR mutants were unable to repolarize at the appressorium pore and could not form penetration pegs (Fig. 4D–F). We conclude that Nox2 and NoxR are necessary for septin-mediated initiation of polarized growth of the penetration peg at the appressorium base, whereas Nox1 is necessary for maintenance of penetration peg elongation.

Discussion

Regulated synthesis of ROS by NADPH oxidases is necessary for a wide variety of developmental processes in mammals, plants, and fungi (1, 3, 6). In filamentous fungi, ROS generation has, for example, been associated with hyphal tip growth, spore formation, infection structure development, conidial anastomosis tube generation, fruiting body formation, fungal–plant mutualistic interactions, and fungal virulence (1, 2). These diverse roles for ROS as signaling molecules in fungi are, however, still not well understood. The best understood NADPH oxidase is the mammalian phagocytic leukocyte gp91phox (or Nox2), which is necessary for the defensive oxidative burst during microbial attack. The gp91phox protein is part of a membrane-spanning complex (flavocytochrome b₅₅₈) with the adaptor protein p22phox and interacts with the cytosolic regulatory proteins p40phox, p67phox, p47phox, and Rac GTPase (3). In total there are five Nox enzymes in humans, which have been implicated in a variety of cellular processes, including cell proliferation, apoptosis, and hormone responses. This has led to the suggestion that ROS production may be a ubiquitous signaling system controlling cellular differentiation in eukaryotes (3–6). Filamentous fungi possess up to three Nox enzymes. Nox 1 (or NoxA) and Nox2 (NoxB) are similar to gp91phox, whereas Nox3 (NoxC) enzymes have E-hand domains that putatively bind Ca²⁺ in the same way as plant respiratory burst oxidases (8, 9) and human Nox5 (3). Fungal Nox complexes seem to involve interactions with cytosolic proteins, previously implicated in the regulation of polarity (1). A recent study of the plant-associated mutualistic fungus Epichloë festucae, for example, showed that NoxR interacts with homologs of the yeast polarity proteins Bem1 and Cdc24 (2) and that this requires the Phox and Bem1 (PB1) protein domain, which is also necessary for localization of these proteins to actively growing hyphal tips. E. festucae mutants that lack Bem1 are defective in hyphal morphogenesis and growth in culture whereas Cdc24 seems to be essential for viability (2). The study therefore provided evidence for an important role for fungal NADPH oxidases in the establishment of polarity and the control of cellular differentiation. This is consistent with recent findings in plant pathogenic fungi, such as Botrytis cinerea, Sclerotinia sclerotiorum, and Claviceps purpurea, in which Nox proteins have been shown to be necessary for fruit body formation and appressorium function (1, 25, 26).
and experiments in *Podospora anserina* that have implicated Nox proteins in polarity establishment and invasive growth (27).

In this report, we have provided evidence that the role of fungal Nox in polarized fungal growth is likely to be based on remodeling of the actin cytoskeleton. The two Nox complexes together, our results are consistent with a model whereby the *M. oryzae* Nox2/NoxR complex is essential for septin-mediated F-actin assembly at the appressorium pore, which is a prerequisite for penetration peg formation and rice infection. In *Saccharomyces cerevisiae*, a recently identified Nox-encoding gene, YNO1, has also been implicated in actin cable formation (30), suggesting that regulation of F-actin dynamics may be one of the key underlying mechanisms by which Nox exert their diverse roles in cellular differentiation. *M. oryzae*, Nox2 is regulated as part of a complex involving NoxR and is also known to interact with Rac1 (31), a small GTPase involved in polarized cell growth and also implicated in F-actin dynamics. Gelsolin is, for instance, a downstream effector of Rac1 (32). We also found evidence that the tetraspanin Pls1 may play a role in the Nox2 complex based on its effect on septin and F-actin organization at the appressorium pore. Nox2 is therefore essential for initiation of polarized growth from the appressorium. Remediation of the effects of the actin depolymerizing agent latrunculin A upon exposure to H2O2 further suggests that the role of Nox2 and NoxR in polarity establishment may be a direct consequence of the regulated synthesis of ROS by NADPH oxidases, perhaps acting directly on actin regulatory proteins such as gelsolin. It has been suggested, for instance, that ROS may increase the actin-severing ability of gelsolin by disulfide bond formation in the protein, leading to greater exposure of barbed ends and increased actin polymerization (22).

By contrast, we found that Nox1 is not required for septin ring assembly at the appressorium pore but is necessary for correct organization of the F-actin network that is scaffolded at this site. Furthermore, Δnox1 mutants are able to initiate penetration peg formation (and therefore establish polarized growth) but cannot elongate invasive hyphae, suggesting that the Nox1 complex may be required for maintenance of polarized growth, which is necessary for host cell entry and pathogenesis. Previously, it was shown that *Δnox1* mutants display increased resistance to calcifluor white, indicating a defect in cell-wall organization affecting chitin deposition. The lighter pigmentation of *Δnox1* mutants also suggests a role in melanin biosynthesis or its polymerization into the cell wall. These phenotypes are notably absent in the other Nox mutants, suggesting that Nox1 may supply ROS to the cell wall for oxidative cross-linking of proteins, and this may be necessary for maintenance of polarized growth.

An important question that arises from this study is how two Nox complexes in the *M. oryzae* appressorium, which are likely to involve the same p67 

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- **Materials and Methods**

**Fungal Strains, Growth Conditions, and DNA Analysis.** The growth and maintenance of *M. oryzae* isolate, media composition, nucleic acid extraction, and fungal transformation were all as previously described (33). Gel electrophoresis, restriction enzyme digestion, gel blots, and sequencing were performed using standard procedures (34).

**Plant Infection Assays.** Pathogenicity assays were performed by spraying seedlings of rice (*Oryza sativa*) cultivar CO-39 with a suspension of 10^9 conidia/mL using an artist’s airbrush (33). Infection-related development was observed by incubating conidia on hydrophobic glass coverslips and allowing appressoria to form after 24 h. Cuticle penetration was measured as described ref. 35.

**Generation of *M. oryzae* Δnox1 Mutant and Strains Expressing GFP Fusions.** Targeted gene replacement of the NOX1 encoding gene was performed using the split-marker strategy. Vectors were constructed using a Hygromycin B-selectable marker, hph, for transformation of *Guy11*. The *hph* gene cassette was cloned into pBluescript (Strategene) as a 1.4-kb EcoRI fragment to amplify the split-templates used were M13 F with HY and M13 R with YG, as described previously (36). *M. oryzae* ΔnoxR was amplified using primers designed to conserved DNA regions identified from an alignment of nox sequences from *M. oryzae*, *E. fuscata*, and *H. sapiens* (Table S1). The Δnox1 sequence was retrieved from the *M. oryzae* genome database of the Broad Institute at the Massachusetts Institute of Technology, Cambridge, MA (www.broad.mit.edu/annotation/fungi/magna/ thatype). The wild-type *Guy11* was transformed with the *nox1:hph* deletion cassette. Transformants were selected in the presence of hygromycin B (200 μg/mL) and assessed by Southern blot analysis. For generation of translational GFP fusions, *SAC6* promoter and terminator sequences were fused to the nox1 sequence. Three-dimensional projections were captured using a Zeiss LMS4 Laser-Merge-System and solid-state lasers (488 nm/70 mW and 561 nm/60-mW diode laser) was modified for use of an OS-IX 71 adaptor (VisiFlow) and controlled by a UGA-40 controller (Rapp OptoElectronic GmbH) (37).

**Analysis of Gene Expression Using High-Throughput SuperSAGE.** A transcriptional profiling dataset (http://cogeme.ex.ac.uk/supersage/) (38) was interrogated to determine the expression of NOX1, NOX2, and NOXR in *Guy11* during appressorium development (4, 6, 8, 14, and 16 h after spore germination). Expression was plotted relative to gene expression in *M. oryzae* mycelium (38).

**Light and Epifluorescence Microscopy and Transmission Electron Microscopy.** For epifluorescence microscopy conidia were incubated on coverslip/leaf sheath and observed at each time point using an IX-81 Olympus inverted microscope connected to a CoolSNAP HQ2 camera. Photobleaching experiments were performed using an IX-81 Olympus inverted microscope with VS-LSM54 Laser-Merge-System and solid-state lasers (488 nm/70 mW and 561 nm/70 mW; VisiFron Systems) and a PlanApo 100×/NA 1.45 Oil TIRF objective (Olympus). The 405-nm/60-mW diode laser was modified by use of a ND 0.6 filter, resulting in 15 mW of output power coupled into the light path by an OSI-IX 71 adaptor (VisiFron Systems) and controlled by a UGA-40 controller (Rapp OptoElectronic GmbH).

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