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Review

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Exploitation of Reactive Oxygen Species by Fungi: Roles in Host-Fungus Interaction and Fungal Development

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In the past, reactive oxygen species (ROS) have been considered a harmful byproduct of aerobic metabolism. However, accumulating evidence implicates redox homeostasis, which maintains appropriate ROS levels, in cell proliferation and differentiation in plants and animals. Similarly, ROS generation and signaling are instrumental in fungal development and host-fungus interaction. In fungi, NADPH oxidase, a homolog of human gp91^{phox}, generates superoxide and is the main source of ROS. The mechanism of activation and signaling by NADPH oxidases in fungi appears to be largely comparable to those in plants and animals. Recent studies have shown that the fungal NADPH oxidase homologs NoxA (Nox1), NoxB (Nox2), and NoxC (Nox3) have distinct functions. In particular, these studies have consistently demonstrated the impact of NoxA on the development of fungal multicellular structures. Both NoxA and NoxB (but not NoxC) are involved in host-fungus interactions, with the function of NoxA being more critical than that of NoxB.

Keywords: Reactive oxygen species, NADPH oxidase, fungal development, host-fungus interaction

Introduction

Reactive oxygen species (ROS) are small molecules containing an unpaired electron that is highly reactive with other molecules such as lipid, proteins, DNA, and carbohydrates [21, 46, 67]. Although reactive oxygen species are produced through several pathways, the starting material for ROS generation is generally superoxide ($O_2^{\cdot-}$). Upon reacting with a hydrogen ion, superoxide then rapidly dismutates to hydrogen peroxide (H_2O_2) in a reaction catalyzed by superoxide dismutase (SOD) (Fig. 1). Diverse reactive oxygen species are also generated spontaneously from superoxide, or in the presence of an appropriate partner *via* several reactions. The hydroxyl radical ($HO^{\cdot}$) can be generated by sequential reduction from superoxide (Fig. 1). Superoxide also reacts with nitric oxide ($NO^{\cdot}$) to produce peroxynitrite (ONO_2^-), a highly reactive oxidizing molecule (Fig. 1). This nitric oxide is synthesized from L-arginine by nitric-oxide synthase (NOS). In the presence of a reduced metal ion such as Fe^{2+} or Cu^{2+}, hydrogen peroxide generates a hydroxyl radical and a hydroxide ion *via* the Fenton reaction (Fig. 1). Two molecules of water and a molecule of oxygen are formed from two molecules of hydrogen peroxide in a reaction catalyzed by catalase enzymes. Singlet oxygen ($^1O_2$), one of the major reactive oxygen species, is produced by excitation of triplet oxygen ($^3O_2$) (Fig. 1). A single electron reduction of triplet oxygen results in the generation of superoxide. Although triplet oxygen is a biradical, it is a stable molecule in nature.

All aerobes must balance oxidative metabolism and the generation of unwanted reactive oxygen species, particularly in mitochondria, chloroplasts, and peroxisomes [4]. Several oxidative enzymes also inadvertently produce small amounts of superoxide [40]. These ROS can attack proteins, lipids, DNA, and carbohydrates in the cell, causing serious problems, including DNA mutation, lipid peroxidation, and protein oxidation [23]. Numerous studies have shown

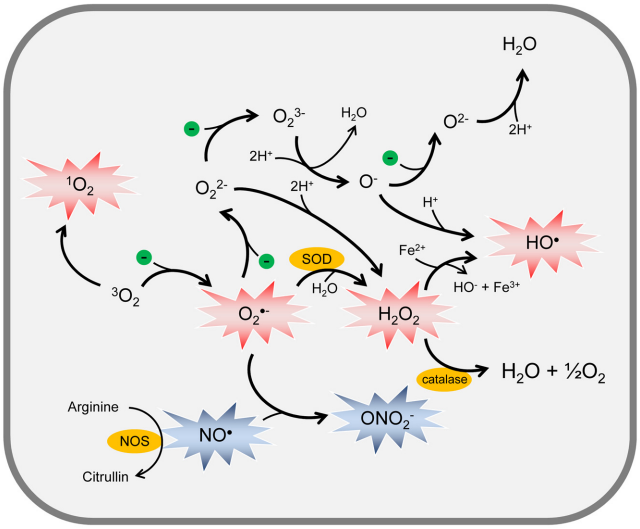

Fig. 1. Generation of different reactive oxygen species (ROS) by energy transfer, sequential reduction, oxidation, and enzyme reaction in cells.

ROS are shown in red stars, and reactive nitrogen species (RNS) are shown in blue stars. The single electrons are depicted in a green circle. Abbreviations: $^1O_2$, singlet oxygen; $^3O_2$, triplet oxygen; $O_2^{\bullet-}$, superoxide radical ion; H_2O_2, hydrogen peroxide; HO•, hydroxyl radical; O_2^{2-}, peroxide ion; O^-, oxene ion; O^{2-}, oxide ion; ONO_2^-, peroxynitrite.

that ROS are detoxified and their levels are controlled by enzymes, including SOD, peroxidase, and catalase, in animals and plants [4, 26]. Non-enzymatic ROS scavenging mechanisms such as the ascorbate-glutathione cycle and antioxidants, including tocopherol, flavonoids, alkaloids, and carotenoids, also protect cells from oxidative damage [4, 9, 71]. Emerging research, however, has suggested that ROS are also necessary for proper growth and development in animals, plants, and fungi [1, 31, 52, 72]. This review article describes the impact of ROS on the proliferation and differentiation of fungi. The focus of the article is mainly on the role of the fungal NADPH oxidase in ROS generation and signaling in fungal development and host-fungus interaction.

ROS as a Signal Molecule

Although very high levels of ROS are toxic to cells, ROS homeostasis is critical in cell proliferation and differentiation [13, 42, 56, 63, 68, 72]. There are two important targets mediating ROS signaling: (i) Proteins containing residue(s) that can be sensitively and reversibly oxidized by ROS; (ii) molecules such as nucleic acids and lipids also produce stable secondary signaling molecules (*e.g.*, 8-nitro-cGMP and nitro-fatty acids) [39, 51].

Numerous proteins have been found to be regulated by ROS. Target proteins for ROS commonly carry cysteine residues that can be oxidized to subsequently activate downstream signal transduction pathways. Protein tyrosine phosphatase (PTP) domain-containing proteins are a well-known target for ROS [33, 41]. The redox regulation of these proteins is achieved through ROS-mediated oxidization of a conserved catalytic Cys residue in the PTP domain, which is involved in phosphatase activity [41]. PTP domain-containing proteins include PTPs, phosphatase and tensin homolog (PTEN), and protein-tyrosine phosphatase 1B (PTP1B). Thioredoxin (TRX) family proteins are also important targets for ROS. TRX family proteins are largely divided into TRX and nucleoredoxins (NRXs). TRX-reductase, possessing selenocysteine, and flavin adenine dinucleotide (FAD) reduce and activate oxidized TRX that contains a pair of redox-active Cys residues. The activated TRX can reduce peroxiredoxin (PRX), which can subsequently reduce H_2O_2 [22]. TRX is implicated in the maintenance of the global redox environment in cells, and it reduces intracellular oxidized proteins to protect cells from oxidative stress. In addition, TRX is known to be a modulator of the ROS-induced cell death signaling pathway mediated by apoptosis signal-regulating kinase 1 (Ask1) [49]. ROS triggers oxidation of TRX, leading to the dissociation of TRX from Ask1. This free Ask1 is activated, and it stimulates the downstream signaling pathway [49]. Similarly, NRX is involved in the Wnt (singles/integrated) signaling pathway. ROS oxidizes NRX, resulting in the dissociation of NRX from Dvl. This activated Dvl form stimulates the downstream signaling pathway [17]. In addition to PTPs and TRX family proteins, PRX family proteins are also targets for ROS [70]. Collapsin response mediator protein 2 (CRMP2) and Lyn, an Src family tyrosine kinase (SFK), were reported as targets possessing a ROS-responsive Cys residue [36].

Recently, several reports revealed that electrophilic compounds such as nucleic acids and lipids could serve as ROS targets to convert unstable ROS signals into stable secondary signaling molecules [39, 50, 51]. In particular, there is considerable evidence indicating that unstable and nonselective ROS signals could be transformed into a controlled signal transduction by 8-nitroguanosine 3′,5′-cyclic monophosphate (8-nitro-cGMP) [39, 50]. Peroxynitrite ($ONOO^-$) generated by the reaction of ROS with NO (see Fig. 1) catalyzes the conversion of GTP to 8-nitro-GTP, which is eventually converted to 8-nitro-GMP by soluble guanylate cyclase (sGC) [2]. In addition, the electrophilic species 8-nitro-GMP can be transformed to 8-SH-cGMP, in

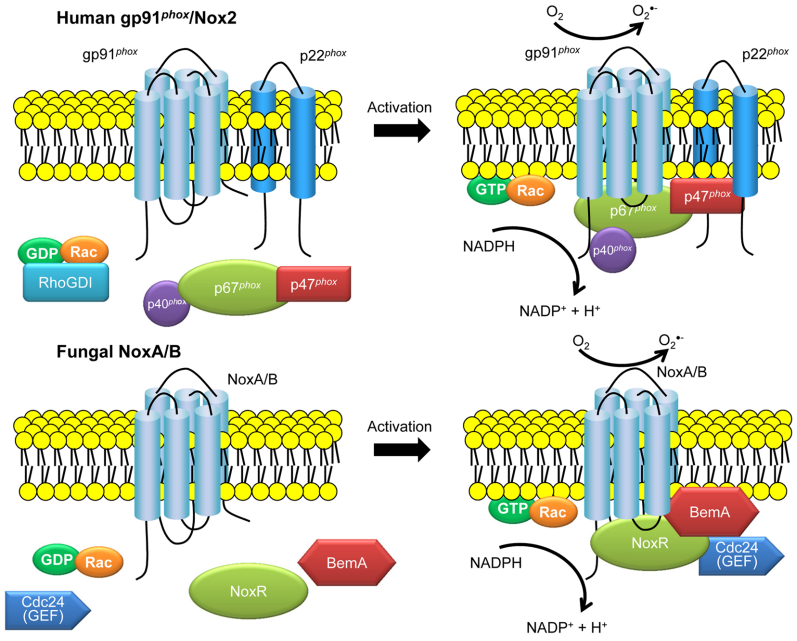

Fig. 2. Assembly and activation of human gp91^{phox}/Nox2 and fungal NoxA/B.

In resting cells, the human gp91^{phox}/Nox2-p22^{phox} complex resides in the extracellular membrane, while the complex of p40^{phox}, p47^{phox}, and p67^{phox} is in the cytosol. Proper stimulation leads to the phosphorylation of p47^{phox} and the entire cytosolic complex is recruited to the membrane. In addition, dissociation of Rac-GDP and RhoGDI is promoted by GEF (guanine-nucleotide exchange) [26]. Although information about the activation mechanism of the fungal NADPH oxidases is limited, a possible model to illustrate the activation mechanism is suggested here based on the findings of a previous study by Scott and his colleagues [57]. In resting cells, the fungal NoxA/B protein appears to be localized in the membrane. By stimulation, Rac and NoxR are recruited to the fungal plasma membrane, resulting in superoxide production. In the fungal cell, BemA appears to serve as a scaffold protein to recruit NoxR and RacA to the plasma membrane as observed with the mammalian p40^{phox} and p47^{phox} [57]. In contrast to gp91^{phox}/Nox2, Cdc24, a GEF for Cdc42, which is the yeast homolog of RacA, appears to be localized with BemA and NoxR in the membrane [14].

a reaction catalyzed by the hydrogen sulfide anion (HS^-) [39]. This 8-SH-cGMP can be involved in the nucleophilic signaling [3, 39]. In line with electrophile-mediated signaling, the electrophilic product 8-nitro-GMP can induce S-guanylation of Keap1 and H-Ras that can further activate downstream signaling pathways [39, 50]. Future studies regarding the role of various electrophile compounds as ROS targets may provide useful information for the development of therapeutics for oxidative stress-related diseases.

Mechanism of NADPH Activation

Superoxide-generating NADPH oxidase is crucial for the generation of ROS in animals, plants, and likely in fungi. In animals, the well-known NADPH oxidase (also designated Nox2) is involved in chronic granulomatous disease and other diseases and in physiological functions [30, 53]. The Nox enzyme consists of a multi-subunit complex: the cytosolic regulatory components Rac, p67^{phox}, p47^{phox}, and p40^{phox}, and the integral membrane protein flavocytochrome b_{558} (Fig. 2) [52]. The core consists of heterodimeric flavocytochrome b_{558}, composed of the catalytic subunits gp91^{phox} and p22^{phox}. Electrons transferred from the electron donor NADPH are translocated to the cytosolic side of the cellular membrane through flavocytochrome b_{558}. These electrons react with the electron acceptor oxygen and produce superoxide. Using this superoxide as starting material, a large variety of ROS are produced, including oxidized halogens, free radicals, and singlet oxygen, as mentioned earlier [4]. The regulation of the Nox protein complex in mammalian phagocytes is well characterized. In resting phagocytes, the complex of p40^{phox}, p47^{phox}, and p67^{phox} is in the cytosol, whereas heterodimeric p22^{phox} and gp91^{phox} are localized in the membranes (Fig. 2) [37]. Upon cell stimulation, the autoinhibitory domain of p47^{phox} becomes heavily phosphorylated, and the entire cytosolic complex is recruited to the membrane (Fig. 2). The cytosolic complex of three proteins, regulated by small GTP proteins such as Rac and RhoGDI, associates with the

two membrane-bound components to generate superoxide from the active oxidase [4, 26, 38, 53]. As NAPDH oxidase activity is transient and requires many stimuli, an alteration in the cellular redox status and changes in the phosphorylation state of $p47^{phox}$ may lead to termination of NADPH oxidase activity [11].

A Burst of ROS from Plant NADPH Oxidase

Plants induce the production of ROS to kill pathogens and to strengthen cell walls *via* oxidative cross-linking of cell wall glycoproteins [7]. ROS have also been shown to be signaling molecules [34, 66]. Owing to its biological importance, plant biologists have attempted to understand the sources of ROS in plants. Using genetics and inhibitor studies, two main sources of ROS generation have been discovered [66]. The plant respiratory burst oxidase homolog (Rboh) protein, known as a homolog of human $gp91^{phox}$, generates superoxide [19, 64]. A cell-wall-bound peroxidase has also been proposed as a source of ROS [5, 6]. ROS generated by the NADPH oxidase of plants are involved in host defense *via* the hypersensitive response [65], in development *via* regulation of plant cell expansion through the activation of Ca^{2+} channels [16], and in several other physiological processes [25]. Furthermore, Torres *et al*. [65] showed that activation of NADPH oxidase inhibited runaway cell death (RCD), an uncontrolled spreading of cell death to uninfected cells surrounding a normal hypersentive response site. They demonstrated that the *Arabidopsis* double-knockout mutants of NADPH oxidase (AtrbohD) and the LSD (lesion simulating disease) zinc-finger protein, a negative regulator of RCD, resulted in the acceleration of RCD [65]. However, the *lsd1* mutant overexpressing AtrbohD reduced RCD as compared with *lsd1* mutants such as *lsd1* single mutant and *lsd1 atrbohD* and *lsd1 atrbohF* double mutants [65]. This suggested that Atrboh and LSD1 are involved in the cell death pathway, depending on salicylic acid, which induces the spread of cell death to cells surrounding infection sites [65].

Fungal NADPH Oxidase

In fungi, ROS studies are increasing, with a significant number of studies probing the role of NADPH oxidase in fungal development. Three different fungal Nox subfamilies have been identified thus far: NoxA (Nox1), NoxB (Nox2), and NoxC (Nox3). NoxA and NoxB are fungal homologs of $gp91^{phox}$, and NoxC has putative calcium-binding EF-hand motifs, which are found in the NH_2-terminal ends of both animal Nox5 and plant Rboh proteins. NoxA is required for the development of the sexual fruiting body in *Aspergillus nidulans*, *Podospora anserina*, and *Neurospora crassa* [52]. Aguirre and his colleagues demonstrated that inactivation of NoxA was responsible for the inhibition of cleistothecia differentiation in *A. nidulans* [27]. However, this gene was not implicated in hyphal growth and asexual development. Consistently, Nox1 (a homolog of NoxA) of *N. crassa* and *P. anserina* is required for sexual development. Nox1 also regulates asexual development and hyphal growth in *N. crassa* [8, 32]. Conversely, NoxB appeared to be responsible for regulation of ascospore germination in the fungi mentioned above [27, 34, 55]. Notably, several fungi, including *Fusarium* spp., *Magnaporthe grisea*, *P. anserina*, *Aspergillus terreus*, and *Phaeosphaeria nodorum*, have the third *nox* gene, *noxC*, although its function is unclear [59].

The regulation of the Nox complex is not well characterized in fungi. NoxR, a homolog of $p67^{phox}$, and Rac, a small GTPase of the Rho subfamily, are required for the regulation of Nox1/NoxA and Nox2/NoxB in fungi (Fig. 2) [52, 58]. In many cases, inactivation or elimination of NoxR or Rac resulted in phenotypes similar to those of mutants that are disrupted in the *nox1/noxA* or *nox2/noxB* genes [54, 55, 58, 61]. This suggests that NoxR and Rac play a pivotal role in the regulation of ROS in fungi. For the activation of fungal NADPH oxidases, it is speculated that fungal Rac and NoxR are recruited to the plasma membrane and they interact with Nox1/NoxA or Nox2/NoxB, leading to the production of superoxide (Fig. 2). Interestingly, a search of fungal genomes for homologs of the mammalian Nox regulatory components revealed the apparent absence of $p47^{phox}$ and $p40^{phox}$ homologs in fungi [59]. Furthermore, the protein-protein interaction domain found in mammalian $p67^{phox}$ for interaction with $p47^{phox}$ and $p40^{phox}$ is absent in the C-terminus of fungal NoxR. These data suggest that NoxR and Rac are major regulators for fungal NADPH oxidase complexes as compared with animal Nox complexes. Moreover, recent research implementing yeast two-hybrid and co-immunoprecipitation assays revealed that fungal NoxR interacted with Bem1 and Cdc24, which are homologs of the yeast polarity proteins [57]. In animals, it has been demonstrated that p38 MAPK and p21-activating kinase are also involved in the regulation of the Nox enzyme complex [19, 58]. Similarly, SakA (stress-activating MAP kinase), a p38 MAPK homolog, of *A. nidulans* and *Epichloë festucae* is associated with expression or modification of NoxA [14, 75]. In *Claviceps purpurea*, Cla4, a homolog of p21-activating kinase (Pak), suppressed expression of the *nox1* gene [47]. Collectively, increasing information regarding NADPH oxidase in filamentous fungi supports the idea that fungal NADPH

oxidases are required for development; however, the underlying mechanisms and pathways of regulation are yet to be elucidated.

Although no NADPH oxidase gene or enzyme had been assigned in hemiascomycetous yeasts, Yno1, an NADPH-oxidase ortholog, was reported in *S. cerevisiae* in a recent study [44]. Rinnerthaler *et al*. [44] showed evidence that Yno1 enzyme possessed all biochemical properties of a fungal NADPH oxidase. Overexpression of Yno1 promoted the production of ROS, causing Yca1p-dependent apoptosis [44]. The *Δyno1* mutant strain displayed resistance against apoptotic stimuli and hypersensitivity to wiskostatin, an inhibitor of F-actin cable nucleation, and latrunculin B, an inhibitor of F-actin cable elongation [44]. This result indicates that Yno1p is involved in the regulation of the actin cytoskeleton.

ROS Signaling in Fungi

Histidine kinases of two-component signal transduction systems, which consist of a histidine kinase and a response regulator, are induced in response to an ROS signal in prokaryotes, fungi, and plants [4, 55]. In fungi and plants, these signals eventually lead to activation of the MAP kinase pathway (*e.g*., Hog pathway in *Saccharomyces cerevisiae* and MPK3 and MPK6 in *Arabidopsis*), whereas the action of enzymes such as protein phosphatases is inhibited by ROS [20, 69]. PRX was reported to stimulate activation of the Hog homolog Sty1 in *Schizosaccharomyces pombe* [70]. It was proposed that H_2O_2 induced activation of Sty1 by disulfide bond formation with Tpx1, the yeast homolog for PRX [70]. The activation of Sty1 also requires the mitogen-activated protein kinase kinase (MAPKK) Wis1 that was activated by the MAPKKKs Wak1 (or Wis4/Wik1) and Win1 [70]. Additionally, the yeast transcription factor Yap1 cooperates with glutathione peroxidase (Gpx3) and regulates expression of genes involved in the detoxification of ROS as well as in drug and heavy metal resistance [12, 30]. Although most fungal homologs of the Yap1 transcription factor are ROS-responsive, thus far, only the biotrophic fungus *Ustilago maydis* and a common necrotrophic fungus, *Alternaria alternata*, have YAP1-like transcription factors that are responsive to ROS and influence virulence [28–30, 62, 75].

Role of Fungal NADPH Oxidase in Plant-Microbe Interactions

Accumulating evidence strongly suggests that ROS play a pivotal role in plant-microbe symbiosis and pathogenesis. *E. festucae* is a fungal mutualist providing stress protection to the host, while the host provides a suitable niche for the fungus. A restriction enzyme-mediated integration (REMI) screen for mutualistic mutants identified a NADPH oxidase gene (*noxA*). When *noxA* was deleted, the fungus unexpectedly exhibited a pathogenic phenotype [60]. Elimination of NoxR and RacA led to a similar phenotype in the plant-*noxA* mutant association [8, 60, 61]. This suggests that ROS production *via* fungal NADPH oxidase is necessary for maintenance of plant-fungus mutualism. Additionally, Talbot and his colleagues showed that an NADPH oxidase of *M. grisea*, a hemibiotroph, is instrumental in the differentiation of the infection-mediating cell, the appressorium: the *Δnox1*, *Δnox2*, and *Δnox1Δnox2* mutants were unable to infect host cells [15]. In a subsequent study, they also showed that NADPH oxidases of *M. grisea* were critical for the septin-mediated remodeling of the F-actin cytoskeleton during plant infection [48]. NADPH oxidases of *Fusarium graminearum*, another hemibiotroph responsible for cereal head blight, have an impact on the fungal development and virulence [73]. The *ΔnoxA* mutant and the *ΔnoxAΔnoxB* double mutant, but not the *ΔnoxB* mutant, are unable to form perithecia and to generate ascospores [73]. Although inactivation of NoxA, but not NoxB, impairs the virulence of *F. graminearum*, both NADPH oxidases are required for complete pathogenicity [73]. Consistent with the findings of these studies, *C. purpurea*, a biotrophic fungus, required Cpnox1 activity for full pathogenicity and the germination of conidia. Cpnox1 is also involved in fungal growth, vegetative differentiation, and the formation of sclerotia [18]. In contrast to Cpnox1, Cpnox2 had no effect on virulence [18]. Similar to that observed with *M. grisea*, however, *Botrytis cinerea*, a necrotrophic fungus, required both *bcnoxA* and *bcnoxB* genes for pathogenesis [54]. Deletion of *bcnoxA* led to slow colonization of the host tissue, although *bcnoxA* mutants eventually were able to penetrate the host tissue. In addition, *bcnoxA* appears to be essential for conidial anastomosis tube (CAT) fusion [45]. The *bcnoxB* and double-knockout mutants (*bcnoxAB*) were unable to penetrate epidermal cells; moreover, double-knockout mutants colonized host tissue slower than *bcnoxA* and *bcnoxB* mutants, and were thus weakly pathogenic. A recent study in *Sclerotinia sclerotiorum*, another important necrotophic fungus, has shown the role of ROS during sclerotial development [24]. Intracellular ROS production from sclerotial initials was monitored by using the ROS indicator 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA) [43]. Fluorescence emission was higher in sclerotial initials, suggesting that sclerotial initiation includes the generation of intracellular ROS [24]. Wild-type *S. sclerotiorum* was treated with either *N*-acetylcysteine

(NAC) or diphenyleneiodonium (DPI), an inhibitor of flavoenzymes such as NADPH oxidase, to further examine ROS involvement in sclerotia formation [10]. NAC and DPI treatment of the fungus hindered sclerotia formation and prevented sclerotia maturation. There were no significant differences in the radial growth of *S. sclerotiorum* in these treatments [24]. In an effort to determine the generator of ROS, two predicted *S. sclerotiorum* NADPH oxidases (SsNox1 and SsNox2) were identified and functionally characterized. The RNA interference technique was employed to generate knockdown mutants for each gene. The genetic study using RNAi showed that both *nox* genes appeared to have roles in sclerotial development. Compared with the wild-type strain and the *Ssnox2*-silenced mutant, the *Ssnox1*-silenced mutant showed a significant reduction in superoxide accumulation during the initial stage of sclerotial formation. In addition, sclerotia formation was impaired in the *Ssnox1*-silenced mutant, whereas the *Ssnox2*-silenced mutant strains only occasionally generated sclerotia. Moreover, SsNox1 appears to be linked to the production of the fungal secondary metabolite oxalate, an important pathogenicity factor in *S. sclerotiorum* disease [24]. These results collectively suggest that the generation of ROS is required for sclerotial development, and NADPH oxidases are responsible for ROS generation in *S. sclerotiorum*. *A. alternata* NADPH oxidase (AaNoxA) also plays an essential role in ROS generation [74]. In addition, inactivation of AaNoxA appears to be critical for resistance to oxidative stress, which is similar to the result observed in the *A. alternata ap1* deletion mutant defective in a YAP1-like transcriptional regulator [74]. AaNoxA also seems to be involved in the virulence and sporulation of *A. alternata* [74]. In the study of NADPH oxidase of *Trichoderma hazianum*, a mycoparasitic filamentous fungus, Montro-Barrientos *et al*. [35] revealed that the *Trichoderma hazianum nox1* gene encoding NADPH oxidase was implicated in the production of ROS and in the secretion of hydrolytic enzymes. They also showed that the increased levels of *nox1* expression in the *nox1*-overexpressed transformants correlated to specific biocontrol activity against *Pythium ultimum* [35].

Concluding Remarks

The discovery of an NADPH oxidase enzyme possessing the sole function of superoxide production has stimulated the study regarding ROS function in the cells. Compared with the NADPH oxidase of plants and animals, fungal NADPH oxidases are instrumental in development and host-fungus interaction. Regardless of the pathogenic lifestyle (biotroph, hemibiotroph, necrotroph, or endophyte), NoxA (Nox1) and NoxB (Nox2) are both important factors for invasion and proliferation in host cells. The role of NoxA, however, appears to be more critical than that of NoxB in the host-fungus interaction. In terms of fungal development, NoxA and NoxB have diverse functions in different filamentous fungi. More importantly, the participation in the development of fungal multicellular structures is a very intriguing function of NoxA. As the fungal kingdom comprises both unicellular and multicellular organisms, and since no NoxA gene or enzyme has been assigned in unicellular fungi such as hemiascomycetous yeasts, the involvement of NoxA in the development of fungal structure may be related to the evolution of multicellularity. Therefore, elucidating the mechanistic details regarding the role of NoxA in the development of fungal structure will be crucial to developing an understanding of the evolution of multicellularity and cellular differentiation in multicellular eukaryotic cells.

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